MOLECULAR ASPECTS OF THE LINK BETWEEN OBESITY, INSULIN RESISTANCE AND BREAST CANCER

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Declaration

This thesis, in its entirety, has been composed by the undersigned candidate and has not been submitted in a previous application for a degree. All analyses described herein have been performed by the candidate. Ideas, contributions and findings from other individuals have been distinguished and are acknowledged in the text. A list of where this information was obtained is also given.

Michael Weichhaus

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A thesis presented for the Degree of Doctor of Philosophy

Abstract

Obesity is a multi-factorial metabolic disease, resulting in increased adipose tissue acquisition by the host. This disease increases the risk for developing co-morbidities, including Metabolic Syndrome and other disorders such as breast cancer. Obesity, and particularly abdominal obesity, is characterised by metabolic changes, including chronically elevated insulin concentrations and aberrant secretion of cytokines released from fat tissue, called adipokines. Epidemiologically, the risk of developing postmenopausal breast cancer is increased in obese individuals. The molecular link between obesity and breast cancer however is not well understood. The study presented here aimed at identifying the molecular mechanisms involved in this link, by testing the hypothesis that high insulin concentration and certain adipokines may promote breast cancer progression and/or breast cancer aetiology.

A cell culture system of breast cancer cells and breast epithelial cells was employed to investigate changes in cell proliferation, activation of cell signalling pathways, cell cycle progression and apoptosis after treatment with insulin, leptin, TNF-α, adiponectin and IL-6.

In MDA-MB-231 breast cancer cells, insulin treatment did not affect cell proliferation, cell cycle or apoptosis. Conversely, IR-phosphorylation, AKT-phosphorylation and ERK1/2-phosphorylation were all significantly increased. Microarray analysis indicated

several important changes in gene expression with insulin treatment. Leptin treatment increased proliferation by 21%. Additional analyses of the effect of leptin indicated that neither the PI3-kinase pathway nor the MAP-kinase pathway was involved in mediating this effect. Treatment with TNF-α increased apoptosis, but did not affect cell proliferation or activation of cell signalling pathways.

In MCF-10A breast epithelial cells, cell proliferation increased after insulin treatment by 180%. IR-phosphorylation, AKT-phosphorylation and ERK1/2 phosphorylation were all significantly increased while early apoptosis decreased after insulin treatment. Analysis of cell cycle however did not indicate a change in progression. Microarray analysis indicated that insulin treatment may increase expression of genes related to cancer growth. Leptin treatment increased cell proliferation and also increased ERK1/2-phosphorylation, while AKT-phosphorylation was not affected. Leptin did not change cell cycle progression. TNF-α treatment increased cell proliferation and also increased ERK1/2 phosphorylation, while AKT-phosphorylation was not changed. TNF-α treatment tended to increase apoptosis, the change however was not statistically significant.

In SK-BR-3 breast cancer cells, cell proliferation did not change after insulin treatment. IR-phosphorylation and AKT-phosphorylation increased after insulin treatment, while ERK1/2-phosphorylation decreased. Gene expression of cyclin D and cyclin E increased with insulin treatment, while apoptotic rate and cell cycle profile were also not affected. Cell proliferation increased by 115% after treatment with 100 ng/ml leptin. ERK1/2-phosphorylation however decreased, while AKT-phosphorylation tended to

increase, but the change was not statistically significant. Cell cycle profile was not affected by leptin treatment, G1-phase however tended to increase, but the change was again not statistically significant. Cell proliferation increased by 59% after 48 h treatment with 10 ng/ml TNF-α. AKT-phosphorylation and ERK1/2-phosphorylation increased with TNF-α treatment. Cell cycle analysis showed a decrease in S-phase and G2-phase, indicative of a decrease in cell cycle progression.

These results indicate that none of the examined obesity-related factors is convincingly identified as the main molecular link between obesity and postmenopausal breast cancer. Conversely, all treatments affected each of the cell lines in, at least, one of the examined aspects. This indicates that many of the obesity-related factors may affect breast cancer and that a single breast tumour may utilise a unique combination of those factors to promote growth. All treatments increased proliferation in MCF-10A breast epithelial cells, with additional analysis generally supporting growth promotion. Insulin treatment particularly increased cell proliferation, while leptin and TNF-α increased MAP-kinase signalling. This may indicate that insulin and adipokines may have a higher impact on breast cancer aetiology than on breast cancer progression.

Key words: Obesity, insulin resistance, breast cancer, adipokines, cell proliferation, cell signalling pathways, cell cycle, apoptosis

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Citations

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List of Abbreviations

7-AAD	7-Aminoactinomycin D
AgRP	agouti-related protein
AKT and PKB	protein kinase B
α-MSH	α-melanocyte-stimulating hormone
AP	alkaline phosphatase
ARC	arcuate nucleus
BAT	brown adipose tissue
BMI	body mass index
BRCA	breast cancer associated gene
BrdU	bromodeoxyuridine
BSA	bovine serum albumin
CART	cocaine amphetamine–regulated transcript
CDK	cyclin dependent kinase
CVD	cardiovascular disease
ddH2O	double distilled water from Milli-Q system
DEXA	dual energy X-ray absorptiometry
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
ER	oestrogen receptor
ERK	extracellular-regulated kinase
FCS	foetal calf serum
FFA	free fatty acids
FITC	fluorescein isothiocyanate
FS	front scatter
FTO	fat mass and obesity associated gene
GABA	γ-aminobutric acid
GH	growth hormone
GLUT4	glucose transporter type 4
HDL	high density lipoprotein
HER2 and ErbB-2	human epidermal growth factor receptor
HIF-1	hypoxic inducible factor-1
HRP	horseradish-peroxidase
IGF	insulin-like growth factor
IGFPB	insulin-like-growth-factor-binding protein
IL-6	interleukin-6
IR	insulin receptor
IRS	insulin receptor substrate
JAK	janus activated kinase
JNK	c-JUN N-terminal kinase
LDL	low density lipoprotein
MAPK	mitogen activated protein kinase
MC4-R	melanocortin-4 receptor

MCAO	medial cerebral artery occlusion
MEK	mitogen activated protein kinase kinase
MRI	magnetic resonance imaging
MSI	microsatellite instability
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NASH	non-alcoholic steatohepatitis
NFLD	non-alcoholic fatty liver disease
NPY	neuropeptide Y
Ob-R	leptin receptor
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCOS	polycystic ovary syndrome
PgR	progesterone receptor
PI	propidium iodide
PI3-K	phosphoinositide-3 kinase
PIP2	phosphatidylinositol-4,5-biphosphate
PIP3	phopshatidylinositol-3,4,5-triphosphate
pO_2	partial pressure of oxygen
POMC	pro-opiomelanocortin
PS	phosphatidylserine
PTEN	phosphatase and tensin homologue deleted on chromosome 10
PVN	paraventricular nucleus
RBP4	retinol binding protein 4
RNA	ribonucleic acid
RT-PCR	reverse-transcription polymerase chain reaction
SD	standard deviation
SDS	sodium dodecyl sulphate
SEM	standard error of mean
SHC	src homology 2 domain-containing
SNP	single-nucleotide polymorphism
SOCS	suppressor of cytokine signalling
SS	side scatter
STAT	signal transducers and activators of transcription protein
T2DM	type 2 diabetes mellitus
TBS	tris buffered saline
TG	triglycerides
TMB	tetramethylbenzidine
TNF	tumour necrosis factor
VEGF	vascular endothelial growth factor
WAT	white adipose tissue

Chapter 1
Introduction

1 INTRODUCTION

1.1 Obesity

1.1.1 Definition and measurement of obesity

Obesity is a disease in which an individual accumulates excessive amounts of body fat. Men are obese if more than 25% of their body weight is fat tissue and women if more than 33% of their body weight is comprised of fat tissue (Haslam and James, 2005). Fat tissue is widely distributed across the body; its main storage is subcutaneous. Fat tissue is however also found intra-abdominally. This makes direct quantification of body fat tissue difficult and several models are in use to estimate body fat percentage (Table 1-1). Some of these models have themselves become definitions for obesity (WHO, 1995). The body mass index (BMI) is one of these models and is defined as the weight of the person in kilograms divided by the square of the height of the person in meters. BMI can then be used to calculate body fat percentage using this equation:

$(1.2 \times BMI) + (0.23 \times age) - 5.4 - (10.8 \times gender)$

with *gender* being 1 for men and 0 for women (Deurenberg *et al.*, 1991).

Rather than using this lengthy equation the World Health Organisation determined BMI cut-off values to be used in order to define individual obesity directly. BMI values to define underweight (<19.5 kg/m^2), normal weight (19.5-25 kg/m^2), overweight (25-30 kg/m^2) and obesity (>30 kg/m^2) have been set (WHO, 1995). While for the general population BMI correlates reasonable close with body fatness, it has some limitations for certain populations (Caterson and Gill, 2002). As BMI cannot distinguish between lean and fat mass, populations with increased muscle mass, such as professional athletes and body builders may be misclassified as overweight or obese. Likewise the elderly may be misclassified as within the normal range, as their lean tissue has decreased

disproportionately to their fat tissue. Additionally, in populations that do not change weight and height in a linear way, *i.e.* in children and adolescent, BMI may lead to exaggerations of rates of overweight and obesity (Speakman, 2004).

Intra-abdominal fat, *i.e.* fat tissue that accumulates around the organs of the abdomen, tends to be more important for obesity-related disease susceptibility than overall obesity as measured by BMI (see section 1.1.5). Thus measurements to estimate this proportion of body fat have been established. Waist circumference and waist-to-hip ratio are commonly used (Table 1-1). Exceeding certain waist circumference values (>102cm for men and >88 cm for women) and waist to hip ratios (>0.9 for men and >0.85 for women) increases the risk of mortality and morbidity for cardiovascular disease (see sections 1.1.5.1 and 1.1.5.3). Waist circumference is also the determining factor in the identification of individuals with Metabolic Syndrome (see section 1.1.5.2). These cut-off points are also used in addition to BMI to define obesity, but especially in the context of identifying individuals at particular risk of developing obesity associated co-morbidities (Reaven, 1988; Reaven, 1993). For the study presented here, the term "obesity" will be used to refer to a BMI of 30 kg/m^2 or above, unless otherwise stated. In addition to these measurements a number of different methods to identify obesity are used (Table 1-1).

Table 1-1: Methods for measuring body composition to evaluate body fatness and obesity, defined as exceeding a gender specific body fat percentage (adapted with extension from Kopelman, 2000)

Method	Definition	Advantages and Limitations
BMI	Weight in kilograms divided by square of height in meters	BMI correlates strongly with densitometry measurements of fat mass, does not distinguish between lean and fat mass, requires only standard equipment for weight and height measurement, low observer error
Waist circumference	Measured (in centimetres) at midpoint between lower border of ribs and upper border of the pelvis	Waist circumference and waist-to-hip ratio provide measurements for assessing upper body fat disposition; neither provide precise estimates of intra-abdominal fat, only tape-measure required, potential for considerable observer error
Waist-to-Hip ratio	Waist circumferences divided by circumference (in centimetres) at widest gluteal hip expansion	
Skinfold thickness	Measurement of skinfold thickness (in centimetres) with callipers at multiple sites of the body	Potential for considerable observer error; only measures subcutaneous fat, requires purchase of specific equipment
Bioimpedence	Based on the principle that lean body mass conducts currents better that fat mass as it is basically an electrolyte solution; the resistance to a weak current across extremities is measured	Simple and practical in use, similar sensitivity of estimating body fat compared to anthropometric measurements, unsuitable for certain individuals, requires purchase of specific equipment, low observer error
Displacement	Body composition is calculated by the amount of water or air displaced	Allows accurate calculation of total body fat, similar to imaging techniques (Fields *et al.*, 2002; Lockner *et al.*, 2000), requires purchase of specific equipment at high cost, low observer error
Imaging	Dual energy X-ray absorptiometry (DEXA) scan; magnetic resonance imaging (MRI)	Allows accurate calculation of total body fat and its distribution; impractical for routine use, very high equipment cost, low observer error

1.1.2 Prevalence of Obesity

There has been a dramatic increase in the prevalence of obesity over the past 30 years, especially in western societies but also in certain "poorer" societies. Obesity is not discriminatory of gender, age or ethnicity, its prevalence is increasing in all population groups. Obesity rates however are higher in females compared to males, increase with age and are higher in certain ethnic minorities compared to the general population. The latest figures from the United States show that, at the end of 2004, 31.2% and 33.2% of US males (♂) and females (♀) are classified as obese, respectively using the BMI definition of >30 kg/m^2 (Ogden *et al.*, 2006). Compared to earlier data, the prevalence of obesity in adults in the various National Health and Nutrition Examination Surveys[1] (NHANES) has increased from 10.4% (♂) and 15.9% (♀) of the population in 1960 to 31.1% (♂) and 33.2% (♀) in 2004 (Figure 1-1). Around two-third (70.8% (♂) and 61.8% (♀)) of the adult US population are now either overweight or obese. Interestingly it seems that in men only obesity rates have increased, while the overweight category has remained unchanged, keeping at around 40% of the US population. In women however both obesity and overweight have increased simultaneously (Figure 1-1 and references therein). Additionally, women in certain sub-populations and ethnic minorities tend to be affected worse by obesity. In the NHANES 2005-2006 survey approximately 53% of non-Hispanic black women and 51% of Mexican-American women, 40-59 years of age, were obese, compared with about 39% of non-Hispanic

[1] The NHANES trials were the National Health Examination Survey (NHES I) in which data was collected between 1960-1962, the National Health and Nutrition Examination Surveys I & II & III (NHANES I, 1971-1974; NHANES II, 1976-1980; NHANES III, 1988-1994), the Hispanic only survey (HHANES, 1982-1984) and three additional surveys with data collected between 1999-2000, 2001-2002, 2003-2004. The population sample is thought to accurately reflect the US-population. All surveys were conducted by the National Center for Health Statistics (NCHS) of the Centers for Disease Control and Prevention (CDC).

white women of the same age. Among women 60 years and older, 61% of non-Hispanic black women were obese compared with 32% of non-Hispanic white women and 37% of Mexican-American women. This ethnicity difference was not observed in men (Gordon-Larsen *et al.*, 1997; Ogden *et al.*, 2007).

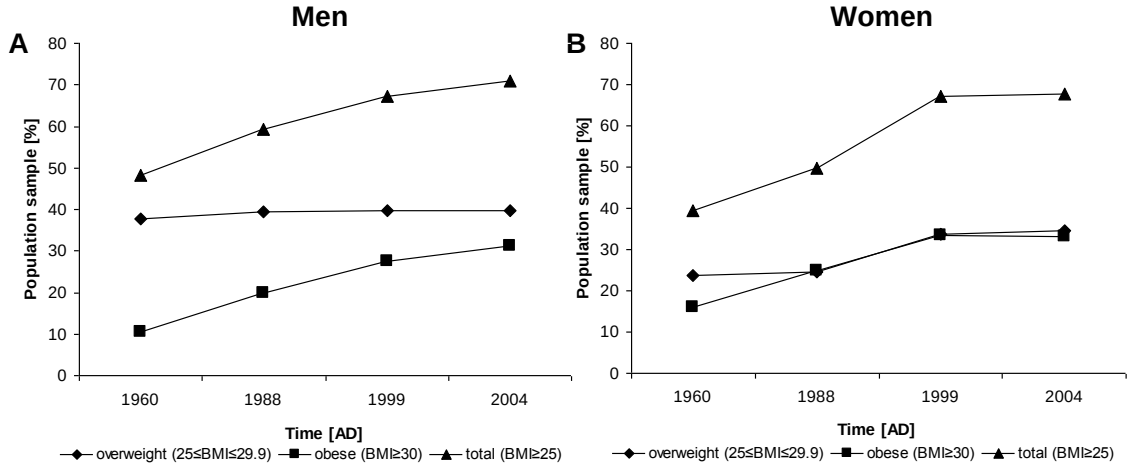

Figure 1-1: Overweight and Obesity prevalence in the NHANES study cohorts in the USA in A) men and B) women between 1960 and 2004 (data used for graphs were from Flegal *et al.*, 1998; Ogden *et al.*, 2006). All BMI-units are in [kg/m^2].

Similar increases have been noted in the UK, where the percentage of obese individuals rose from 7% in 1980 to 20% in 2000 (WHO, 2000); likewise the overweight category (25kg/m^2≤BMI≤29.9kg/m^2) has increased from 20% of the population in 1980 to 45% in 2000 (WHO, 2000), indicating that the majority of UK adults are also either overweight or obese. The Foresight Report found that in 2004 nearly a quarter of men (23.6%) and women (23.8%) in England were obese (Butland *et al*., 2007). The authors also suggested that by 2010 the obesity rate will increase to 33% of men and 28% of women (Butland *et al*., 2007).

Recent data from an American population showed that the increase of the obese and overweight population has slowed (Ogden *et al.*, 2007). There has not been a statistical increase in the number of obese men and women between 2003-2004 and 2005-2006. Among men, the prevalence was 31.1% in 2003–2004 and 33.3% in 2005–2006. Among women, the prevalence in 2003–2004 was 33.2% and in 2005–2006 it was 35.3%. The annual estimates for both men and women were not statistically different from each other (Ogden *et al.*, 2007). Despite the non-significance in these results total numbers of obese and overweight individuals still increased. Thus the authors were reluctant to conclude a definite stop in the increase of obesity rates in the United States has occurred and invited further research to be conducted on the validity of their findings. Even as obesity rates level out, western civilisations are exposed to around 1/3 of their population being affected by the obesity disease for the foreseeable future.

1.1.3 Causes of Obesity

1.1.3.1 Energy balance

Body weight and composition is in balance if energy intake equals energy expenditure. Energy intake may be defined as any form of food intake, while the metabolic rate and exercise are energy expenditure features. The first law of thermodynamics defines the principle of energy conservation, *i.e.* energy cannot be created or destroyed. The consequence of this principle is that, if any energy equation is not in balance, an additional variable is needed to comply with this principle. This additional variable is energy storage. In mammals energy can be stored by tissue growth, both lean and fat tissue. Storage of energy can thus balance any imbalance between energy intake and

energy expenditure. Thus energy balance in mammals may be represented in this equation:

Energy intake = Energy expenditure + Energy storage.

If energy intake equals energy expenditure the equation is in balance and no input from energy storage is necessary. Energy intake and energy expenditure can actively be changed by what is eaten or drunk or by changed activity levels, respectively. In response, energy balance changes passively to balance the equation. Everyone (*i.e.* athletes, body builders, the obese) trying to alter their energy storage (*i.e.* their lean and fat body mass), changes their intake and expenditure with the effect that their energy storage changes in order to balance the equation.

The consideration of an imbalance in the energy balance equation may be viewed as the physiological cause of obesity. If an individual has a higher energy intake than an energy output, the excess energy is stored, mostly in form of adipose tissue. Much of the modern and westernised lifestyle facilitates this energy imbalance, *e.g.* the continuous availability of cheap energy dense food and the wide-spread use of mechanical devices in transport and in the household. The molecular causes for energy imbalance however are not as easily accessible. In the following the current understanding of energy balance regulation and findings in this field are introduced.

1.1.3.2 Regulation of energy balance

Small changes in energy balance over a long period of time could have detrimental effects on the above energy balance equation (Weigle, 1994). For example, if, over a period of 25 years, an individual's intake would exceed its daily energy expenditure by

only 200 kJ (the energy conserved in an average apple), the resulting imbalance would accumulate to 1825 MJ (25 x 365 x 200 kJ). Assuming the total imbalance would all be stored as fat and since 1 kg fat tissue contains about 33.1 MJ energy (Forbes, 1987), the accumulation of 1825 MJ would result in about 55 kg of fat tissue (Weigle, 1994). The situation that an individual weighing 70 kg at age 20 should increase their weight to 125 kg at age 45 by only eating a single apple a day more than required for energy balance is extremely unusual. In fact, energy balance seems to be tightly matched to energy expenditure with only a mean compensatory change of 0.2% in energy storage over 25 years (Friedman, 2000; Weigle, 1994). This extremely tight match between energy intake and expenditure led to the assumption that energy balance in mammals must be a regulated mechanism, *i.e.* excessive energy intake is not sustained over long periods of time, but counteracted by decreasing appetite and increasing energy expenditure. Indeed as early as the 1950's Kennedy (1953) observed that young rats are able to regulate their food intake precisely to the challenges put to them. Food intake increased in response to exposure to cold and during lactation, leaving their fat depots virtually unaltered. Alternatively, body weight did not increase significantly with unrestricted access to food. Interestingly body weight started to increase and obesity developed only in response to hypothalamic injuries. The author suggested the possibility that the hypothalamus is able to recognise and to react to fat tissue derived products to achieve "lipostasis". During "lipostasis", energy balance is accomplished solely by altering energy intake and expenditure, without the need to deposit or remove from energy storage. This "lipostatic" model of energy regulation proposes that energy storage (adipose tissue mass only) is monitored by the central nervous system, which in

turn adapts energy intake and expenditure to keep adipose tissue mass constant (Kennedy, 1953).

Shortly after this hypothesis was formulated, a mouse that exhibits hyperphagia and grows enormously obese, without hypothalamic injury, appeared spontaneously in The Jackson Laboratories in the US (Coleman, 1978). The effect was attributed to a homozygous deletion in what was termed the "obese"-gene (ob) and the mice strain became known as the *ob/ob* mouse, as it has lost both alleles of the ob-gene. Subsequently leptin (λεπτοσ (leptos) = thin) was found as the product of the ob-gene (Zhang *et al.*, 1994) and suggested to be the adipose derived factor, hypothesised by Kennedy (1953). The finding that an adipose derived factor plays a role in energy balance regulation is further supported by the idea that energy balance is achieved by a lipostatic model[2]. The quantitative signal from fat tissue, *i.e.* amount of secreted leptin, is compared to a genetically determined "set-point" of body fatness and energy balance is adapted accordingly[3].

While this model explains the consistency of body weight over long periods of time, it lacks the ability to explain why we do not continuously feel hungry, when our leptin

[2] In addition to the lipostatic model of energy balance regulation other models have been proposed. The "glucostatic" model suggests nutrient content in the blood is the stimulus for energy intake and expenditure (Mayer, 1955). A model in which the content of lean body mass is also taken into consideration, a "proteostatic" model, has also been proposed (Mellinkoff *et al.*, 1956). Uncertainty exists about whether different body tissues have different regulatory system, or whether a single regulatory system exists, which compartmentalises distribution of resources according to a set-target (Speakman *et al.*, 2002). For comprehensiveness only the lipostatic model will be considered further.

[3] See Schwartz and Seeley (1997), Friedman and Halaas (1998) and Auwerx and Staels (1998) for comprehensive overviews of leptin action and its feedback to the central nervous system and its support of a lipostatic model for energy balance regulation in humans.

concentrations are low, but still have an eating habit of discrete meals. Evidently a second short acting regulation mechanism of appetite must be in place (Gibbs *et al.*, 1973; Mayer, 1955). Several factors have been linked to play a role in feeding behaviour, including insulin (Pliquett *et al.*, 2006), ghrelin (Horvath *et al.*, 2001; Klok *et al.*, 2007; Kojima *et al.*, 1999) and glucose (Levin, 2002). Short-term appetite and satiety seem to be regulated by gastric distension and changes in the excitability of the *Vagus Afferents* nerve (Schwartz, 2000). In addition the gastric peptide ghrelin (Kojima and Kangawa, 2005) and the intestinal peptide YY_{3-36} (Batterham *et al.*, 2002) have been found to play a role in short-term feeding behaviour. Thus the familiar feelings of hunger and satiety seem to underlie a different regulatory system than the long-term regulation of energy balance of the lipostatic model based on adipose-derived leptin secretion (Speakman *et al.*, 2002). As obesity is a slowly developing disease, it may be that the lipostat and leptin play a more important role than additional regulatory mechanisms.

1.1.3.3 The leptin signalling mechanism

The leptin signal is a quantitative signal, *i.e.* the amount of fat tissue determines the amount of leptin secreted. Indeed serum leptin concentrations correlate with BMI (Considine *et al.*, 1996). Similarly to leptin, whose gene knockout produced the *ob/ob* mouse, the leptin-receptor knockout produced an obese, diabetic mouse, the *db/db* mouse (Coleman, 1978). The product of the *db* gene was the leptin receptor, whose human analogue has since been found (Tartaglia *et al.*, 1995). The leptin receptor (Ob-R) is a class-I cytokine receptor, which exists in several splice variants including a short (Ob-Ra) and a long (Ob-Rb) form of the receptor (Tartaglia, 1997). Of all splice

variants, only the long form (Ob-Rb) contains the intra-cellular signalling domain. As Kennedy (1953) demonstrated in his early rat experiments, obesity develops after injuries to the hypothalamus and indeed in hamsters expression of the long form of the leptin receptor has predominantly been located to cells of the arcuate nucleus (ARC) within the hypothalamus (Mercer *et al.*, 1996a; Mercer *et al.*, 1996b). Further studies identified two types of neurons expressing Ob-Rb within the ARC, one are neuropeptide Y/Agouti-related protein (NPY/AgRP) co-expressing neurons (Hahn *et al.*, 1998), the second type of neurons are pro-opiomelanocortin/cocaine amphetamine–regulated transcript (POMC/CART) neurons (Figure 1-2). NPY/AgRP neurons project signals to the brain stem into the paraventricular nucleus (PVN) and to POMC/CART neurons.

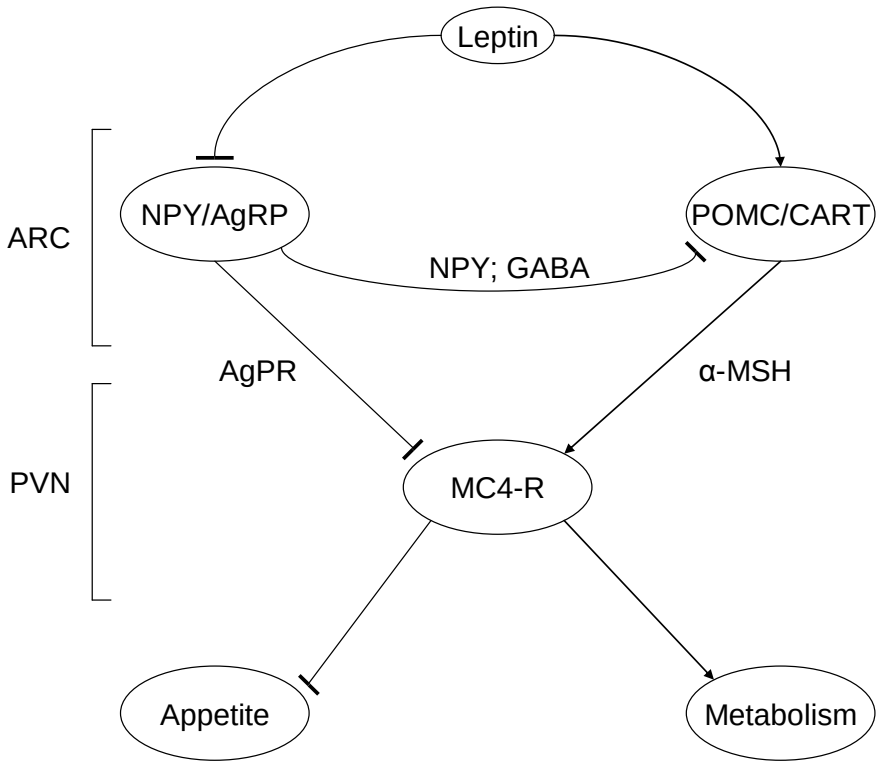

Figure 1-2: Simplified schematic overview of leptin signalling in hamsters. AgRP: Agouti-related protein; α-MSH: α-melanocyte-stimulating hormone; ARC: arcuate nucleus; CART: cocaine amphetamine-regulated transcript; GABA: γ-aminobutric acid; MC4-R: melanocortin-4 receptor; NPY: neuropeptide Y; POMC: pro-opiomelanocortin; PVN: paraventricular nucleus

Both NPY/AgRP neurons and POMC/CART neurons project to cells in the PVN expressing melanocortin-4 receptors (MC4-R) and to cells in the ventromedial nucleus expressing melanocortin-3 receptors (MC3-R). NPY/AgRP neurons secrete AgRP which is an antagonist to both MC4-R and MC3-R, whereas POMC/CART neurons secrete α-melanocyte-stimulating hormone (α-MSH), an agonist of both these receptors (Zemel and Shi, 2000). Leptin activates the signalling cascade by docking to its receptor on NPY/AgRP neurons. This reduces the secretion of NPY and γ-aminobutric acid (GABA) at the synaptic connection with POMC/CART neurons and additionally decreases the release of AgRP at the synaptic connection with MC4-R expressing neurons in the PVN. POMC/CART neurons on the other hand are stimulated by leptin to increase secretion of α-MSH and additionally POMC/CART inhibition by GABA is decreased (Yokosuka *et al.*, 1998). Stimulation of MC4-R, by increased α-MSH and decreased AgRP, promotes two downstream events. First the sympathetic system is stimulated resulting in increased secretion of noradrenalin and second food intake is inhibited. If the leptin stimulus decreases GABA and NPY concentrations increase at the POMC/CART cells, which results in decreased α-MSH secretion, which in turn, in association with increased AgRP secretion at MC4-R positive cells of the PVN, decrease sympathetic activity and stimulate feeding behaviour (Figure 1-2).

Some of the factors potentially involved in short-term energy balance regulation have also been shown to interact with the ARC. Insulin has been shown to suppress NPY secretion with the effect of inducing POMC/CART neurons' activity (Schwartz *et al.*, 1992). Similarly receptor for gut-derived peptides ghrelin (Horvath *et al.*, 2001) and peptide Y_{3-36} (Batterham *et al.*, 2003) were detected in the ARC. POMC/CART neurons

also receive signals from serotonin expressing neurons and carry serotonin-receptors. These may act as leptin independent short-term signals indicating satiety (Halford and Blundell, 2000).

1.1.3.4 Energy balance regulation impairments

Combining the above information, it may be concluded that energy balance and thus body weight is a tightly regulated mechanism. With an energy balance mechanism, *i.e.* a lipostat, in place, it seems unlikely that individuals should be susceptible to increased accumulation of body fat as observed during obesity development. It has therefore been suggested that impairments within the lipostatic model[4] may occur during the development of obesity. As mentioned the *ob/ob* mouse cannot produce any leptin, while the *db/db* mouse carries a defective leptin-receptor (Ob-Rb). Both mice are susceptible to an accumulation to body fat, because of a defect in the lipostat. Broken lipostats, as in these mice, were also identified in humans. Two massively obese siblings (the older weighing in at 110 kg at age 10) presented at a Cambridge hospital and showed no detectable leptin concentrations. In fact both carried a homozygous defect in their leptin alleles, eliminating the production of any leptin, essentially presenting with the identical feature as *ob/ob* mice (Montague *et al.*, 1997). Regular

[4] A critical review of the lipostatic model, addressing its weaknesses and an alternative non-lipostatic model using elegant computer simulation models is presented by Speakman and colleagues (2002). Since an increase in body weight to the point of morbid obesity seems unlikely with such a tight mechanism as the lipostat in place, with mutations in lipostasis mediating genes virtually non-existent and with leptin concentrations not impaired in the obese, the idea of a lipostat faces certain scrutiny. Most of the existing data from mice and rats studies, however, support the lipostatic model of energy balance regulation. A further evaluation of the validity of the lipostatic model in obesity is beyond this thesis as its existence or non-existence is irrelevant for the thesis' aims (see section 1.7). For the further contemplation, it will be assumed that a lipostatic model of body weight regulation in mice, rats, hamsters and humans is in place.

leptin treatment decreased their appetite dramatically and reduced their body weight over time (Farooqi *et al.*, 1999).

Single site mutations in the leptin gene or in genes associated with cerebral leptin signalling are rare and only a handful of families have been identified (Clement *et al.*, 1998; Montague *et al.*, 1997). Estimates however suggest that indeed up to 90% of the variability in body weight in individuals sharing identical environments have a genetic background (Barsh *et al.*, 2000; Maes *et al.*, 1997). Thus, if the lipostat is not entirely broken, it may be argued that the set-point, to which the actual fat mass is compared, is set at an inappropriately high level. The lipostatic model keeps energy in balance by adjusting energy intake and energy output, without the use of the energy storage to balance the equation. It is thought that the level at which to keep the energy storage, *i.e.* body fatness is genetically determined and natural selection would keep the genes that hold energy storage at levels that do not predispose to starvation on the one hand and do not increase susceptibility to predation with increased energy stores on the other[5]. One can now see that, if the genetically determined set-point for the lipostat is high, obesity develops. Energy balance is altered in such a way that this inappropriately high set-point is achieved.

[5] Modelling such scenarios for humans is difficult. However Brodin (2007) described several models, which "the little bird in winter " has to adapt in order to survive Scandinavian winters including reaching optimum body fatness to minimise risk of starvation and risk of predation. Risk of predation is thought to increase with body weight due to a growing inability to escape quickly enough. Body weight needs to be tightly controlled between these two markers to keep the risk of starvation and predation as low as possible (Brodin, 2007). This idea shall play a role in the hypotheses discussed in section 1.1.3.7.

Alternatively it may be suggested that the leptin signalling mechanism is somehow aberrant in obese individuals. Completely broken leptin signals are rare, such as genetic mutations in the leptin gene or the leptin receptor gene. If leptin production however is not completely broken but decreased, one would still expect an increase in body weight. Likewise, if the signal cannot be clearly identified in the hypothalamus, a similar situation is conceivable. Unfortunately, leptin concentrations correlate extremely well with body weight (Considine *et al.*, 1996) contradicting the former idea. Instead the idea of leptin resistance, either by impairment of leptin crossing the blood-brain barrier or exerting a sufficient intra-neuronal signal, is a better supported hypothesis. Leptin signalling can be inhibited by activation of suppressor of cytokine signalling (SOCS)-3 (Bjørbæk *et al.*, 1999). In a negative feedback loop leptin itself is an inducer of SOCS3 mRNA expression (Bjørbæk *et al.*, 1998). Additionally the obesity-associated increase in inflammatory cytokine secretion (see section 1.1.4.4) may play a role, as for example IL-6 increases SOCS-3 expression (Cirillo *et al.*, 2008). Leptin resistance however may not play a role in the initial phases of obesity development, as it seems to occur in response to obesity induced molecular changes. Thus the idea of the inappropriately lipostatic set-point may be the initial phase, while leptin resistance eventually potentiates its effect on body fatness, possibly increasing body weight beyond the genetically determined lipostatic setpoint.

1.1.3.5 The obesogenic environment

If the lipostatic set-point is genetically fixed, it has to obey the rules of Mendelian inheritance and natural selection. Obesity-rates in the population however only increased significantly since the early 1980s. Most people that were alive in 1985 are

still alive today, so the argument that the genetically determined lipostatic set-point has changed to inappropriate high levels, within this timeframe, is not supported by this hypothesis as there is not enough time, in evolutionary terms, to change the genetic composition of the entire population. As mentioned, the genetic composition may account for up to 90% of the obesity in the population (Barsh *et al.*, 2000; Maes *et al.*, 1997). Thus, since the genetic composition of the population cannot have changed in this short period of time, it has to be a change in environment that phenotypically manifests an already underlying genotypic predisposition to obesity, *i.e.* past events have created a population in which some individuals should become obese, but environmental constraints were not favourable to such an outcome until about 1980[6]. The environment of western societies has changed dramatically in the last thirty years, coinciding with the timeframe and the location of increased obesity rates. These changes induced a decrease in energy expenditure for everyday living and decreased restriction on the availability of nutrition. In industrialised societies labour-saving devices such as cars, elevators, escalators and remote controls have decreased day-to-day energy expenditure. Increased agricultural mechanisation and technology have increased and stabilised food supply. Food technology and mass food production have increased energy density, palatability and storability of food (Binkley *et al.*, 2000; Putnam and Allshouse, 1999). These changes have greatly increased individual survival

[6] The general population and some researchers (Chisholm *et al.*, 1998; Ogden and Flanagan, 2008; Poston and Foreyt, 1999) tend to misunderstand the influence of genetics on body weight. There is no direct link between our genes and body weight. It is the individual's genetically fixed lipostat that predisposes to obesity and keeps an imbalance on energy regulation. Thus the individual genetic composition has influences on energy expenditure and stimulation of energy intake, which in turn causes body weight to change and not on body fatness itself. A statement such as "I am obese, because of my genes", is therefore not valid. Furthermore the idea that obesity cannot be caused by genetic disposition but has solely an environmental cause (Poston and Foreyt, 1999) is not valid and violates the delicate gene-environment interaction that is needed for obesity to develop. The saying that "genes load the gun, but the environment pulls the trigger" has never been more valid than in the case of obesity.

and health in addition to political and economic stability in industrial nations. These changes have decreased however any environmental restrictions that have kept genetically predisposed individuals from reaching their inappropriately high lipostatic set-point.

To illustrate this effect of the environment on obesity, Eaton and colleagues (1996) compared the lipostat to a domestic thermostat and the heat produced by the radiators to body weight. If several houses with different settings on their thermostats are placed far north in the Arctic Circle, the houses will always be cool as no matter how high the thermostat is set, the heat in the house will never reach above a certain level. Now placing the same houses in tropical regions, one expects to see a very different picture. The houses with a thermostat set at the correct level will keep their temperature within normal levels, while those houses with an inappropriately high set thermostat will get boiling hot. Our environment has now changed from a Palaeolithic hunter/gatherer (arctic climate) environment 10.000 years ago to an agricultural settled sedentary (tropic climate) environment[7] in which individuals (houses) with inappropriately set lipostats (thermostats) become increasingly overweight and obese (boiling hot). This illustration is a good example of how an obesogenic environment common to industrialised nations has an effect on individuals differently pre-disposed to obesity (Eaton *et al.*, 1996).

[7] Such an environment was achieved at different points in time for different populations. Palaeolithic figurines, such as the Venus of Willendorf, suggest that obesity was known even in the hunter-gatherer area (30.000 years ago). The aristocrats of the past millennia were also characterised as displaying obesity. The major change in the environment however, the increased automation and motorisation of the 1980s, changed the lifestyle of the majority of the population to increase sedentary.

The effect of the changing environment can also be seen by examining populations which are only partly exposed to the obesogenic environment of the industrialised, western societies. The average BMI of Japanese men living in the continental United States is significantly higher than of those living in Japan across all age groups (Curb and Marcus, 1991). A well studied group of Pima Indian women have a significantly increased mean BMI, if they live in Arizona (mean BMI = 35.5) compared to women living in rural Mexico (mean BMI = 25.1; Ravussin *et al.*, 1994).

1.1.3.6 Pre-disposing obesity genes

Thus it is the genetic variation between individuals that predisposes to the development of obesity. Genome-wide association studies to correlate genetic variation with BMI have recently produced the first successes in identifying these genetic variations. Genome-wide association studies examine large amounts of individuals and associate gene variants with, in this case, BMI. A common variant in the fat mass and obesity associated (FTO) gene was associated with a 3 kg increase in body weight and a 1.67-fold increased odds risk of obesity for homozygous carriers in humans (Frayling *et al.*, 2007). The common variant of FTO was originally strongly associated with type-2 diabetes, but this association was abolished when adjustments for BMI were taken into account, leading to the conclusion that the association with diabetes was mediated through BMI changes. Human FTO gene function is unknown. It was identified as the human analogue to mutated genes in the fused-toe (FT) mouse phenotype, which carries a deletion of part of chromosome eight, missing genes *ftm*, *ftn* and *fto* in addition to other genes (Peters *et al.*, 2002). Homozygous deletions of this kind are embryonically lethal in mice and the large number of genes deleted in this mouse model makes it a bad

candidate for studying FTO action. Overexpression or single FTO knock-out in mice have not been reported (Frayling *et al.*, 2007).

An additional study identified a region on the human chromosome 18, 188 kb downstream of the melanocortin-4 receptor (MC4-R) gene, associated with obesity (Loos *et al.*, 2008). The importance of functioning MC4-R in normal leptin signalling became apparent when the screening of severe, early onset obese individuals revealed miss-sense (Farooqi *et al.*, 2000; Gu *et al.*, 1999) non-sense (Hinney *et al.*, 1999) and frameshift (Vaisse *et al.*, 1998; Yeo *et al.*, 1998) mutations in the MC4R gene. In fact common variant and mutation in the MC4-R gene may account for 3-5% of morbid obesity, making it the most common single gene defect associated with obesity (Vaisse *et al.*, 1998).

The most recent genome-wide study in humans found three loci that were associated with increased waist circumference and increase waist-to-hip ratio (Lindgren *et al.*, 2009). Two loci (*TFAP2B* and *MSRA*) were associated with waist circumference, and the third locus, near *LYPLAL1*, showed a relationship with waist-to-hip ratio in women, but not in men (Lindgren *et al.*, 2009).

Homozygous carriers of the FTO risk allele were on average 3 kg heavier individuals carrying the non-risk allele (Frayling *et al.*, 2007). In all these genome-wide association studies, the influence of FTO on obesity was the strongest, with all further discovered genes having less of an influence on body weight. The influence of these genes is rather

small, compared to deletion of the leptin gene or the leptin receptor gene in mice and human. Considering the genetic influence on a lipostatic set-point, findings from a small rodent study may be of interest, instead. In this study hamsters were subject to different light periods during a normal 24 h day, simulating long-day or short-day periods (Mercer and Speakman, 2001). A hamster transferred from long-days to short-days started to lose weight until a new constant body weight is achieved. This weight loss occurred independently of food restriction, *i.e.* a hamster transferred to short-day periods will lose weight, even if food intake is unrestricted. On the other hand, when the hamster was transferred back to long-day periods, it will increase its body weight to the same levels as before the first transfer[8].

These findings indicate a transition in the lipostatic "set-point", allowing these hamsters to adapt their body fatness to environmental changes. Gene mutations and polymorphisms cannot explain a shifting lipostatic set-point in the same hamster. Gene expression changes in the ARC during short-day weight loss in these hamsters on the other hand, could explain this phenomenon. Hence it might be that the lipostatic "set-point" is dependent of expression of yet unknown genes. The study, indicating that environmental changes were able to shift the lipostatic "set-point" in a hamster (Mercer and Speakman, 2001), could provide a new aspect of obesity research, yet to be explored.

[8] Mercer and Speakman, 2001, p.107 Fig. 3: The "sliding set-point" of body weight regulation.

Conclusively, gene polymorphisms that affect the lipostatic "set-point" have been demonstrated. Furthermore studies in hamsters indicate that expression of genes involved in response to environmental changes can lead to a shift in the lipostatic "set-point". As the argument is continued it may be asked how these inappropriately lipostatic "set-points" have developed and are prevailing in the human population. An attempt to answer this question has been made by the hypotheses of a "thrifty"- or "drifty" genotype.

1.1.3.7 Thrifty vs. Drifty genotype

Over the years two ideas of inappropriately high lipostatic set-points have been proposed. One, the thrifty genotype hypothesis, suggests that genes that are responsible for increasing the lipostatic "set-point" were advantageous at some point in the human evolutionary past (Neel, 1962). This is based on the suggestion that the human historic environment was punctuated by periods of famine (Chakravarthy and Booth, 2004; Prentice, 2001). During periods in between famines, those individuals with the thrifty genotype of high lipostatic "set-points" were able to accumulate more body fat than those without and would therefore be more likely to survive a succeeding famine. Thus these individuals had an evolutionary advantage and natural selection would tend to favour these individuals, increasing their proportion within the population. While this genetic make-up may have been beneficial in the past, protecting from starvation, in the modern, western society, where this periodic feast-famine pattern does not exist, this "thrifty" genotype now promotes continuous accumulation of fat.

The second hypothesis is called the "predation release" or "drifty genotype" hypothesis (Speakman, 2007; Speakman, 2008). Its concept is based on the suggestion that the acquisition of social behaviour, the harnessing of fire and the developing of weaponry in human evolutionary history has dramatically decreased the risk to fall victim to predation by large carnivores. This development has decreased the selective pressure to the upper limit of the body fatness (see also footnote 5). Thus without natural selection, the genetically determined lipostatic "set-point" was allowed to drift upwards in the population. In Figure 1-3 the selective pressure of starvation and predation is illustrated. With the release from predation risk, only the selective pressure from starvation remains[9], allowing body weight to drift upward.

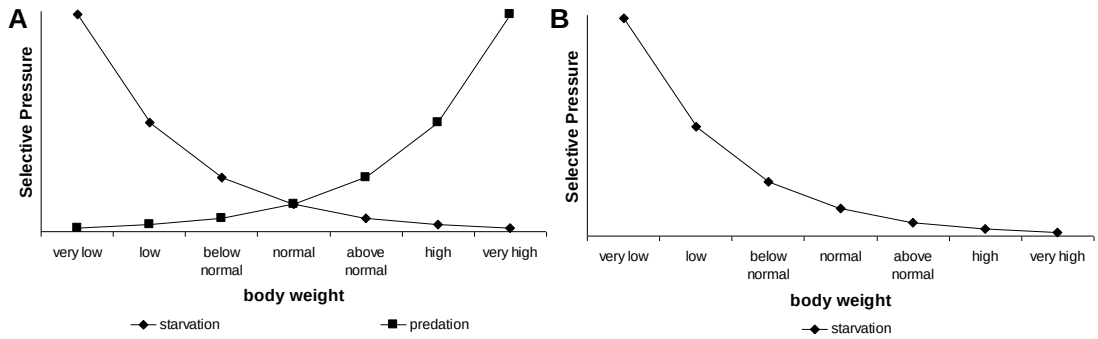

Figure 1-3: Illustration of selective pressure of starvation and predation on body weight regulation in A) proposed original setting and B) after predation-release as proposed by the drifty genotype hypothesis[10].

[9] Speakman's original hypothesis does not seem to suggest that there is a continuous selective pressure from starvation, thus the genetic drift to higher body weight is at random and not under selective pressure. Instead he suggests a "lower intervention level [being] set by the risk of starvation" (Speakman, 2008, p.1615), equalling to a cut-off point for selective pressure above which the selective pressure for starvation is negligible. In this case the exponential relationship between body weight and starvation risk assumed in Figure 1-3 is wrong and would have to be represented by a steep linear increase below Speakman's cut-off point.

[10] Graph was drawn for illustrative purposes only, assuming the following: Selective pressure of starvation and predation were assumed to be equal. Selective pressure of both factors was assumed to increase or decrease exponentially with body weight changes. The lowest combined risk, i.e. the point were the curves cut, was assumed to be at normal body weight (BMI~20).

Both hypotheses have come under scrutiny from supporters of the other side. The thrifty-genotype hypothesis has been criticised to overestimate the rate of mortality during famine. Most deaths during famine do not occur due to starvation, but from infectious diseases such as diarrhoea and cholera (Tauxe *et al.*, 1988). Furthermore deaths during famine occur primarily in the very young and the very old. Deaths in children under the age of 10 could not have been biased towards obesity as childhood obesity is a condition only found in the very recent past. Likewise the elderly will most likely already have procreated, thus their genotype, whether "thrifty" or not has been passed on. Additionally the rate at which famines occurred (about once every 150 years) reduced the selective force of starvation to every 5^{th}-7^{th} generation, giving ample time for the non-"thrifty" genotype to "catch-up" under normal conditions in which the thrifty genotype is not advantageous.

The main criticism against the drifty genotype is that it does not take into account the influence of varying fat depots on female fertility during times of food shortage. In rural agricultural populations, like The Gambia and Bangladesh, body weight oscillates during the season, being lowest just before harvest (Prentice, 2005). Simultaneously birth rates oscillate in the same pattern, with a 9-months shift, reflecting a 30-50% reduction in fertility (Prentice *et al.*, 2008). Thus it is claimed that a regular seasonal shift in fertility together with the occasional severe famine, during which fertility is virtually absent, would create sufficient positive selective pressure to favour and thereby support the idea of the "thrifty" genotype. Prentice and colleagues (2008) however fail to mention, if heavier women are more resilient to the seasonal reduction in fertility and during severe famine. This would be necessary to validate their critique. Furthermore

the occurrence of polycystic ovarian syndrome (Pasquali *et al.*, 1997) and reduced female (Jokela *et al.*, 2008) and male fertility (Jarow *et al.*, 1993) being closely related to obesity is another point that should have been addressed.

Thus both theories provide an interesting and comprehensible explanation for the causes of high lipostatic "set-point" without being totally convincing or being supported by all the available data. Both theories are indeed supported by historical facts. Famines have been an important characteristic of the human past and provide sufficient natural selection for individuals that are better equipped to survive, it merely being to decrease susceptibility to disease or being able to continue producing offspring, which is the basis for the thrifty-genotype hypothesis. Likewise there is hardly an argument to be made that modern humans have managed to escape large-scale predation and the idea that this reduced the selective pressure to keep body fatness low is conceivable, supporting the conception of a drifty-genotype hypothesis. Despite both sides claiming that their theories are mutually exclusive, mainly stating that the same genes cannot be under selective pressure (thrifty hypothesis) and drifting randomly (drifty hypothesis; Speakman, 2008), the polygenic nature of the obesity disease may allow indeed for both to have happened in the past.

An interesting recent idea (Bhattacharya, personal communication) is that the increased susceptibility to disease with increased obesity may act as the "new" predation selective pressure to keep obesity low in the population (Figure 1-3). Obese individuals have reduced life expectancy (Allison *et al.*, 1999), reduced fertility (Jokela *et al.*, 2008) and

higher susceptibility to debilitating disease (Kopelman, 2000) and therefore may die before, be unable to or too ill to produce offspring, thereby reducing the gene pool of obesity predisposing genes, creating a new equilibrium.

1.1.4 Changes in adipose tissue as obesity develops

While the exact causal effect of the development and the distribution and selectiveness of obesity remains elusive for the moment, the insight into the changes during adipose tissue formation are increasing. Considering the case of obesity development, the energy balance equation (section 1.1.3.1) between energy intake and energy expenditure is not equal and needs to be balanced by energy storage. This energy is stored by increasing lean and fat body mass. The distribution value between increasing lean and fat body mass is determined by genetic factors, exercise and overall body fitness (Payne and Dugdale, 1977). Since obesity is a disease characterised by excessive accumulation of body fat, only the expansion of fat tissue will be regarded further. In the following sections the changes in the expansive adipose tissue are described with particular focus on the changes in white adipose tissue (WAT[11]) secretion profile.

1.1.4.1 Fat distribution

WAT tissue is found in distinct depots in mammals. Subcutaneous fat depots are the most characteristic fat depots as they are responsible for body shape. In humans, they are responsible for the distinct shapes of male and female. Additional fat tissues are

[11] All further considerations reflect the changes in white adipose tissue. It shall be noted that a second type of adipose tissue exists in mammals, the brown adipose tissue (BAT). Its cells are characterised by containing multiple lipid droplets and an increased amount of mitochondria. BAT is of particular interest in thermogenesis in rodents. In humans BAT is only present in newborns, regulating thermogenic processes (Gesta *et al.*, 2007).

found in the abdomen as visceral fat (mesenteric and omental) and in the retroperitoneum. Visceral fat protects intra-abdominal organs from physical forces and holds organs in their distinct places within the abdomen. The place at which fat is deposited as obesity develops is highly variable between individuals. Women however tend to store fat preferably around the buttocks and thighs, while men preferably store fat around the waist. Total body fat is distributed as ~85% subcutaneous fat and ~15% visceral fat (Abate *et al.*, 1997). The rate, at which the two distinct fat tissues (subcutaneous and visceral) expand during obesity development, is however highly variable[12] (Tchernof, 2007). Furthermore, many obesity-related adverse health effects are more closely related to visceral fat-tissue growth rather than overall fat growth. The effect of the molecular changes within fat tissue during obesity development seem to be more pronounced in visceral fat than in subcutaneous fat, possibly due to the proximity of mesenteric fat to the liver, *i.e.* normal metabolic mechanisms in the liver are impaired by mesenteric fat tissue (Klein *et al.*, 2007).

[12] The phenomenon of lipodystrophy shall be mentioned here. This is a condition in which distribution of fat does not represent the 85%:15% fat distribution between subcutaneous and visceral stores. In its severest congenital form an individual suffering from this disease cannot form subcutaneous adipocytes and their subcutaneous fat is virtually empty. However ectopic fat content is extremely high combined with all the increased predisposition to adverse health effect related to obesity and increased visceral fat. Several gene defects have been associated with lipodystrophy. According to the OMIM database (2009) "congenital lipodystrophy" may be caused by a single gene mutation in one of three genes (AGPAT2, BSCL-3, CAV1), leading to congenital generalized lipodystrophy type 1, 2 or 3, respectively. Its inheritance is autosomal recessive. Other forms of lipodystrophy are known.

1.1.4.2 Adipocyte hypertrophy and ectopic fat

Fat tissue, rather than being a mere storage organ, has emerged as a metabolically active, highly endocrine, diverse organ. It contains several different cell types, generally divided into adipocytes and stromal cells, which also contain cells associated with the immune system. Adipocytes are cells that store fat in form of a single (see footnote 11) lipid droplet in the cytoplasm, containing triglycerides and fatty acids (FA). During obesity development this droplet increases in size to the extent that the lipid droplet is the dominant feature within the adipocyte, leading to adipocyte hypertrophy, an expansion of the adipocyte to the limit of its storage capacity. Adipocytes mature from mesenchymal stem cells to pre-adipocytes, to mature adipocytes. Thus, if the lipid storage capacity of existing adipocytes reaches its limit, further storage capacity should be achieved through maturing additional adipocytes.

In adulthood however, the ability to mature new adipocytes from pre-adipocytes declines, leaving only the existing adipocytes to accommodate more fat (Karagiannides *et al.*, 2001). Maturing pre-adipocytes into adipocytes still exists to some degree in adults and it is suggested that the inability to recruit and mature adipocytes sufficiently is a major contributor to an individual's susceptibility to obesity-related metabolic diseases (Dubois *et al.*, 2006). Thus the expansion in fat tissue is caused by an expansion of adipocytes (hypertrophy) rather than an increase in the number of adipocytes (hyperplasia) to accommodate more fat. In fact it has been suggested that the number of mature adipocytes is set during childhood and adolescence and remains constant during adulthood (Spalding *et al.*, 2008). The rate at which new adipocytes can

mature from pre-adipocytes is therefore only able to uphold the natural turnover rate of adipocytes.

While hypertrophy of adipocytes creates a number of additional consequences for the adipose tissue itself (see following sections), there is an obvious need to examine the possibility of what happens, if an individual simply "runs out of space" to store additional triglycerides. Hypertrophic adipocytes cannot absorb any more triglycerides and, if there is insufficient capacity to increase the number of adipocytes, the excessive triglycerides are stored in non-adipose tissue, especially liver, muscle[13] and heart tissue as well as in pancreatic islets cells.

These atypical fat stores have been termed "ectopic" fat and are thought to exert a lipotoxic effect on the affected tissue, leading to cell death (van Herpen and Schrauwen-Hinderling, 2008). This "ectopic" fat is very likely part of the associated health effect of obesity, including steatohepatitis of the liver, insulin resistance in muscle, cardiomyopathy and type 2 diabetes. While "ectopic" fat may play a major role in promoting several of the obesity related adverse health effects, it is the effects within the white fat tissue that will attract further attention for this project.

[13] It should be noted that after exercise and in athletes, lipid content in muscle is also elevated, presumably to promote glycogen regeneration. However the lipid profile in muscle after exercise is different from insulin resistant muscle, suggesting that these two phenomena are not related, explaining why insulin-resistant "ectopic" muscle fat is detrimental (Kelley and Mandarino, 2000; Schrauwen-Hinderling *et al.*, 2003).

1.1.4.3 Hypoxia

Hypertrophic adipocytes can reach a size of up to 150-200 μm (Skurk *et al.*, 2007), thus lying within the maximal diffusion range for oxygen of 100-200 μm (Brahimi-Horn and Pouyssegur, 2007) and suggesting that adipocytes, that are not situated directly next to arterial capillaries, will not receive sufficient oxygen. Indeed in fast growing tumours the partial pressure of oxygen (pO_2) can drop to zero within only 70 μm from the blood vessel (Vaupel, 2004). These observations led to the suggestion that adipocyte hypertrophy, as a result of obesity development, could lead to localised hypoxic areas in WAT (Trayhurn and Wood, 2004). Hypoxia is defined as a deficiency of oxygen in tissues. Partial oxygen pressure (pO_2) progressively declines from air at sea level (160 mmHg), to the lungs (150 mmHg), to the arterial saturation (104 mmHg), to the venous saturation (40 mmHg) to tissue saturation (4-20 mmHg, tissue dependent). Usually oxygenation of tissue is directly related to its degree of vascularisation. Different from other tissues, which expand predominantly by increasing cell division, thus allowing for adequate neo-vascularisation, adipose tissue expands primarily by cell expansion, not allowing for increased vascularisation. The key growth hormone for neo-vascularisation is vascular endothelial growth factor (VEGF). In obese mice however adipocyte VEGF expression is decreased compared to adipose expression in lean mice (Hausman and Richardson, 2004; Lijnen *et al.*, 2006), which is another indicator that vascularisation of WAT decreases with obesity development.

In a comparative study between obese and non-obese surgical patients with artificial arterial pO_2 of 150 mmHg, the subcutaneous, *i.e.* fat, tissue perfusion was 44 mmHg pO_2 in obese patients and 57 mmHg pO_2 in normal weight patients (Fleischmann *et al.*,

2005). Furthermore in genetic and diet induced obese mice models, pO_2 was 48 mmHg in adipose tissue of lean mice and 15 mmHg in adipose tissue of obese mice (Ye *et al.*, 2007). These recent findings strongly support the idea of hypoxia development in obese adipose tissue.

The response of cells to a hypoxic environment is the activation of hypoxia specific transcription factors, especially the hypoxic inducible factor-1 (HIF-1) (Semenza and Wang, 1992; Wang and Semenza, 1993). HIF-1 consists of two subunits (α and β). Both are continuously expressed. During normoxia HIF-1α is immediately targeted for proteasomal degradation, by ubiquitination. During hypoxia however prolyl hydroxylase domain dioxygenases, which provide hydroxylated ubiquitin binding sites on HIF-1α during normoxia, are inhibited. Thus HIF-1α can complex with its β-subunit to form functioning HIF-1 (Stuart Wood *et al.*, 2009). HIF-1 has been shown to be involved in the transcriptional regulation of over seventy genes, including genes involved in angiogenesis, glucose metabolism, apoptosis and inflammation (Semenza, 1998). Crucially, HIF-1 has also been linked to increase transcription of the leptin gene (Ambrosini *et al.*, 2002; Grosfeld *et al.*, 2002).

At a similar time as the hypoxia theory was first introduced, Bornstein and colleagues (2000) discovered the presence of immune cells in the adipose tissue. Recently Pasarica and colleagues (2009) suggested a link between hypoxia in human WAT and macrophage infiltration. In fact macrophages were found in localised hypoxic areas within the adipose tissue in mice (Rausch *et al.*, 2008). Additionally adipocytes grown

in vitro in hypoxic conditions, release monocyte chemoattractant protein 1 (MCP-1, a macrophage chemoattractant) and macrophage migration inhibitory factor-1 (MIF-1, prevents macrophage release from the tissue) (Gerhardt *et al.*, 2001; Skurk *et al.*, 2005; Trayhurn *et al.*, 2008).

1.1.4.4 Macrophage infiltration and adipose tissue inflammation

Macrophages infiltrate expanding adipose tissue during the development of obesity, thus account for an increase in stromal cells in WAT (Weisberg *et al.*, 2003; Xu *et al.*, 2003). The amount of macrophages in WAT was 2% of stromal cells in wild-type mice and 30% in *ob/ob* mice (Xu *et al.*, 2003). Adipocyte hypertrophy and WAT hypoxia may trigger the attraction of macrophages to adipocytes (see section 1.1.4.3). In support of this hypothesis, primary mice macrophages grown *in vitro* under hypoxic conditions increased their expression of inflammatory markers such as TNF-α and IL-6, which create an immune response (Ye *et al.*, 2007). Additionally, adipocyte size has been directly correlated to increased serum concentrations of inflammatory markers TNF-α, IL-6 and C-reactive protein (Bahceci *et al.*, 2007). A different hypothesis suggests that macrophages are recruited by dead or dying adipocytes to facilitate phagocystosis. Adipocyte death is a rare feature in lean adipose tissue, it is however common in hypertrophic adipocytes (Cinti, 2005; Murano *et al.*, 2008; Strissel *et al.*, 2007). A causal relationship between hypoxia and increased adipocyte cell death has also been suggested (Surmi and Hasty, 2008). The third hypothesis for increased macrophages in WAT during obesity is the increased secretion of leptin from adipocytes. Leptin has been demonstrated to be a chemoattractant for neutrophiles (Ottonello *et al.*, 2004) and smooth muscle cells (Oda *et al.*, 2001) as well as macrophages (Gruen *et al.*, 2007).

These suggestions are not mutually exclusive and may even act synergistically to increase macrophage infiltration. In conclusion the attraction of macrophages to WAT may contribute to the chronic inflammation observed in WAT in obese individuals (Xu *et al.*, 2003).

1.1.4.5 Adipokine deregulation

Infiltrated macrophages increase secretion of inflammatory cytokines, which act paracrinic on the surrounding adipocytes (Suganami *et al.*, 2005). This inflammatory milieu together with the other features of obesity (adipocytes hypertrophy and hypoxia) are likely responsible for the change in adipokine secretion in obese adipose tissue. The term "adipokines" has been suggested to be used summarily to include all factors secreted by adipocytes (Trayhurn and Wood, 2004)[14]. Since the discovery of leptin (Zhang *et al.*, 1994) and the finding that adipocytes secrete TNF-α (Hotamisligil *et al.*, 1993), the number of factors discovered to be secreted by adipose tissue has increased to more than seventy-five (Stuart Wood *et al.*, 2009). Adipokines are a diverse group of agents, exerting a variety of different functions (Table 1-2) This change in adipokine secretion has been suggested to be the key factor in mediating many of the

[14] Thus technically inflammatory markers (TNF-α and IL-6) secreted by macrophages are not adipokines, yet a distinction fails to be made in a majority of the available literature. However TNF-α and IL-6 are also secreted from adipocytes (Hotamisligil *et al.*, 1993; Mohamed-Ali *et al.*, 1997), making it difficult to routinely distinguish between adipocyte-derived and macrophage-derived TNF-α and IL-6. In fact most adipose tissue derived inflammatory markers are produced by non-adipocytes (Fain *et al.*, 2004). Keeping this discrepancy in mind, it shall be noted that the term "adipokine" in the study presented here will include all agents derived from adipose tissue irrespective of their cellular origin.

accompanying health effects (Kopelman, 2007; Kopelman, 2000), which will be introduced in the following section[15].

Table 1-2: Non-exhaustive list of adipokines secreted by adipocytes and affected by adipose tissue expansion during obesity development.

Adipokine	**Function**	**correlation with adipose tissue expansion**	**Reference**
Leptin	energy balance regulation	positive	(Zhang *et al.*, 1994)
Adiponectin	glucose metabolism, insulin sensitivity	negative	(Maeda *et al.*, 1996)
TNF-α	Inflammation	positive	(Hotamisligil *et al.*, 1993)
Interleukin-6	Inflammation	positive	(Mohamed-Ali *et al.*, 1997)
Resistin	Insulin sensitivity (mice only)	positive	(Steppan *et al.*, 2001)
Appelin	Angiogenesis	positive	(Beltowski, 2006; Tatemoto *et al.*, 1998)
Visfatin	Insulin-independent glucose metabolism	positive	(Beltowski, 2006; Fukuhara *et al.*, 2005)
Plasminogen activator inhibitor-1 (PAI-1)	Haemostasis	positive	(Lundgren *et al.*, 1996)
Cholesteryl ester transfer protein (CETP)	Lipid metabolism	positive	(Jiang *et al.*, 1991)
Angiotensinogen	Blood pressure regulation	positive	(Saye *et al.*, 1989)
Vascular epithelial growth factor (VEGF)	Angiogenesis	positive (with hyperinsulinaemia only)	(Mick *et al.*, 2002)

[15] Although it was attempted to present the changes in the WAT in a chronological order, the actual occurrence of these events is likely to happen simultaneously or in a different order than presented. For example hypoxia alone may trigger changes in adipokine secretion (*e.g.* leptin expression is HIF-1 responsive), which in turn may attract macrophages and increase inflammation. However these events cannot occur at random. For instance hypoxia only occurs in response to hypertrophy and adipokine deregulation seems to always be associated with macrophage infiltration.

1.1.5 Obesity related adverse health effect

1.1.5.1 Obesity related mortality

While obesity itself is a metabolic disease, it is the accompanying co-morbidities that are thought to cause the most danger to health (Kopelman, 2000). Mortality increases with BMI and excessive waist circumference, as established in epidemiological studies. The INTERHEART study concluded that abdominal obesity was one of the leading risk factors for acute myocardial infarction[16] (Yusuf *et al.*, 2004; Yusuf *et al.*, 2005). In the USA the number of deaths attributable to obesity is 280,000 deaths (Allison *et al.*, 1999). The latest study on actual causes of death rated poor diet and physical inactivity as the second most likely cause of death in the USA in 2000, only surpassed by smoking related deaths[17] (Mokdad *et al.*, 2004). In a Europe-wide study about one in 13 recorded deaths may be attributable to obesity, bringing the total number of obesity related deaths to an excess of 300,000 annually (Banegas *et al.*, 2003). In the UK about 30,000 premature deaths are attributed to obesity-related illnesses (Bourn, 2001). Compared to an individual with a normal BMI (20-22 kg/m^2) lifespan on average decreases by nine years in obese persons (BMI>30 kg/m^2) (Bourn, 2001). Mortality of obesity is largely due to cardiovascular disease and diabetes, through the consequences of obesity-

[16] This case-control study (Yusuf *et al.*, 2004) of acute myocardial infarction examined ~30,000 (15152 cases, 14820 controls) individuals in 52 countries. The risk factors examined were history of smoking, hypertension, diabetes, waist/hip ratio, dietary patterns, physical activity, consumption of alcohol, blood apolipoproteins and psychosocial factors. Together these factors account for over 90% of the risk of an initial myocardial infarction. Smoking and abnormal lipid concentrations were the most promising risk factors, followed by psychosocial factors, abdominal obesity, diabetes and hypertension (Yusuf *et al.*, 2004).

[17] Taking into account the previous report a decade earlier (McGinnis and Foege, 1993) the "poor diet and physical inactivity" attributable category increased by over 100.000 deaths annually (from 300,000 in 1990 to 400,000 in 2000). By comparison deaths related to smoking increased "only" by 35.000 (from 400,000 in 1990 to 435,000 in 2000). This led the authors to predict that the "poor diet and physical inactivity" category will soon overtake the "smoking" category as the leading cause of death in the USA (Mokdad *et al.*, 2004).

induced Metabolic Syndrome. Obesity also increases risk for development of several other diseases (Table 1-3).

Table 1-3: Health risk associated with increasing body mass index (adopted from Kopelman, 2007)

Health complication	Risk associated with increasing BMI
Metabolic syndrome	30% of middle aged people in developed countries have features of Metabolic Syndrome (according to IDF definition)
Type 2 diabetes	90%of type 2 diabetics have a body mass index (BMI) of >23 kg/m^2
Hypertension	5-fold increased risk with obesity; 66% of hypertension cases are linked to excess weight 85% of hypertension is associated with a BMI >25 kg/m^2
Coronary artery disease (CAD)	3.6-fold increased risk of CAD for each unit change in BMI Dyslipidaemia progressively develops as BMI increase from BMI = 21 kg/m^2 70% of obese women with hypertension have left ventricular hypertrophy Obesity is a contributing factor of cardiac failure in >10% of patients
Stroke	Overweight/obesity-related hypertension is associated with increased risk of ischemic stroke
Respiratory effects	Neck circumference of >43 cm in men and >40.5 cm in women is associated with obstructive sleep apnoea, daytime somnolence and development of pulmonary hypertension.
Cancers	10% of all cancer deaths among non-smokers are related to obesity (30% of endometrial cancers)
Reproductive function	6% of primary infertility in women is attributable to obesity Impotency and infertility are frequently associated with obesity in men
Osteoarthritis	Frequent association in the elderly with increasing body weight – risk of disability attributable to osteoarthritis equal to heart disease and greater than any other medical disorder in the elderly
Liver and gall bladder disease	Overweight and obesity associated with non-alcoholic fatty live disease (NFLD) and non-alcoholic steatohepatitis (NASH); 40% of NASH patients are obese; 20% have dyslipidaemia; 3-fold increased risk of gall bladder disease in women with a BMI or >32 kg/m^2; 7-fold increased risk if BMI of >45 kg/m^2

1.1.5.2 Metabolic Syndrome

The negative health effects of obesity correlate well with BMI, but for some co-morbidities excess waist circumference is a better marker. Waist circumference provides a stronger link than BMI to CVD risk (Balkau *et al.*, 2007; Park *et al.*, 2009; Smith and Haslam, 2007; Welborn and Dhaliwal, 2007; Yusuf *et al.*, 2004) and insulin resistance (Balkau *et al.*, 2007; Lee *et al.*, 2006; Westphal, 2008). Other researchers however found no difference in predicting increased CVD risk between waist circumference and BMI (Freiberg *et al.*, 2008). It had been observed that total fat mass *per se* did not indicate the imminent development of diabetes, but the excess accumulation of fat in the upper body did (Vague *et al*., 1980). It was repeatedly observed that a cluster of metabolic changes (insulin resistance, impaired fasting glucose, hypertension, dyslipidaemia) appeared in patients with increased waist circumference. These symptoms were so closely linked that their appearance was given its own name: "Metabolic Syndrome" (Alberti and Zimmet, 1998). Several attempts were made to provide cut-off values to define patients suffering from Metabolic Syndrome. The recent consensus by the International Diabetes Federation (2006), for example, establishes the existence of Metabolic Syndrome in two stages: First by an ethnically specific increase in waist circumference and secondly by identifying at least two of four further abnormalities (raised triglycerides, reduces HDL cholesterol, impaired fasting glucose, and hypertension). This interpretation places abdominal obesity at the centre of the syndrome and relates additional factors to it. Other definitions for Metabolic Syndrome have been proposed (WHO, EGIR, NCEP), all including abdominal obesity as a main characteristic of Metabolic Syndrome. Accordingly the same individual may or may not be classified as having Metabolic

Syndrome and thus requiring treatment. More stringent definitions also increase the amount of the population suffering from Metabolic Syndrome and thus requiring medical intervention. Hence the use of Metabolic Syndrome in a clinical setting has come under some scrutiny by its original describer. In its original form "syndrome X" should identify those patients at risk of cardiovascular events (Reaven, 1988; Reaven, 1993). He now argues that diagnosis of Metabolic Syndrome has no clinical, educational or pathophysiological value for the treatment of the patient, as such definition and diagnosis of Metabolic Syndrome is irrelevant (Reaven, 2006). Instead each of the components of the syndrome should be diagnosed, assessed and treated independently to reduce CVD risk (Reaven, 2005). Others however have come to the aid of Metabolic Syndrome, defending its position as a tool for the global prevention of CVD and diabetes (Cameron *et al.*, 2009). For the study presented here, the finding that a series of obesity induced metabolic changes cluster together emphasises the adverse health effect of obesity.

1.1.5.3 Cardiovascular disease

Due to the strong connection between obesity and CVD, Metabolic Syndrome has been suggested as a marker of individual risk of obesity associated CVD. Indeed all four associated markers of Metabolic Syndrome (hypertension, insulin resistance, triglycerides and HDL) have independently been associated with CVD.

Obese patients are at increased risk of developing hypertension (Johnson *et al.*, 1975; Kannel *et al.*, 1967). Results from the Framingham Heart Study have indicated that increased blood pressure between 130/85 to 139/89 mmHg, considered to be on the high

end of the normal range, increased risk of developing cardiovascular diseases two-fold compared to blood pressure at the optimum of 120/80 mmHg (Vasan *et al.*, 2001). As with any muscle that is subjected to increased exercise, heart muscle increases in size to overcome hypertension. As such left ventricle mass itself is directly associated with BMI (de la Maza *et al.*, 1994). As heart muscle is different to skeletal muscle however an increase in size leads to dilatation of the ventricle and decreased output (ejection fraction). This decreased output is not caused by a weakness in heart muscle, but by a dysfunction in the diastole, decreasing normal ventricle filling when the heart muscle is relaxed (Lalande and Johnson, 2008).

Additional features of Metabolic Syndrome, increased insulin resistance and elevated glucose concentrations, have been associated with increased risk of cardiovascular disease. High blood glucose concentrations may be an independent risk factor for CVD (Balkau *et al.*, 1998) and CVD is the major cause of death in diabetics (Wingard and Barrett-Connor, 1995). Wilson and colleagues (1991) showed high blood glucose concentrations to be an independent risk factor for CVD in women, but not in men in the Framingham Study Population (Wilson *et al.*, 1991). In fact more recent data suggests that lowering blood glucose alone in diabetic patients did not reduce CVD (Cheng and Leiter, 2009). They examined data from three randomised control trials, indicating that intensive glucose lowering treatment to <7% HbA1C (glycosylated haemoglobin[18]) did not decrease risk of CVD in diabetic patients. These findings from

[18] Glucose concentrations in blood fluctuate during the day, making an assessment of how much glucose an individual is exposed to difficult. Haemoglobin becomes aberrantly glycosylated in a glucose environment and the amount of glycosylated haemoglobin is proportional to the amount of glucose in its environment, i.e. blood. Additionally the glycosylation of haemoglobin remains constant, allowing for increased sensitivity of an individual's glucose exposure.

diabetes trials indicate that high blood glucose concentrations may not account alone for the increased risk of CVD in obese individuals unless additional features of Metabolic Syndrome are present.

A lipoprofile indicative of Metabolic Syndrome (high triglycerides, high LDL, low HDL) is also a strong link between obesity and CVD. High LDL-cholesterol and triglyceride (TG) concentrations and low HDL-cholesterol concentrations have been linked to increased arteriosclerosis and its associated increase in CVD (Castelli, 1996). Decreasing LDL-cholesterol concentrations is beneficial for CVD risk (Nissen, 2005). No evidence however has been presented at present showing specifically that lowering TG-concentrations are beneficial for cardiac health. This may be caused mainly by the tight clustering of additional features (abdominal obesity, impaired glucose concentrations, increased concentrations of lipoprotein remnants) around increased TG-concentrations. Trials to increase HDL concentrations are also underway; their main result however is still outstanding (Schaefer and Asztalos, 2007).

1.1.5.4 Cerebrovascular disease

The underlying mechanism for ischemic stroke and ischemic heart disease are very similar in being caused through arterial blockage. Additionally a connection between hypertension and haemorrhagic stroke has long been established (Mizukami *et al.*, 1972; Phillips *et al.*, 1977). Several studies examined the correlation between obesity and the two forms of stroke. The northern Manhattan stroke case control study found that abdominal obesity, measured by waist-to-hip ratio increased risk of ischemic stroke (Suk *et al.*, 2003). In fact individuals with a waist-to-hip ratio above the mean of the

examined population had a 3-fold increased risk of stroke. Interestingly waist-to-hip ratio was a stronger risk factor of stroke than BMI. Additionally the association between abdominal obesity and stroke risk was higher in younger individuals than in older ones, but risk was equal among all examined ethnic groups (Suk *et al.*, 2003). An interesting finding was obtained when examining cerebral artery vessels in diet-induced obese rats after induced medial cerebral artery occlusion (MCAO) (Deutsch *et al.*, 2009). Obese rats showed increased damage to the vessel wall caused by increased stiffness due to increased collagen disposition during overfeeding. Overfeeding was started in three-week old rats from which the authors concluded that obesity beginning in childhood has an effect of cerebral vessel remodelling, increasing stroke risk in later life, thus supporting the findings of the Manhattan study, where the association between stroke risk and waist-to-hip ratio was higher in younger individuals (Suk *et al.*, 2003). The link between abdominal obesity and stroke risk was also evaluated in an Asian study, showing increased risk of ischemic stroke with increased abdominal obesity (Chen *et al.*, 2006b). General obesity again was not as strongly correlated to ischemic stroke as abdominal obesity. Likewise a further study showed increase risk of ischemic stroke with increased waist circumference, but no correlation of increased stroke risk and BMI was observed (Winter *et al.*, 2008).

Interestingly, despite the strong connection between obesity and hypertension, as well as hypertension and haemorrhagic stroke, the connection between obesity and ischemic stroke is far higher than between obesity and haemorrhagic stroke. This finding may be explained by a weight-independent dietary factor, such as sodium intake, which could be responsible both for increased blood pressure and haemorrhagic stroke (Haslam and

James, 2005). There are no studies however examining this possible connection. From these studies it can be deduced that stroke risk is increased in obese individuals, especially in abdominally obese and early onset obese persons.

1.1.5.5 Respiratory effects

Effects of obesity on respiration are of physical rather than molecular nature. The increased fat mass around the chest wall and the abdomen has marked effects on the mechanisms relating to diaphragm movement, reducing respiratory capacity. Compensation requires increased respiratory muscle force to overcome elastic recoil of the fat mass. These effects are exaggerated when lying flat, taking greater effect during sleeping than being awake, when increased breathing resistance can be overcome consciously. This phenomenon leads to a temporary cessation of breathing (apnoea) accompanied by a decrease in arterial oxygen saturation (hypoxia) during sleeping. This effect seems to be related exclusively to obese men and obese postmenopausal women, while premenopausal obese women do not show a decrease in nocturnal arterial oxygen saturation (Kopelman *et al.*, 1985; Kopelman *et al.*, 1986). Sleep disruption leads to increased daytime somnolence and fatigue. Indeed most patients, referred to sleep disorder centres for fatigue, are eventually diagnosed with sleep apnoea (Vgontzas *et al.*, 2003). While increased daytime sleepiness increases risk of traffic or work accidents (Haslam and James, 2005), sleep apnoea causes severe physiological changes during nocturnal hypoxia. Hypoxia accompanied by hypercapnia is compensated by pulmonary hypertension and thus associated with increased risk of right ventricle failure (Kopelman, 2000).

Interestingly, patients with sleep apnoea show increased leptin, TNF-α and IL-6 concentrations, independent of BMI (Vgontzas *et al.*, 2000). Additional studies found increased fasting blood glucose concentrations and plasma insulin concentrations in apnoeics compared to obese non-apnoeics (Ip *et al.*, 2002; Manzella *et al.*, 2002; Vgontzas *et al.*, 2000). Some researchers also suggest that sleep apnoea should be included as another factor for the definition of Metabolic Syndrome (Lam and Ip, 2009). There is some inconsistency as to the causal link between sleep apnoea and insulin resistance, *i.e.* whether sleep apnoea causes insulin resistance or *vice versa*. Insulin resistance is present in patients with mild sleep apnoea (Punjabi *et al.*, 2002), and treatment for sleep apnoea has failed to significantly increase insulin sensitivity (Vgontzas *et al.*, 2003). Thus it seems more likely that insulin resistance causes sleep apnoea or has no effect, instead of the other way round.

1.1.5.6 Reproductive abnormalities

Oestrogen concentrations are higher in obese women, compared to normal weight women, caused by a strong association of adiposity to peripheral aromatase activity in white adipose tissue (Longcope *et al.*, 1986). Excess oestrogen causes impaired feedback mechanisms to the hypothalamus, deregulating ovulatory cycle and possibly leading to female infertility. A total of 6% of ovulatory dysfunction infertility is attributable to obesity (Green *et al.*, 1988). Males with erectile dysfunction (ED) are generally found to have higher body weight, have higher incidences of hypertension and hypercholesterolemia (Esposito *et al.*, 2006) suggesting that some features of Metabolic Syndrome are associated with ED.

The disturbance of sex hormones in obese women is also associated with hirsutism and the development of polycystic ovarian syndrome (PCOS) (Laven *et al.*, 2002). This syndrome is additionally characterised by increased insulin resistance and increased androgen production (Haslam and James, 2005). It responds favourably with weight loss (Pasquali *et al.*, 1997), indicating a close relation to obesity.

1.1.5.7 Non-alcoholic fatty liver disease and Non-alcohol steatohepatitis

Free fatty acid (FFA) concentrations are generally higher in the obese, independent of insulin resistance. Additionally obese intra-abdominal fat tissue may deliver excess FFAs to the liver on a direct route through the portal vein (Scheen and Luyckx, 2002). These factors lead to an increase in fat accumulation in liver cells (steatosis). This feature is called non-alcoholic fatty liver disease (NFLD), as steatosis in liver cells is frequently observed with excess alcohol consumption. Metabolic Syndrome is not the only cause of this disease; several inborn metabolic errors are also known to predispose to this phenomenon. Additionally certain types of bariatric surgery[19] (jejunoileal bypass and gastroplasty) have been associated with higher risk for NFLD (Raman and Allard, 2006). Steatosis itself is not thought to affect normal liver function and usually does not cause symptoms in the majority of patients. Increased concentrations of transaminases, with modestly elevated concentrations of alanine aminotransferase and aspartate aminotransferase[20] are signs of NFLD, but only in the context of additional risk factors, most notably insulin resistance. According to Raman and Allard (2006), NFLD is the

[19] Newer methods of bariatric surgery (roux-en-Y gastric bypass, gastric band and sleeve gastrectomy) do not seem to predispose or worsen NFLD and NASH, but may be favourable in reducing steatosis, steatohepatitis and fibrosis (Mattar *et al.*, 2005).

[20] In NFLD patients the ratio of aspartate aminotransferase to alanine aminotransferase is less than one, distinguishing NFLD patients from alcohol-induced liver disease patients (Bacon *et al.*, 1994).

first hit in their proposed two-hit hypothesis in developing non-alcoholic steatohepatitis (NASH). The second hit, an inflammation of the liver cells caused by lipid toxicity (steatohepatitis), can lead to scarring, fibrosis and cirrhosis of the liver. A progression of NFLD to NASH cannot be diagnosed, except through liver biopsy, making routine screening more difficult. Different mechanisms for the progression to NASH have been proposed. Raman and Allan (2006) favour several mechanisms that increase oxidative stress on hepatocytes and include Cyp2E1 (a pro-oxidant cytochrome P450 isoform) overexpression, increased lipid peroxisome catalysis, decreased expression of PPAR-α and mitochondrial abnormalities. Rivera (2009) alternatively proposes involvement of the innate immune system, especially toll like receptor-4 (TLR-4) signalling activated by FFAs in the development of NASH. Whichever way NASH develops the influence of FFA and thus obesity and Metabolic Syndrome is evident (Rivera, 2008).

1.2 Obesity and insulin resistance

Type-2 diabetes mellitus, which can develop as a result of insulin resistance, is the closest related and most severe co-morbidity of obesity (Kahn and Flier, 2000). In fact 90% of diabetes patients show a BMI of 23 kg/m^2 or more (Stevens *et al.*, 2001). Diabetes[21] develops as pancreatic β-islet cells increase production of insulin to overcome hyperglycaemia as a result of insulin resistance. Eventually these β-islet cells fall victim to this process either by overexertion or through insulin resistance/obesity related mechanisms that inhibit β-cells function, such as accumulation of ectopic fat in the pancreas causing lipotoxicity or through action of inflammatory cytokines secreted

[21] Unless otherwise stated, "diabetes" shall be understood to refer to type-2 diabetes mellitus specifically. Type-1 diabetics by comparison cannot produce any pancreatic insulin. This can occur congenitally, as a germline defect in genes involved in insulin production or as a result of pancreatic injury. They do not show features of hyperinsulinaemia and insulin resistance.

from hypoxic fat tissue (Kahn, 1998; Kahn *et al.*, 2006). Likewise ectopic fat in muscle and particularly liver cells has been suggested as a link to insulin resistance and diabetes (Stefan *et al.*, 2005). Lettner and Roden (2008) however suggested that ectopic fat is not the causal effect but rather a biomarker of insulin resistance.

Normal intra-cellular insulin signalling involves activation of the insulin receptor, its target the insulin receptor substrate and activation of the phosphoinositide-3 kinase (PI3-kinase) pathway[22] (Figure 1-4). Chronic activation of this pathway by hyperinsulinaemia has been linked to impaired activation of protein kinase B (PKB/AKT), a downstream target of PI3-kinase, leading to insulin resistance (Bertacca *et al.*, 2005).

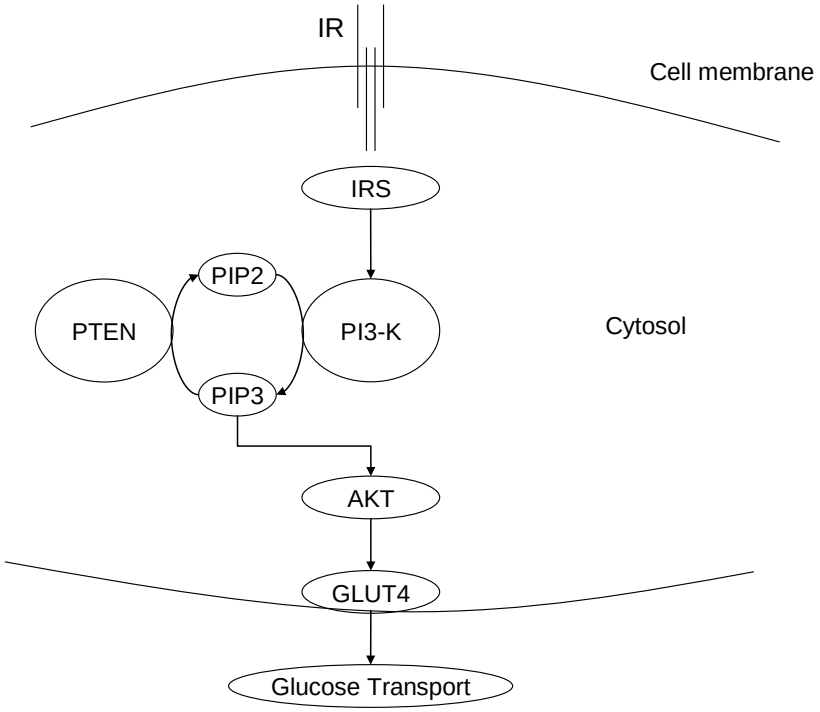

Figure 1-4: Schematic overview of the PI-3 kinase pathway and its specific involvement in mediating normal insulin signalling.

[22] The exact mechanism of this pathway, especially in the context of its involvement in breast cancer is introduced in section 1.6.1.

The change in adipokine secretion with obesity (section 1.1.4.5) has also been suggested to have a significant role in promoting obesity-induced insulin resistance. Antuna-Puente and colleagues (2008) summarised findings on the influence of several adipokines on insulin resistance. The main effect is exerted by the production of inflammatory cytokines, mainly and IL-6 (Antuna-Puente *et al.*, 2008). Indeed the effect of inflammation within the adipose tissue in obese individuals itself has been considered as a connection between obesity and insulin resistance (Bastard *et al.*, 2006; Steinberg, 2007). TNF-α exerts its insulin resistance inducing effects by serine-phosphorylation of insulin receptor substrate 1 (IRS1), thereby blocking its interaction with the insulin receptor or its regular tyrosine phosphorylation needed to activate the PI3-kinase signalling pathway (Antuna-Puente *et al.*, 2008; Bastard *et al.*, 2006; Hotamisligil, 2003). This mechanism turns IRS1 effectively into an inhibitor for IR tyrosine kinase activity, blocking normal insulin response, leading to insulin resistance (Nieto-Vazquez *et al.*, 2008; Pirola *et al.*, 2004; White, 2003). This serine phosphorylation of IRS is mediated by TNF-α induced inhibitor-kappa B kinase (IKK) (Gao *et al.*, 2002) and other inflammation responsive serine kinases such as c-JUN N-terminal kinase (JNK) (Hirosumi *et al.*, 2002), protein kinase C (PKC) (Griffin *et al.*, 1999) and ribosomal protein kinase S6 kinase (S6K) (Tremblay *et al.*, 2007).

Chronic elevated concentrations of IL-6 have also been linked to promote muscular insulin resistance (Bastard *et al.*, 2002). The IL-6 receptor belongs to the class I family of cytokine receptors that use Janus kinases (JAKs) as intra-cellular second messengers (Ihle *et al.*, 1995). IL-6 has also been shown to increase expression of suppressors of cytokine signalling (SOCS) (Senn *et al.*, 2003). The SOCS family inhibits cytokine

signalling, especially of the JAK2/STAT3 cell signalling pathway (Starr *et al.*, 1997), thus may act as a negative feedback to IL-6 activation of JAKs. SOCS are known however to also inhibit the insulin receptor in response to IL-6 recognition (Senn *et al.*, 2003). Thus long-term exposure to elevated concentrations of IL-6 may fail to create a sufficient feedback loop to stop IL-6 signalling and decrease insulin receptor phosphorylation instead.

Controversially, IL-6 has also been shown to activate AMP-activated protein kinase (AMPK) (Ruderman *et al.*, 2006). AMPK activation in turn has been demonstrated to increase insulin sensitivity (Hardie and Carling, 1997). Thus it has been suggested that acute IL-6 exposure, which is present after physical exercise, could be beneficial for insulin sensitivity, while chronic exposure, such as evident in obese individuals, may promote the opposite, *i.e.* insulin resistance (Kim *et al.*, 2009). This could be explained by the negative feedback loop that IL-6 mediated SOCS activation creates.

Another interesting connection between adipose tissue and insulin resistance has recently been discovered. As obesity develops expression of the insulin-responsive glucose transporter GLUT4 decreased in adipocytes (Shepherd and Kahn, 1999). This change is selective for the adipocytes only and does not affect GLUT4 expression in muscle cells, the principle site for glucose uptake (DeFronzo, 1997). Adipocyte-selective knock-out of GLUT4 however increased insulin resistance in liver and muscle cells in a mouse model (Abel *et al.*, 2001). Furthermore *ex-vivo* analysis of muscle cells indicated normal insulin action, indicating an adipocyte-derived circulating factor, *i.e.* an unidentified adipokine, may be responsible for the observed increase in muscle and

liver insulin resistance (Abel *et al.*, 2001). Indeed a subsequent study showed increased expression of retinol binding protein 4 (RBP4) in adipose tissue of adipose specific GLUT4 knockout mice (Yang *et al.*, 2005). Moreover RBP4 concentrations correlated with BMI, waist-to-hip ratio and serum triglyceride concentrations. Additionally only in individuals in whom exercise induced an improvement in insulin sensitivity, serum concentrations of RBP4 decreased (Graham *et al.*, 2006). Thus RBP4 may be an adipokine directly involved in mediating obesity induced insulin resistance in muscle cells. RBP4 is the main transport protein for hydrophobic retinol (vitamin A) in the circulation and may inhibit intra-muscular insulin signalling at the level of PI3-kinase (Yang *et al.*, 2005).

Several additional adipokines have been suggested to be involved in the mediation of obesity-induced insulin resistance, including resistin (Steppan *et al.*, 2001), serum amyloid A (Leinonen *et al.*, 2003), plasminogen activator-1 (Bastard *et al.*, 2000), visfatin (Fukuhara *et al.*, 2005), vaspin (Hida *et al.*, 2005) and omentin (de Souza Batista *et al.*, 2007). The mechanism of how many of these factors increase insulin resistance is still under investigation. As a matter of fact the current findings linking resistin and visfatin to insulin resistance are very controversial (Antuna-Puente *et al.*, 2008; Nieto-Vazquez *et al.*, 2008).

1.2.1 Obesity and insulin-like growth factor-1: a potential controversy

Since the evidence for a connection between obesity and insulin resistance with resulting hyperinsulinaemia is so overwhelming, the focus on a possible connection between obesity and the insulin-like growth factor-1 (IGF-1) intensified. This is an

interesting suggestion, especially on the background of the study presented here as IGF-1 is a known mitogen for breast cancer[23] (Sachdev and Yee, 2001). Insulin and IGF-1 share a high degree of homology (Rotwein, 1991). In fact insulin and the two known insulin-like growth factors (IGF-1 and IGF-2) belong to the same gene family (Blundell and Humbel, 1980). IGF-1 mediates the effects of Growth Hormone (GH) in increasing foetal skeletal growth (Daughaday and Rotwein, 1989). While insulin is exclusively produced by the pancreas in response to an increase in blood glucose concentrations, IGF-1 is continuously produced by hepatic cells. Only a part of the total circulating IGF-1 is free and bio available. Much is bound, and thus metabolically inactive, to insulin-like growth factor binding proteins (IGFPBs). The IGF-family consists of IGF1 and IGF-2 and their respective trans-membrane receptors IGF-1R and IGF-2R, six IGFBPs and their respective proteases (IGFBP-protease) (Sachdev and Yee, 2001).

The connection of IGF-1 with obesity is not straight forward. GH from the pituitary gland is the major factor that increases hepatic secretion of IGF-1 (Veldhuis and Iranmanesh, 1996). Additionally, insulin also stimulates hepatic production of IGF-1 (Boni-Schnetzler *et al.*, 1991), and reduces production of IGFBP-1 (Suikkari *et al.*, 1989). As obesity decreases pituitary GH secretion and correlates also with increased insulin concentrations, IGF-1 secretion is under two conflicting influences during the development of obesity. Consequently, correlating total IGF-1 concentrations to BMI has produced conflicting results (see references 5-17 in Frystyk *et al.*, 1995). Serum

[23] The connection of IGF-1 to breast cancer is briefly introduced in section 1.5.2.1 and evaluated in section 8.4.2.

concentrations of total IGF-1 were highest in individuals with BMIs between 26-28 kg/m^2 and lower for patients with body weights above or below (Allen *et al.*, 2003). Thus some research has focused on the connection between BMI and IGFBP-1 concentrations to examine, if free IGF-1 increases with BMI. Free IGF-1 concentrations however were not different in simple obese and diabetic obese individuals and were also not elevated compared to lean individuals. Similarly, total IGF-1 was not different between all three groups. IGFBP-1 however was elevated in the simple obese group compared to the diabetic obese and lean group, between which no difference was observed (Frystyk *et al.*, 1999). Thus the hypothesis that obesity increases free IGF-1 concentrations through hyperinsulinaemia was not validated by this study. The ratio of free IGF-1/total IGF-1 however was higher in obese individuals compared to overweight and lean subjects, possibly due to a decrease of IGFBP-3 (Frystyk *et al.*, 2009). Despite the conflicting findings of obesity's contribution to IGF-1 concentrations many authors suggest an obesity induced increase in IGF-1 as the main contributor to the obesity-insulin resistance-breast cancer connection (Boyd, 2003; Godsland, 2009; Lorincz and Sukumar, 2006). Interestingly many of the reviews examining the molecular changes with insulin resistance and hyperinsulinaemia do not mention IGFs (DeFronzo, 1997; Pessin and Saltiel, 2000; Shulman, 2000).

The complex relationships of obesity decreasing GH secretion and increasing insulin secretion may explain the non-linear connection between obesity and IGF-1 concentrations. During obesity development insulin secretion increases, while a decrease in GH secretion does not significantly affect IGF-1 secretion, thus IGF-1 concentrations rise until the BMI of 28 kg/m^2. As BMI increases further GH secretion

becomes markedly reduced, while insulin secretion increases further. If one assumes GH to be a more potent stimulant for IGF-1 secretion, one has a convincing explanation for an IGF-1 concentration maximum at a BMI of 28 kg/m^2.

1.3 Obesity and cancer

Obesity has been suggested as a risk factor for endometrial cancer for some time (Cavanagh *et al.*, 1984). The impact of obesity on the cancer burden however has become evident with the publication of a large cohort study (Calle *et al.*, 2003). Their conclusions estimate that morbidly obese men and women (BMI>40 kg/m^2) had an increased risk of dying from cancer of 52% and 62%, respectively. Additionally with the current pattern of obesity in the USA, they estimated that 14% and 20% of all cancer deaths were attributable to obesity, for men and women, respectively.

In addition the recent second expert report of the World Cancer Research Fund (WCRF, 2007) identified overweight and obesity as the major preventable risk factor for cancer prevention. Most of their recommendations (p. 368ff) are based on body weight modification and "healthy" life-style choices. Their examinations concluded that incidences of cancers of the colorectum, oesophagus (adenocarcinoma), endometrium, pancreas, kidney, and breast (postmenopausal) are convincingly associated with obesity. A short overview of the connection of obesity with these cancers follows. The connection between obesity and breast cancer will be examined in section 1.5

Colorectal cancer incidence and mortality is increased in obese individuals (Calle *et al.*, 2003). Subsequent meta-analyses have confirmed this connection on an epidemiological

level (Dai *et al.*, 2007; Huxley *et al.*, 2009; Larsson and Wolk, 2007; Moghaddam *et al.*, 2007). Similarly large cohort studies have indicated an increased risk of colon cancer with obesity (Moore *et al.*, 2004; Pischon *et al.*, 2006). These studies also conclude that waist circumference is a stronger marker for colon cancer risk and that the association between abdominal obesity and colon cancer is stronger in men than in women. While the epidemiological evidence for a connection between abdominal obesity and colon cancer is overwhelming, the molecular connections are not well explored. Recently single-nucleotide polymorphisms (SNP) in genes coding for adipokines and insulin have been linked to colon cancer risk (Pechlivanis *et al.*, 2009). Unfortunately the authors failed to explore the connection of these SNPs to obesity. Thus while the SNPs may indeed have an effect on promoting colon cancer, it may also be the case for lean subjects carrying this mutation. This study however provides an insight into a possible molecular mechanism between obesity and increased risk of colon cancer. Besides obesity, colorectal cancer feature a number of additional risk factors, including low intake of dietary fibre and increased consumption of red meat (WCRF, 2007). Likewise obesity relies on an intake of energy-dense food, which is naturally low in fibre. Thus the connection could lie at a nutritional level linking both obesity and colorectal cancer.

Obesity is associated with adenocarcinoma of the oesophagus (WCRF, 2007). The association between obesity and oesophageal adenocarcinoma has been demonstrated to involve the connection of both of them to increased gastroesophageal reflux disease (El-Serag, 2008). Reflux of gastric fluid (acidic) into the distal part of the oesophagus has been linked to increased inflammation of the oesophageal endothelium, which can lead to metaplasia of the endothelium (Barrett's oesophagus) (Eisen *et al.*, 1997;

Shaheen and Richter, 2009), even though the idea of a direct connection between reflux and the development of Barrett's oesophagus is not unanimous (Pondugula *et al.*, 2007). Barrett's oesophagus in turn is a known pre-cursor of oesophageal adenocarcinoma (Pera *et al.*, 2005). Obesity is thought to mechanistically increase gastric pressure and lead to transient lower oesophageal sphincter relaxation, thus facilitating reflux (El-Serag, 2008). As expected, the relationship between abdominal obesity and reflux was even higher than the relationship to BMI (Pandolfino *et al.*, 2006). An interesting meta-analysis examining the potential of a connection between BMI and Barrett's oesophagus found no association, if corrected for reflux disease (Cook *et al.*, 2008). Thus obesity is an indirect marker for Barrett's oesophagus and oesophageal adenocarcinoma, by increasing gastric fluid reflux. Further evidence to the assumption that obesity affects oesophageal adenocarcinoma by mechanical, rather than molecular means comes from the finding that adipokine polymorphisms were not associated with increase in oesophageal adenocarcinoma (Doecke *et al.*, 2008). In contrast a connection between obesity-associated increased oestrogen levels and oesophageal adenocarcinoma has been suggested by others (El-Serag, 2008).

Endometrial cancer shows the strongest link between incidence rate and BMI (Calle *et al.*, 2003; Ito *et al.*, 2007; Lacey *et al.*, 2008; Potischman *et al.*, 1996). In fact up to 90% of endometrial cancer patients are obese (von Gruenigen *et al.*, 2005). An endometrial precursor is endometrial hyperplasia, a condition of chronic thickening of the uterine wall. In pre-menopausal women the endometrium changes during the menstrual cycle, thickening to receive a fertilised oocyte and shedding this build-up during menstruation. The menstrual cycle is under hormonal control, particularly oestrogen and progesterone,

with oestrogen being responsible for increased endometrial cell proliferation (Siiteri, 1978). Chronic oestrogen exposure is a feature of obesity, with high turn-over rates for oestrogen in obese fat tissue, due to aromatase overexpression [24] (Key and Pike, 1988). High oestrogen levels are favoured as the main mechanistic component of the connection between obesity and increased endometrial cancer risk (Fader *et al.*, 2009). Additionally insulin (O'Mara *et al.*, 1985), IGF-1 (Talavera *et al.*, 1990), leptin (Cymbaluk *et al.*, 2008) and adiponectin (Cong *et al.*, 2007) have been suggested to effect endometrial carcinoma.

Increased risk of development of carcinoma of the pancreas has been associated with increased body fatness (WCRF, 2007). A meta-analysis linking obesity and pancreatic cancer incidences found an increased relative risk of 1.19 for individuals with BMIs over 30 kg/m^2 (Berrington de Gonzalez *et al.*, 2003). An interesting molecular experiment had human pancreatic cancer cell lines placed in lean and obese mice, with the result that tumours growing in obese mice were greater and developed more metastases (Zyromski *et al.*, 2009). Additionally tumours grown in obese mice had higher adipocyte mass within their tumours than non-obese mice. The current understanding in humans links obesity and increased pancreatic cancer risk by obesity increasing the risk for development of pancreatitis. Pancreatitis, a chronic inflammation of the pancreas, is a major pre-cursor of pancreatic cancer (Gumbs, 2008). As mentioned in section 1.2, obesity increases the accumulation of ectopic pancreatic fat

[24] Oestrogen exposure also plays an important role in breast cancer and thus the connection between obesity, oestrogen production and breast cancer is discussed further in section 1.5.1 and section 8.4.1

and the secretion of inflammatory adipokines TNF-α and IL-6, all of which have been linked to increased risk of pancreatitis (Greer and Whitcomb, 2009; Pitt, 2007).

Renal carcinoma has been determined as one of the cancers convincingly associated with obesity (WCRF, 2007). Recent meta-analyses linking obesity and kidney cancer in men (Ildaphonse *et al.*, 2009) and women (Mathew *et al.*, 2009) have indicated a small increase in kidney cancer risk with increased BMI of 1.05 (95% CI=1.04-1.06) and 1.06 (95% CI=1.05-1.07), respectively. Another study estimated the odds ratio for obesity increased risk on kidney cancer incidence at 3.3 for men and 2.2 for women (Shapiro *et al.*, 1999). The mechanistic relation of obesity and renal cancer is not well researched, however several possibilities have been proposed. Vitamin D levels are lower in obese compared to normal weight individuals. It has been shown that high vitamin D concentrations inhibit growth of renal cancer cells *in vitro* (Amling, 2004), suggesting that vitamin D levels could play a role in this connection. Increased arterionephrosclerosis may also provide a mechanistic link, as well as obesity induced hypercholesterolaemia, as cholesterol lowering drugs were protective against renal cancer in mice studies (Pascual and Borque, 2008).

The mechanisms of obesity's involvement in these cancers are very cancer specific. Mechanistic changes with obesity on organs and an increase in inflammation seem to be the most re-occurring features. Obesity-related hormonal changes however, hyperinsulinaemia and adipokine deregulation, may have an important role in mediating the obesity-cancer connection.

1.4 Breast Cancer

Breast cancer is the most common cancer in women in the UK, with one in nine women developing breast cancer in their lifetime and almost one third of all cancers diagnosed in women are breast tumours (Cancer Research UK, 2006). In terms of mortality, breast cancer accounts for 16% of all cancer deaths in UK women, making it the second highest contributor to cancer deaths, after lung cancer deaths (20% of all cancer deaths) (Cancer Research UK, 2007). Though uncommon, in men 314 breast cancer case have also been diagnosed in the UK in 2006 (Cancer Research UK, 2006), with 92 male deaths attributable to breast cancer in the UK in 2007 (Cancer Research UK, 2007). In a study on the population of the 25-member European Union in 2006, breast cancer was the most common form of cancer (30.9% of all cases) and the leading cause of cancer death (16.7% of all cancer deaths) in European women (Ferlay *et al.*, 2007), with similar numbers reported in 2008 (Ferlay *et al.*, 2010).

Breast cancer arises from epithelial cells lining the mammary ducts. Compared to mice, human mammary ducts branch into terminal ductal lobular units, a cluster of small ductules (Figure 1-5 A and B). It is here that the vast majority of breast cancers occur (Figure 1-5 C).

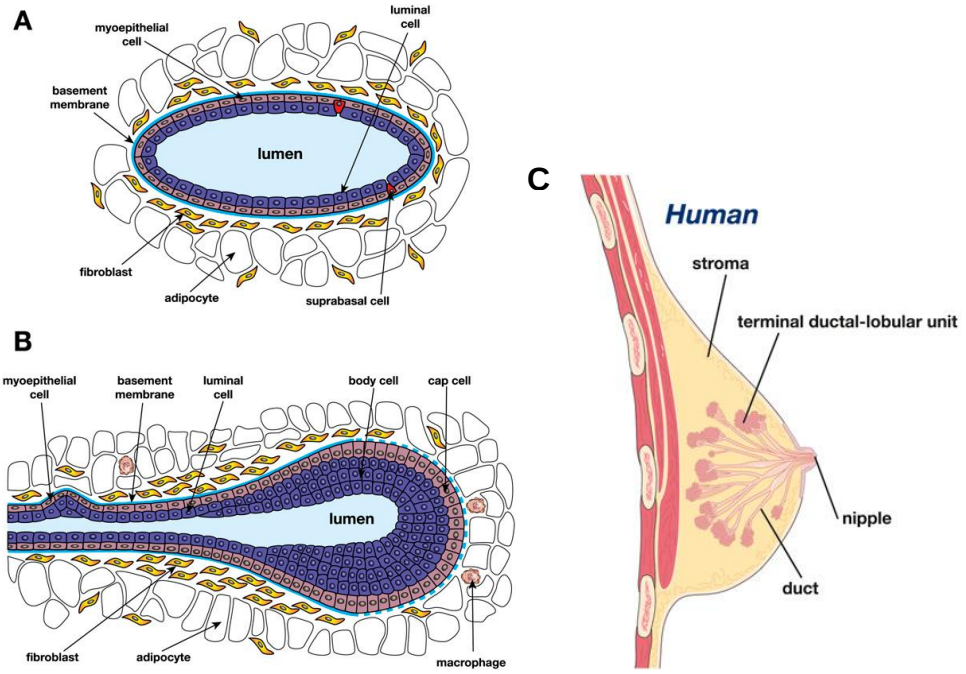

Figure 1-5: Schematic cross section of A) a mammary duct and B) a terminal end bud. C) Schematic longitudinal section of the human breast (Visvader, 2009).

Depending on the area of interest different ways of classifying breast cancer are possible. Breast cancer may be classified according to site of origin, stage of disease progression or on the basis of histological markers and gene expression. Breast cancer may originate in the duct or the lobe of the human breast (ductal or lobular breast cancer). Of 100 breast cancer cases 70-80 would be classified as ductal breast cancer, with the rest being lobular breast cancer. Several rare forms of breast cancer exist (*e.g.* inflammatory breast cancer or Paget's disease), which comprise less than 2% of breast cancer cases (Cancer Research UK, 2009a). Depending on the progression of the disease, a staging of the tumour development might be possible. A simple model divides breast cancer in local or invasive cancer. Local breast cancer means the cancer cells are contained within the lumen of the duct or lobe, while invasive breast cancer has spread

beyond this confinement. Other stages involve the physiological presentation of the breast cancer. One of those clinical staging systems is the Manchester System (Table 1-4).

Table 1-4: Staging of breast tumour according to the Manchester System (Nachlas, 1991)

Stage	Clinical manifestation
1	The primary tumour is confined to the breast, is movable, with the only skin attachment being at the tumour site.
2	Similar tumour features, but there are palpable mobile lymph nodes in the axilla of the same side.
3	The primary growth is more extensive, involving a wider skin area with muscle fixation, but tumour and lymph nodes are not fixed to the chest wall.
4	Extension beyond the breast as shown by chest wall fixation of the tumour and axillary nodes, supraclavicular node involvement, and distant metastases.

The presence or absence of certain genetic markers is another way of classifying breast cancer. The most important determinants are the expression of the oestrogen receptor (ER) with or without progesterone receptor (PgR) expression, and the amplification and overexpression of the human epidermal growth factor receptor (HER2/neu) (Visvader, 2009). Another distinction in breast cancer can be made according to menopausal status of the affected individual. Indeed the differences between post- and pre-menopausal breast cancer are such that it was proposed to describe the two cancers as different diseases (de Waard, 1979). Recently it has been noted that cancers which are classified using the above methods have arisen from very different molecular origins and that gene expression profiling using microarray technology may soon give rise to new molecular classifications of breast cancers (Cianfrocca and Gradishar, 2009).

With breast cancer being a heterogenic disease, with many possible origins and progression, determining clear causes has so far been impossible. Several risk factors have been identified. The most common risk factors are age, lifetime oestrogen exposure (measured by early onset of menarche, late onset of menopause and hormone replacement therapy, oestrogen containing contraceptive), late primiparous (late age at first time giving birth), nulliparous (never having given birth), post-menopausal obesity, low levels of physical activity and alcohol consumption (Table 1 in Feigelson and Henderson, 1996). Breast density is a further risk factor (Boyd *et al.*, 2005; Harvey and Bovbjerg, 2004). Increased calcification of breast ducts, which leads to higher breast density may a factor attributable to obesity. Breast density has also been suggested as a possible heritable factor of breast cancer risk (Boyd *et al.*, 2009).

An investigation into the heritability of breast cancer discovered the functional mutation of breast cancer associated genes-1 and-2 (BRCA-1 and -2) to be associated with increased risk to develop breast and ovarian cancer. If the mutation arises in the germline, hereditary breast cancer occurs. BRCA-1 has been proposed to act as a "scaffolding" protein in the "BRCA1-associated genome surveillance complex" (BASC), which includes the DNA damage sensing gene ATM (Wang *et al.*, 2000). Without proper functioning of this complex, DNA damage can go unnoticed as the cell progresses into mitosis. The tumour suppressor gene TP53 is involved in arresting cell cycle in the presence of DNA damage (Harris and Hollstein, 1993). But in over 90% of BRCA deficient tumours TP53 is dysfunctional through mutation, which may indicate that TP53 mutation is required for BRCA-1 mediated breast cancer and functioning TP53 may be a BRCA-redundant mechanism of DNA damage detection (Schuyer and

Berns, 1999). Mutations in BRCA genes have also been linked to ovarian cancer (Merajver *et al.*, 1995). BRCA mutations explain the existence of hereditary breast cancer, which comprises between 4-9% of breast cancer cases (Blackwood and Weber, 1998; Ellisen and Haber, 1998). Most breast tumours however seem to develop sporadically, which during carcinogenesis does involve the mutation of tumour suppressor genes (like BRCA genes and TP53) and activation (by mutation or epigenetic events) of oncogenes.

1.5 Obesity and breast cancer

Due to the fact that pre- and postmenopausal breast cancers are essentially two different diseases, their connection with obesity is equally different. Several studies have identified either no or even an inverse relationship between obesity and premenopausal breast cancer (Stephenson and Rose, 2003). Reduced ovarian production of oestrogen in premenopausal obese women has been suggested as a reason for this association (Abrahamson *et al.*, 2006). Obesity has however been suggested as a risk factor for premenopausal breast cancer because of the relationship between adiposity and ER-negative breast cancers (Rose and Vona-Davis, 2009). Indeed Daling and colleagues (2001) found that, while premenopausal obese women do not have an elevated risk of developing cancer, obese premenopausal breast cancer patients had higher incidences of ER-negative, thus more malignant tumours, which resulted in longer treatments with less favourable outcomes. Hence obesity may not increase premenopausal breast cancer incidence rates, but may instead increase the severity of premenopausal breast cancer. Obese postmenopausal women have an increased risk of developing breast cancer

(Abrahamson *et al.*, 2006; Lahmann *et al.*, 2004). All further reference to breast cancer will relate to postmenopausal breast cancer specifically, unless otherwise stated.

It has been estimated that women with a BMI over 40 kg/m^2 are more than twice as likely to develop breast cancer compared to women with normal BMI (Calle *et al.*, 2003). In this study breast cancer mortality risk increased by 34% in patients with BMIs between 25-30 kg/m^2, by 63% for BMI 30-35 kg/m^2, by 70% for BMI 35-40 kg/m^2 and by 112% for BMI over 40 kg/m^2 compared to patients with normal BMI between 18.5-25 kg/m^2. McTiernan (2005) concluded that this study alone linked obesity convincingly with breast cancer incidence and mortality. Furthermore the World Cancer Research Fund (2007) found ample evidence to conclude that "there is […] a clear dose-response relationship with robust evidence […] that greater body fatness is a cause of postmenopausal breast cancer" (WCRF, 2007, p293). Additionally a link of increased waist circumference and breast cancer risk has been observed (Huang *et al.*, 1999). Obesity has been linked to several stages of breast cancer increasing susceptibility to, progression of and mortality from breast cancer (Berclaz *et al.*, 2004; Calle *et al.*, 2003; Carmichael, 2006).

Several molecular causes for this relationship have been suggested (Lorincz and Sukumar, 2006). First, obesity may increase the production of oestrogen, which in turn can predispose to and promote progression of (certain types of) breast tumours (the oestrogen connection). Second, obesity mediated hyperinsulinaemia may promote breast cancer, caused by the mitogenic potential of insulin (the insulin connection).

Third, obesity-related deregulation of adipokine secretion may promote breast cancer by adipocytes acting as mitogens or promoting an inflammatory state (the adipokine connection). It is unlikely that only one connection will be solely responsible for mediating the molecular connection. All three connections are probable contributors to the overall obesity-breast cancer relationship. Individually, one may outweigh the others, especially concerning the on-off contribution of the oestrogen receptor. Lastly synergistic effects of these three connections may contribute to increase breast cancer risk. The three connections are now introduced in further detail.

1.5.1 The oestrogen connection

As mentioned breast cancer can be classified into two groups, according to their expression of the oestrogen receptor (ER). Different to other breast cancer associated growth factor receptors (*e.g.* HER2/neu), ER expression is not graded, meaning a particular breast tumour either expresses this receptor or it does not. The ER is a soluble nuclear ligand-activated transcription factor (Dahlman-Wright *et al.*, 2006). Two subtypes with high degree of similarity at the amino acid level are known, termed ERα and ERβ. Upon activation by oestrogen, the ER forms dimers and complexes with hormone-response elements, before promoting transcription of proliferative and survival genes (Klinge, 2000; Safe, 2001).

Oestrogens are a group of steroid hormones, with oestriol, oestradiol and oestrone being the most important ones (Figure 1-6). Oestradiol is the most prominent form of oestrogens, mediating many of the effects of oestrogen on the menstrual cycle.

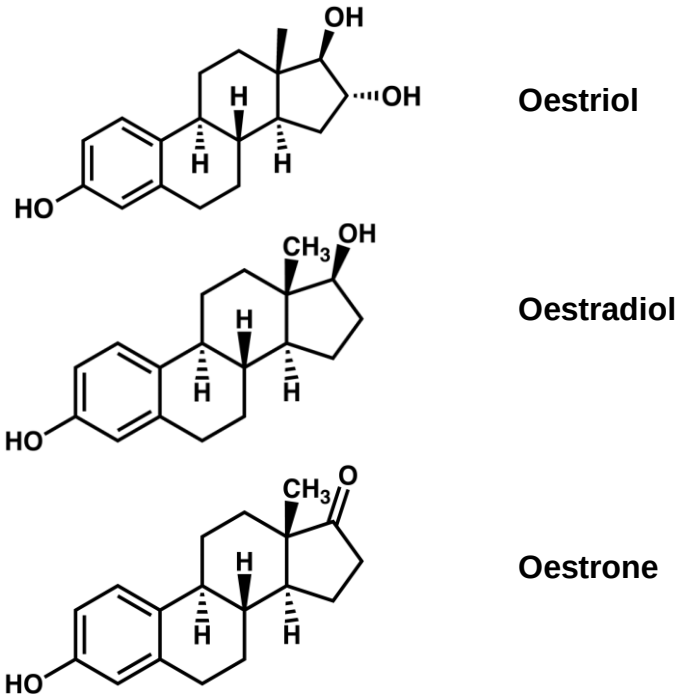

Figure 1-6: Chemical structure of common oestrogens

Before menopause most circulating oestrogen is periodically produced by ovaries, according to menopausal status. Oestrogen concentrations increase in response to luteinising hormone and follicle stimulating hormone secreted by the hypothalamus and decrease in response to progesterone secreted by the corpus luteum (Nussey and Whitehead, 2001). It stimulates differentiation of the endometrium and sexual drive, preparing the female body for the reception of a fertilised oocyte. Menopause is characterised by cessation of the menstrual cycle, due to termination of the delicate interplay of oscillating reproductive hormones, including oestrogen. Oestrogen is still present in the postmenopausal female body, in form of oestrone (Nelson and Bulun, 2001). Oestrogen (oestradiol[25]) is synthesised by conversion of testosterone, catalysed by the enzyme aromatase (Nussey and Whitehead, 2001). Interestingly in

[25] All further references to oestrogen shall be understood to refer to oestradiol specifically, unless otherwise stated.

postmenopausal obese women adipose tissue is the main source of oestrogen, and serum oestrogen concentrations are directly related to BMI (Grodin *et al.*, 1973). This increase in oestrogen concentrations with increased BMI is caused by an overexpression of the aromatase enzyme in stromal adipose tissue cells (Cleland *et al.*, 1985).

Breast cancer risk is increased with increased exposure to oestrogen. Oestrogen exposure is measured by age at menarche and menopause, hormone replacement therapy and the use of oestrogen containing contraceptive (Feigelson and Henderson, 1996). Cohort studies have positively linked oestrogen concentrations with breast cancer risk (Berrino *et al.*, 1996; Toniolo *et al.*, 1995). Exposure of healthy breast epithelial cells to oestrogen increased their potential to become malignant (Dickson and Lippman, 1986). Furthermore breast cancers of obese postmenopausal women are more often ER-positive (Rose *et al.*, 2004).

The exact actions by which oestrogen predisposes to breast cancer development and contributes to breast cancer progression are not entirely known. Oestrogen increases cell proliferation in healthy epithelial cells and ER-positive breast cancer cells[26] (Dickson and Stancel, 2000). Thus anti-oestrogens and ER-inhibitors decrease growth of ER-positive breast tumours and are used in treatment of these tumours (Pearson *et al.*, 1982). Since breast tissue is composed of up to 90% of adipose tissue, a direct cross-talk between adipose cells and breast cancer cells has been suggested (Bulun *et al.*, 1994).

[26] Another concept to explain the action of oestrogen and breast cancer has been the idea that oestrogen metabolites, especially catchol oestrogens, increase the oxidative stress in breast epithelial cells and increase the transcriptional activity of the ER compared to oestrogen alone (Yager, 2000). This would make oestrogen act similarly to an environmental carcinogen.

Indeed oestrogen concentrations inside breast tumours are as much as ten times higher than circulating oestrogen concentrations in post-menopausal women (van Landeghem *et al.*, 1985). Furthermore cancer cells may cross-talk to the surrounding adipose tissue to produce more oestrogen or even produce it themselves (Brodie *et al.*, 1997; Chen *et al.*, 2002).

Breast cancer progression is also stimulated by oestrogen. The vascular endothelial growth factor, responsible for stimulating angiogenesis has been shown to be under the regulation of the ER in ER-positive breast cancer cells *in vitro* (Applanat *et al.*, 2008). Furthermore oestrogen is thought to act as a mitogen on the breast cancer cell increasing its cell cycle and proliferation. Conversely, the presence of the ER in breast cancer cells has also been linked to decreased potential of invasion and metastasis (Platet *et al.*, 2004).

This finding has given rise to a controversy about the origin of ER-negative breast tumours. One suggestion is that all breast tumours start of expressing the ER. As the disease progresses ER expression is consecutively decreased. Thus the cancer has achieved progression to a more malignant state, enabling it to become more invasive and create metastases (Habel and Stanford, 1993). A second idea is that ER status represents different entities of breast tumours, *i.e.* breast tumours start of either expressing the ER or not (Zhu *et al.*, 1997). Several observations support both hypotheses. The finding of ER-mosaicism, *i.e.* ER-positive and ER-negative breast cancer cells in the same tumour, supports the first hypothesis. The finding that

preventive tamoxifen (an ER-inhibitor) treatment only protects against development of ER-positive breast cancer supports the second hypothesis. If both ER-positive and ER-negative breast cancer would start as ER-positive breast cancer, tamoxifen should protect against development of both types of breast cancer. Thus a third hypothesis, combining the other two has been suggested (Kurbel, 2005). ER-positive and ER-negative breast tumours may start off differently, however the ER-positive tumours have the ability to selectively decrease ER-expression. The origin of ER expression is of interest from a treatment perspective. If ER expression is determined at the stage of carcinogenesis, the aetiology of the two types of tumours would be different and thus treatment may have to be adapted.

It has been suggested that so called "triple negative breast cancers" are more prevalent in women with Metabolic Syndrome (Maiti *et al.*, 2009). A triple negative breast tumour does not express ER, PgR and does not overexpress HER2/neu (Irvin and Carey, 2008). Furthermore, while the prevalence of ER-positive breast tumours in obese postmenopausal breast cancers is increased (Rose *et al.*, 2004), there is also an increase in ER-negative breast cancers in postmenopausal obese women compared to postmenopausal women of normal weight (Boyd, 2003). These findings may suggest that obesity induced increased postmenopausal oestrogen production is not the only connection between obesity and breast cancer to affect breast cancer risk (Daling *et al.*, 2001).

1.5.2 The insulin connection

In section 1.2 the current understanding of the insulin resistance–obesity connection has been introduced. Several authors have indicated that the resulting hyperinsulinaemia may be the indicator of the connection between obesity and breast cancer (Boyd, 2003; Lorincz and Sukumar, 2006). Besides its role in glucostasis, insulin is an important growth hormone and can stimulate cell proliferation of a number of tissues (Hill and Milner, 1985; Straus, 1984).

Several studies examined the correlation between C-peptide[27] concentrations and breast cancer. In a meta-analysis, examining C-peptide concentrations and breast cancer risk, only case-control studies showed a positive correlation, while prospective cohort studies did not (Pisani, 2008). The largest of the cohort study however indicated that in the non-fasting postmenopausal sub-group of their study, a positive correlation was observed (Verheus *et al.*, 2006). Additionally, measuring fasting insulin concentrations directly showed an increased risk of breast cancer with high insulin concentrations (Gunter *et al.*, 2009). Similarly in a case control study of Chilean women insulin resistance was identified as a risk factor for developing breast cancer in postmenopausal women (Garmendia *et al.*, 2007). Interestingly, hyperinsulinaemia has also been linked to premenopausal breast cancer (Del Giudice *et al.*, 1998; Goodwin *et al.*, 2002), although this association was not consistently observed (Eliassen *et al.*, 2007). A meta-analysis indicated an increased risk of development of and mortality from breast cancer in

[27] The insulin gene translates not into mature insulin directly, but into a longer peptide, proinsulin, which requires post-translational modification. It needs to be cleaved to obtain mature metabolically active insulin. The other resulting peptide is called C-peptide, which lies in stoichiometric relation to insulin. However different from insulin it has a longer half-live. Thus, theoretically, measuring C-peptide provides a more consistent measurement of insulin resistance (Becker *et al.*, 2009).

diabetic women (Larsson *et al.*, 2007). Additionally recent findings suggest the combined effects of obesity and insulin resistance elevated biochemical markers indicative of increased risk of developing premenopausal breast cancer (Alokail *et al.*, 2009).

The breast cancer cell line MCF-7 is a well-established model of an ER-positive breast cancer. Stimulation with insulin increased its ability to incorporate leucine and thymidine (Osborne *et al.*, 1976), increased fatty acid synthesis (Monaco and Lippman, 1977), increased cell cycle progression (Gross *et al.*, 1984), protected from apoptosis (Geier *et al.*, 1992) and downregulated protein degradation and promoted cell size (Faridi *et al.*, 2003). Insulin treatment of MDA-MB-231 and other ER-negative breast cancer cell lines however did not increase cell proliferation or effect apoptosis (Costantino *et al.*, 1993; Godden *et al.*, 1992), even though, in case of MDA-MB-231 cell line, the insulin receptor content was increased (Papa *et al.*, 1997). This suggested the possibility of these cell lines still responding to insulin in an augmented fashion.

The insulin-receptor (IR) is expressed in many different cell types and studies suggest an over-expression of IR in breast cancer cells (Belfiore *et al.*, 1996a; Milazzo *et al.*, 1992; Papa *et al.*, 1990). In breast cancer cell lines, insulin mediates its effects by activation of the phosphoinositide-3 kinase (PI3-kinase) (Backer *et al.*, 1992; Brown *et al.*, 1999) and the extracellular-regulated kinase (ERK) member of the mitogen-activated protein kinase (MAP-kinase) cell signalling pathways[28] (O'Malley and

[28] see section 1.6 for details on the function of cell signalling pathways and their involvement in breast cancer

Harvey, 2004). In the study presented here, the impact of insulin on breast cancer cells and breast epithelial cells is investigated in chapter 3 and chapter 7.

1.5.2.1 Insulin resistance, insulin-like growth factor-1 (IGF-1) and breast cancer

In section 1.2.1 the complex relationship between obesity and IGF-1 has been introduced. Apart from the obesity-hyperinsulinaemia-IGF-1 controversy, IGF-1 plays an important role in breast cancer development and progression (Moschos and Mantzoros, 2002; Yu and Rohan, 2000). Similar to insulin, *in vitro* and animal studies suggested that IGF-1 activates cell signalling pathways through which it exerts its mitogenic and anti-apoptotic potential. In MCF-7 breast cancer cells, IGF-1 induces activation of PI3-kinase and MAP-kinase pathways (Jackson *et al.*, 1998). Also in this cell line, IGF-1 increased expression of cyclin D, which promotes cell cycle progression (Dufourny *et al.*, 1997). Overexpression of IGF-1 and/or its receptor are particularly effective in promoting tumour growth and one study found that half of all diagnosed primary tumours overexpressed the IGF-1R (Shimizu *et al.*, 2004). In agreement with this finding, Sachdev and colleagues (2003) found reduced tumour growth in MCF-7 xenografts after inhibiting IGF-1 receptor (Sachdev *et al.*, 2003). A mouse model, overexpressing IGF-1, showed abnormal mammary gland development and showed signs of mammary tumour development eight weeks after birth (Carboni *et al.*, 2005).

Several meta-analyses of epidemiological data and cohort studies examined the relationship between IGF-1 and IGFBP-3 (the main IGF-1 binding protein) concentrations and breast cancer risk. A positive correlation between high IGF-1

concentrations and high IGFBP-3 concentrations with premenopausal breast cancer risk was found in two meta-analyses (Renehan *et al.*, 2004; Shi *et al.*, 2004). Furthermore another meta-analysis found a correlation between high IGF-1 concentrations and premenopausal breast cancer, but no correlation with IGFBP-3 concentrations (Sugumar *et al.*, 2004). Fletcher and colleagues (2005) observed no correlation between IGF-1 gene polymorphisms and breast cancer risk. None of these studies would support a connection between obesity, IGF-1 and breast cancer as obesity only affects postmenopausal breast cancer risk and mortality. Interestingly the first two meta-analysis studies found a positive correlation between IGFBP-3 and breast cancer risk (Renehan *et al.*, 2004; Shi *et al.*, 2004), even though bound IGF-1 is thought not to be able to bind its receptor and thus produce a mitogenic signal (Renehan *et al.*, 2006). Conversely results from large cohort studies indicate a correlation between IGF-1, IGFBP-3 and breast cancer in postmenopausal, but not in premenopausal women (Baglietto *et al.*, 2007; Rinaldi *et al.*, 2006; Schernhammer *et al.*, 2006). Interestingly gene polymorphisms in the gut hormone ghrelin were linked to increased concentrations of IGF-1, increased BMI and increased breast cancer risk, providing an insulin independent mechanism for an obesity-IGF-1 connection (Dossus *et al.*, 2008). The authors however were cautious of this particular finding as a similar connection had not been observed previously and needed to be further validated by additional studies. Recently a connection of IGF-1 concentrations and mammary density has been suggested (Diorio *et al.*, 2005). This connection however has been demonstrated in premenopausal women only.

The IR and the IGF-1R are heterodimeric receptors, both of which are overexpressed in breast cancer (Papa *et al.*, 1990; Papa *et al.*, 1993). Thus it has been hypothesised and subsequently demonstrated that their great homology allows these receptors to form hybrid receptors (Belfiore *et al.*, 2009; Moxham and Jacobs, 1992; Pandini *et al.*, 1999; Pandini *et al.*, 2002; Soos *et al.*, 1990). Interestingly these hybrid receptors seem to have a similar affinity for insulin as the IR, but their intra-cellular signalling is similar to the IGF-1R (Belfiore and Frasca, 2008). Thus insulin may mediate certain enhanced mitogenic effects through these hybrid receptors, especially as both receptors are frequently overexpressed in breast cancer cells. It has also been suggested that the IR-IGF-1R hybrid receptor may mediate increased cell proliferation in response to IGF-1 binding (Belfiore *et al.*, 2009).

Not all recent data concerning the insulin and/or IGF-1 connection with breast cancer is supportive of the idea that hyperinsulinaemia, either direct or through IGF-1 stimulation, is the cause for increased breast cancer risk. Insulin alone has little effect on ER-negative breast cancer cells and seems to need a supportive factor to increase breast cancer cell growth (*e.g.* oestrogen; see section 1.5.4). IGF-1 stimulates breast cancer cell proliferation *in vitro* and in mouse models, but epidemiological data is conflicting. Furthermore its connection to obesity is not linear and a correlation to postmenopausal breast cancer was not found. To add to this conflict, insulin-like-growth factor binding protein-3, which decreases free IGF-1 concentrations, has also been positively correlated to breast cancer risk.

1.5.3 The adipokine connection

In addition to the increase in insulin resistance, the hypertrophic-hypoxic-macrophage infiltration axis occurring in obese individuals also promotes abnormal adipokine expression in obese patients (see section 1.1.4.5). It has been suggested that the potential for adipokines to affect breast cancer risk is to increase cell proliferation and/or inhibiting apoptosis[29] (Housa *et al.*, 2006). Adipokine expression and secretion is impaired in obese individuals. Most adipokine concentrations (*e.g.* leptin, TNF-α and IL-6) are proportional to BMI, except adiponectin which is inversely proportional to BMI (Considine *et al.*, 1996; Korner *et al.*, 2005).

1.5.3.1 Leptin and breast cancer

Besides its main involvement in body weight regulation and energy homeostasis, leptin's other functions include stimulation of immune responses (Lam and Lu, 2007; Lord *et al.*, 1998), angiogenesis (Sierra-Honigmann *et al.*, 1998) and reproduction (Masuzaki *et al.*, 1997). Additionally, it has been observed that leptin is also secreted by other cell types, including the stomach (Bado *et al.*, 1998) and the placenta (Masuzaki *et al.*, 1997) but also by breast epithelial cells (O'brien *et al.*, 1999) and has been observed in human milk (Casabiell *et al.*, 1997; Houseknecht *et al.*, 1997). Importantly leptin is also responsible for normal breast development (Hu *et al.*, 2002; Kiess *et al.*, 1999; Neville *et al.*, 2002), suggesting that leptin may be involved in the metabolism of breast epithelial and breast cancer cells. Indeed, leptin has been shown to induce cell proliferation in the human MCF-7 breast cancer cell line by activation of cell signalling pathways associated with cell proliferation and cell survival (Dieudonne *et al.*, 2002).

[29] Adiponectin would constitute the exception, as it has been suggested to protect against breast cancer (see section 1.1.4.5).

This particular study concluded that leptin would act in an endocrine-like or paracrinic fashion on breast cells as leptin may be produced in fat cells around the tumour or further apart and be transported to the tumour site through the blood stream. Additional research found that many breast cancer cell lines are also able to express and secrete leptin themselves (O'brien *et al.*, 1999). This suggests leptin may increase breast cancer growth also in an autocrine manner (see also section 8.5). This interesting suggestion was further validated by the findings of Ishikawa and colleagues (2004), which showed increased leptin receptor expression in 83% of examined breast cancer cells, but not in normal mammary epithelial cells. Additionally leptin expression was detected in 92% of breast cancer samples, but not in normal mammary tissue. Interestingly, 34% of tumours expressing both leptin and leptin-receptor were found to have metastasised, while none of the tumours lacking either leptin-receptor expression or leptin expression developed metastases. Further *in vitro* experiments found consistently that leptin increased cell proliferation in MCF-7, T47D and ZR75-1 breast cancer cell lines (Catalano *et al.*, 2003; Dieudonne *et al.*, 2002; Garofalo *et al.*, 2004; Hu *et al.*, 2002; Laud *et al.*, 2002; Okumura *et al.*, 2002). Interestingly leptin also increased the activity of cell signalling pathways, including the MAP-kinase pathway (Catalano *et al.*, 2003) and the PI3-kinase pathway (Garofalo *et al.*, 2004) and the leptin target JAK2/STAT3 pathway.

In epidemiological studies, correlations between increased leptin concentrations and postmenopausal breast cancer risk have been noted (Cust *et al.*, 2009; Petridou *et al.*, 2000; Tessitore *et al.*, 2004).. Conversely, other studies found no correlation between leptin concentrations and postmenopausal breast cancer risk (Stattin *et al.*, 2004; Woo *et*

al., 2006). Furthermore polymorphisms in the leptin and leptin-receptor genes were also related to an increase in breast cancer risk for pre- and postmenopausal women (Liu *et al.*, 2007; Okobia *et al.*, 2008; Snoussi *et al.*, 2006). Not all studies however confirmed these findings (Teras *et al.*, 2009; Woo *et al.*, 2006).

The findings examining circulating leptin concentrations and breast cancer risk were not unanimous. Ishikawa and colleague's (2004) findings of leptin and leptin receptor expression in breast cancer cells however gave rise to the idea of the possibility of leptin autocrine signalling within the tumour tissue (Vona-Davis and Rose, 2007). This would alleviate the explanation for the inconclusive findings from circulating leptin concentrations, which may not be necessary to increase leptin stimulation, if leptin is produced locally. Indeed increased production of leptin was observed in breast cancer cell lines and breast tumours (Garofalo and Surmacz, 2006; Garofalo *et al.*, 2006; O'brien *et al.*, 1999; Revillion *et al.*, 2006). In the study presented here, the impact of leptin on breast cancer cells and breast epithelial cells is investigated in chapter 4.

1.5.3.2 Tumour necrosis factor-alpha (TNF-α) and breast cancer

Serum TNF-α concentration is positively correlated with BMI and abdominal obesity (Olszanecka-Glinianowicz *et al.*, 2004; Park *et al.*, 2005; Samaras *et al.*, 2010). This cytokine was named after its ability to induce necrosis in the murine fibrosarcoma L-929 cell line, while normal epithelial cells remained unaffected by this action (Carswell *et al.*, 1975; Pennica *et al.*, 1984; Shirai *et al.*, 1985). Similarly, in MCF-7 and T47D breast cancer cells, TNF-α inhibited cell growth (Pagliacci *et al.*, 1993; Pusztai *et al.*, 1993; Rozen *et al.*, 1998). Mice tumour xenografts however did not

reduce significantly in growth after the mice were treated with TNF-α (Balkwill *et al.*, 1986). Furthermore it was noted that TNF-α induced apoptosis in MCF-7 breast cancer cells was depending on functioning TP53 and TP53 knockout led to resistance to TNF-α initiated apoptosis (Cai *et al.*, 1997). TP53 gained interest as more than half of all diagnosed breast tumours had malfunctioning p53 protein, caused by mutations in TP53 (Harris and Hollstein, 1993). Interestingly, TNF-α decreased the expression of the ER in MCF-7 breast cancer cells, which led to a decrease in cell viability (Lee and Nam, 2008). ER down regulation however has been suggested as a sign of increased malignancy (Kurbel, 2005). Furthermore TNF-α may indirectly promote breast cancer growth, as it was observed to be an aromatase inducer, thus increasing oestrogen production and secretion (Purohit and Reed, 2002; Purohit *et al.*, 2002). Additionally TNF-α stimulated angiogenesis, a vital process for tumour progression (Fajardo *et al.*, 1992).

While *in vitro* experiments demonstrated in part the cytotoxic effect of TNF-α on breast cancer cells, epidemiological studies linked increased serum concentrations of TNF-α with increased breast cancer incidences (Mallmann *et al.*, 1991; Sheen-Chen *et al.*, 1997). Additionally, in metastatic breast cancer patients, TNF-α concentrations were negatively correlated with progression free survival and TNF-α concentrations were positively correlated to breast cancer staging (Bozcuk *et al.*, 2004). In patients with locally advanced breast cancer, TNF-α concentrations were negatively correlated to partial and complete responses to neoadjuvent chemotherapy (Berberoglu *et al.*, 2004). Ardizzoia and colleague (1992) observed higher TNF-α concentrations in patients with solid cancer tumour compared to healthy individuals and further increased TNF-α

concentrations in patients in which metastases had formed. Others however did not observe a correlation between TNF-α concentrations and breast cancer incidences (Krajcik *et al.*, 2003). Interestingly the failure of immune cells, particularly macrophages to increase TNF-α production in response to lipopolysaccharide (LPS) stimulation has been linked to decreased survival in breast cancer patients, suggesting a pleiotropic role of TNF-α in cancer biology (Jablonska, 1998; Zielinski *et al.*, 1990). This may suggest that short acting localised bursts of TNF-α secretion by immune cells counteract tumour development and progression, while chronically increased circulating concentrations of TNF-α, as observed with obesity, predisposes to tumour development and enhances tumour growth. In the study presented here the impact of TNF-α on breast cancer cells and breast epithelial cells is investigated in chapter 5.

1.5.3.3 Adiponectin and breast cancer

Besides leptin, adiponectin is the other adipokine that has attracted considerable attention in the obesity breast cancer connection. Adiponectin is an anti-inflammatory and anti-proliferative adipokine and is thought to decrease breast cancer risk and mortality. While other adipokines are also expressed in non-adipose tissues, adiponectin is exclusively expressed and secreted by adipocytes. In fact it is the most abundantly expressed protein of adipocytes (Koerner *et al.*, 2005; Scherer *et al.*, 1995). Adiponectin plasma concentrations are between 3 µg/ml-30 µg/ml (Heliovaara *et al.*, 2006). Interestingly while concentrations of the majority of other adipokines are positively correlated with BMI, adiponectin concentrations are negatively correlated with BMI (Arita *et al.*, 1999). This is also represented in research on adiponectin concentrations in breast cancer patients. Serum adiponectin concentrations were reduced in breast cancer

patients for both premenopausal (Miyoshi *et al.*, 2003) and postmenopausal women (Mantzoros *et al.*, 2004; Miyoshi *et al.*, 2003; Tian *et al.*, 2007; Tworoger *et al.*, 2007). Conversely direct measurement of adiponectin concentrations in breast tissue suggested a positive correlation between adiponectin concentrations and breast cancer risk (Karaduman *et al.*, 2007). In *in vitro* studies on MCF-7 breast cancer cells, it was discovered that adiponectin had an anti-proliferative effect on these cells (Arditi *et al.*, 2007; Dieudonne *et al.*, 2006). One study found that adiponectin increased apoptosis in MCF-7 cells (Dieudonne *et al.*, 2006), while a subsequent study did not (Arditi *et al.*, 2007). In addition, Brakenhielm and colleagues (2004) found that adiponectin induces apoptosis by inhibiting angiogenesis *in vitro* and in mouse xenografts. Breast tumour samples showed expression of the adiponectin receptors, suggesting an involvement of adiponectin in breast cancer growth (Korner *et al.*, 2007). Additionally adiponectin receptor polymorphisms were associated with increased breast cancer risk (Kaklamani *et al.*, 2008). A recently published study on co-treatment of breast cancer cells with different ratios of leptin and adiponectin additionally emphasised the importance of the leptin–adiponectin interplay in the obesity-postmenopausal breast cancer connection (Nkhata *et al.*, 2009). In the study presented here the influence of adiponectin on cell proliferation in breast cancer cells and breast epithelial cells is investigated in chapter 6.

1.5.3.4 Interleukin-6 (IL-6) and breast cancer

Interleukin-6 (IL-6) is another cytokine related to obesity. BMI correlates with circulating IL-6 concentrations and adipose tissue expresses IL-6 (Fried *et al.*, 1998; Vgontzas *et al.*, 1997; Vidal, 2001). IL-6 is a mediator of inflammation initiation, however there is some discrepancy regarding the effect of IL-6 on breast cancer. Both

tumour-promoting and growth-inhibiting effects of IL-6 on breast cancer cell lines and primary breast cancer cells are reported (Knupfer and Preiss, 2007). For example, Basolo and colleagues (1996) reported no increase in cell proliferation after treatment with 10 ng/ml IL-6 for 3 days in primary breast cancer cells. IL-6 treatment of MCF-7 breast cancer cells increased their resistance to doxorubicin, a chemotherapeutic anti-cancer drug (Conze *et al.*, 2001). Similarly IL-6 treatment of ER-negative MDA-MB-231 breast cancer cells increased expression of GP96, a stress protein related to chemotherapy drug resistance (Haverty *et al.*, 1997). Conversely IL-6 induced apoptosis measured by DNA fragmentation, in ER-positive MCF-7 and ZR-75-1 breast cancer cells (Chiu *et al.*, 1996). While the influence of IL-6 on cell survival could not be decisively concluded, additional studies showed that treatment with IL-6 promoted an aggressive phenotype in MCF-7 cells by reducing the expression of ER (Bhat-Nakshatri *et al.*, 2004) and decreasing E-cadherin expression (Asgeirsson *et al.*, 1998). Furthermore IL-6 treatment of breast cancer stem cells and MCF-7 breast cancer cells promoted a hypoxia-resistant and invasive phenotype (Sansone *et al.*, 2007). These authors also demonstrated that these aggressive features were sustained in MCF-7 cells by initiating an autocrine IL-6 signalling loop that was self-sustained for up to two weeks after the initial exposure to IL-6.

Epidemiological studies demonstrated a correlation between high IL-6 concentrations and increased breast cancer risk (Hussein *et al.*, 2004; Kozlowski *et al.*, 2003; Salgado *et al.*, 2003) In fact IL-6 serum concentrations have been correlated with poor disease outcome and reduced prognosis of survival (Salgado *et al.*, 2003; Zhang and Adachi, 1999). Benoy and colleagues (2002) found higher IL-6 serum concentrations in

metastatic breast cancer patients compared to patients suffering from a localised tumour. Similarly high IL-6 concentrations were positively related to decreased survival in metastatic breast cancer patients (Bachelot *et al.*, 2003). Additionally it has been suggested that a combination of hyperinsulinaemia and increase IL-6 concentrations may act synergistically[30] in increasing breast cancer risk (Gonullu *et al.*, 2005).

Single nucleotide polymorphisms in the promoter region of the IL-6 gene were positively correlated to breast cancer risk and disease outcome (Slattery *et al.*, 2007). Particularly the G/C polymorphism at position -174 is associated with breast cancer risk, with C being the recessive risk allele (Gonullu *et al.*, 2007; Hefler *et al.*, 2005; Saha *et al.*, 2003; Slattery *et al.*, 2007). Several other studies however did not find a similar association (Cherel *et al.*, 2009; Litovkin *et al.*, 2007). Since these SNPs are in the promoter region of the gene, it is suggested that their function may have an effect on IL-6 expression and secretion. None of these authors however measured IL-6 concentration between different SNP carriers. In the study presented here the influence of IL-6 on cell proliferation in breast cancer cells and breast epithelial cells is investigated in chapter 6.

1.5.4 The oestrogen, insulin, adipokine interplay

In the obese individual all three possible connections (oestrogen, insulin, and adipokines) may contribute to the link between obesity and breast cancer. It is however increasingly recognised that oestrogen, insulin and leptin may interact in their contribution to the obesity breast cancer connection.

[30] See also section 1.5.4

For example, insulin seems to increase cell proliferation only in ER-positive breast cancer cells. Epidemiologically, insulin resistance, characterised by increased insulin concentrations, has been is linked to increased risk of breast cancer independent of its ER-status (Goodwin *et al.,* 2002). At the same time in *in vitro* studies no connections between ER-negative breast cancer cell and changes in cell proliferation or apoptosis after insulin treatment have been found (Costantino *et al.,* 1993; Godden *et al.,* 1992). On the other hand ER-positive breast cancer cell lines showed increased proliferation after IGF-1 treatment (Karey and Sirbasku, 1988) and insulin (Osborne *et al.,* 1976). Furthermore combined treatment of ER-positive MCF-7 breast cancer cells with IGF-1 and oestrogen promotes cell proliferation synergistically (Dupont and Le Roith, 2001). Additionally IGF-1 can activate the transcriptional activity of ER (Cho *et al.,* 1994). It has also been suggested that IGF-1 is necessary for maximal transcriptional activity of ER (Lee *et al.,* 1999) and the synergistic effect of oestrogen and IGF-1 treatment increased ER activity to a higher extent than either ligand alone (Yee and Lee, 2000). Alternatively, blocking either IGF-1 or oestrogen signalling, decreased growth stimulation of the other in breast cancer cells (Fagan and Yee, 2008). Furthermore IGF-1 was able to increase non-mitogenic processes, such as migration and invasion, in ER-positive and –negative breast cancer cells, but only ER-positive breast cancer cells also showed growth stimulation with IGF-1 treatment (Bartucci *et al.,* 2001; Surmacz and Bartucci, 2004).

One protein that is continually mentioned in the oestrogen-insulin cross talk is insulin receptor substrate (IRS), which is necessary for insulin and IGF-1 signalling (Sachdev and Yee, 2001). In fact ER is a transcription factor for IRS expression (Molloy *et al.,*

2000). When ER-negative MDA-MB-231 breast cancer cells were transfected to express ER, IRS expression stabilised (Morelli *et al.*, 2003). In fact in MCF-7 cells ER and IRS form a complex, as they were found to co-precipitate (Sisci *et al.*, 2007). Conversely insulin signalling increased ER content in ER-positive MCF-7 breast cancer cells (Panno *et al.*, 1996), which was dependent on normal IRS functioning (Ando *et al.*, 1998). Oestrogen treatment also increased insulin sensitivity in MCF-7 breast cancer cells, which increased activation of PI3-kinase and MAP-kinase cell signalling pathways (Mauro *et al*., 2001).

Trastuzumab (herceptin), a novel anti-cancer agent, is a monoclonal antibody that targets the HER2/neu receptor and blocks human epidermal growth factor mediated proliferation signals, in HER2/neu overexpressing breast cancer cells. Combined treatment with herceptin and IGF-1R inhibitors synergistically decreased proliferation in HER2/neu overexpressing breast cancer cells, compared to either treatment alone (Camirand *et al.*, 2002). Furthermore IGF-1 interferes with herceptin action and may contribute to herceptin resistance (Lu *et al.*, 2001). Inhibition of IGF-1R restored sensitivity to herceptin. In a follow-up study it was shown that IGF-1 contribution to herceptin-resistance is predominantly mediated by activation of the PI3-kinase pathway, but not the MAP-kinase pathway (Lu *et al.*, 2004). These findings suggest a connection between activation of HER2/neu receptor by IGF-1. IGF-1 is able to protect from anti-cancer properties of herceptin and may account in part for observed treatment failures using herceptin. Additionally IGF-1 (Knowlden *et al.*, 2005) and high insulin concentrations (Hryniuk *et al*., 2001) may also contribute to tamoxifen resistance.

Leptin increased cell proliferation only in ER-positive breast cancer cell lines (MCF-7; T47D; ZR75-1) (Dieudonne *et al.*, 2002; Hu *et al.*, 2002; Laud *et al.*, 2002; Somasundar *et al.*, 2003). Similarly to insulin and IGF-1 the cell proliferative effects of leptin seem to be connected to the ER, leading to the suggestion of increased cross-talk between either leptin and the ER or Ob-R and ER. Leptin treatment of MCF-7 breast cancer cells decreased the growth inhibitory effect of the novel anti-oestrogen Faslodex™ (ICI 182,780), decreasing ubiquitination and increasing the half-life of ER (Catalano *et al.*, 2004). Additionally, leptin enhanced the expression of aromatase, the enzyme responsible for the production of oestrogen, in MCF-7 breast cancer cells (Catalano *et al.*, 2003; Robertson, 2001). Furthermore in rat adipocytes, oestrogen treatment was able to increase leptin mRNA expression (Machinal *et al.*, 1999). In addition to this, the rate of oestrogen aromatisation and circulating concentrations of oestradiol and oestrone sulphate were positively correlated with leptin concentrations in obese postmenopausal breast cancer patients (Geisler *et al.*, 2007). Conversely, in ER-positive breast cancer cells, oestrogen stimulation also increased the leptin-induced activation of STAT3 (Binai *et al.*, 2009).

Interestingly insulin treatment of insulin unresponsive ER-negative MDA-MB-231 breast cancer cells stimulated increased expression of leptin mRNA, indicating a cross-talk between insulin and leptin (Garofalo *et al.*, 2006). This increase was mediated by HIF-1 (Bartella *et al.*, 2008). MDA-MB-231 cells are “triple-negative” breast cancer cells and their growth stimulation is unknown. With the finding that these cells produce leptin and express the leptin receptor (Garofalo *et al.*, 2006; O'brien *et al.*, 1999; Revillion *et al.*, 2006), it may be possible to conclude that insulin could trigger an

autocrine loop of leptin signalling in these cells. This may be a novel way to explain ER-negative breast cancer growth in general, however no studies examined this possibility, which could be done, for example, by inhibiting Ob-R in breast cancer cells known to express Ob-R and leptin.

1.6 Cell signalling pathways and breast cancer

Insulin, IGF-1 and most adipokines are mitogens, stimulating cell growth. It is highly likely that these factors exert their mitogenic effect by activation of cell signalling pathways known to enhance cell proliferation, survival and decrease apoptosis. Two of the major cell signalling pathways involved in mitogenic mitigation of extra-cellular signals are the phosphoinositide-3 (PI3) kinase pathway and particularly the mitogen-activated protein (MAP) kinase pathway (Figure 1-7).

1.6.1 The Phoshoinositide-3 Kinase (PI-3 kinase) pathway

The PI3-kinase pathway is an intra-cellular kinase cascade, responsible for mediating the effect of extra-cellular signals to stimulate appropriate intra-cellular responses. The interesting function of this pathway is its mediation of insulin signalling in target cells. The PI3-kinase cell signalling pathway is activated by ligand–induced activation of specific receptors at the cell membrane. The type 1A PI3-kinase can be activated by the insulin receptor. When insulin binds the insulin receptor, the insulin receptor substrate (IRS) is phosphorylated (Figure 1-4 and Figure 1-7). Through recognition of the SH_2-binding site, IRS activates type 1A PI3-kinase. PI3-kinase comprises of two subunits, the p110 catalytic subunit and the p85 regulatory subunit. The p85 subunit is localised at the IRS and upon activation complexes with the p110 subunit to form

functioning PI3-kinase. It then phosphorylates phosphatidylinositol (4,5) phosphate (PIP2) to phosphatidylinositol (3,4,5) phosphate (PIP3). The phosphatase PTEN is the corresponding phosphatase (PIP3 to PIP2), effectively regulating PIP3 signalling (Figure 1-4). PIP3 acts as second messenger to activate pleckstrin homology (PH) domain containing protein kinases (PDK) and protein kinase B (PKB/AKT)[31]. AKT is activated by translocation to the membrane and phosphorylation of Thr308 and Ser 473 by PDK1 and 2, respectively (Brader and Eccles, 2004). There are three isoforms of AKT (AKT1, AKT2 and AKT3). AKT-1 was originally found as human homologue to the retroviral oncogene v-akt (Staal, 1987). AKT proteins are serine/threonine kinases, with numerous downstream targets. The insulin-inducible downstream target is GLUT-4 vesicles, which then translocate to the cell membrane to import glucose through endocytosis. Other targets include caspase 9 and BAD, which inhibit apoptosis, MDM2, an inhibitor for p53, and GSK-3, linked to cell survival (Brader and Eccles, 2004; Fresno Vara *et al.*, 2004; McCubrey *et al.*, 2006; Scheid and Woodgett, 2001b).

1.6.1.1 PI-3 kinase pathway and breast cancer

Several studies have examined the role of members of the PI-3 kinase pathway in breast cancer aetiology and progression (Fresno Vara *et al.*, 2004; McCubrey *et al.*, 2006; Scheid and Woodgett, 2001a). Overexpression of AKT1 was frequently found in human breast cancer samples and was required for carcinogenesis of NIH3T3 cells (Sun *et al.*, 2001). Bellacosa and colleagues (1995) observed an increase in AKT2 mutations in breast carcinomas, which was associated with ER-negative tumours, indicating an association with more aggressive tumours (Bellacosa *et al.*, 1995). Additionally

[31] "PKB" and "AKT" are used synonymously for describing protein kinase B in the literature. In the study presented here "AKT" will be used in all further references to protein kinase B.

overexpression of AKT-3 was found in ER-negative breast tumours (Nakatani *et al.*, 1999). Loss of the heterozygosity of the PIP3 phosphatase PTEN[32] (10q23) was found in breast carcinomas expressing markers of poor prognosis (Garcia *et al.*, 1999).

1.6.1.2 PI-3 kinase pathway and obesity

While alterations mediating increased signalling in the PI3-kinase are likely linked in breast cancer progression it is hard to see how this pathway could play a role in linking obesity-mediated signals to increased risk of breast cancer. The reason is that PI3-kinase pathway signalling is downregulated in obese insulin resistant individuals (Asano *et al.*, 2007).

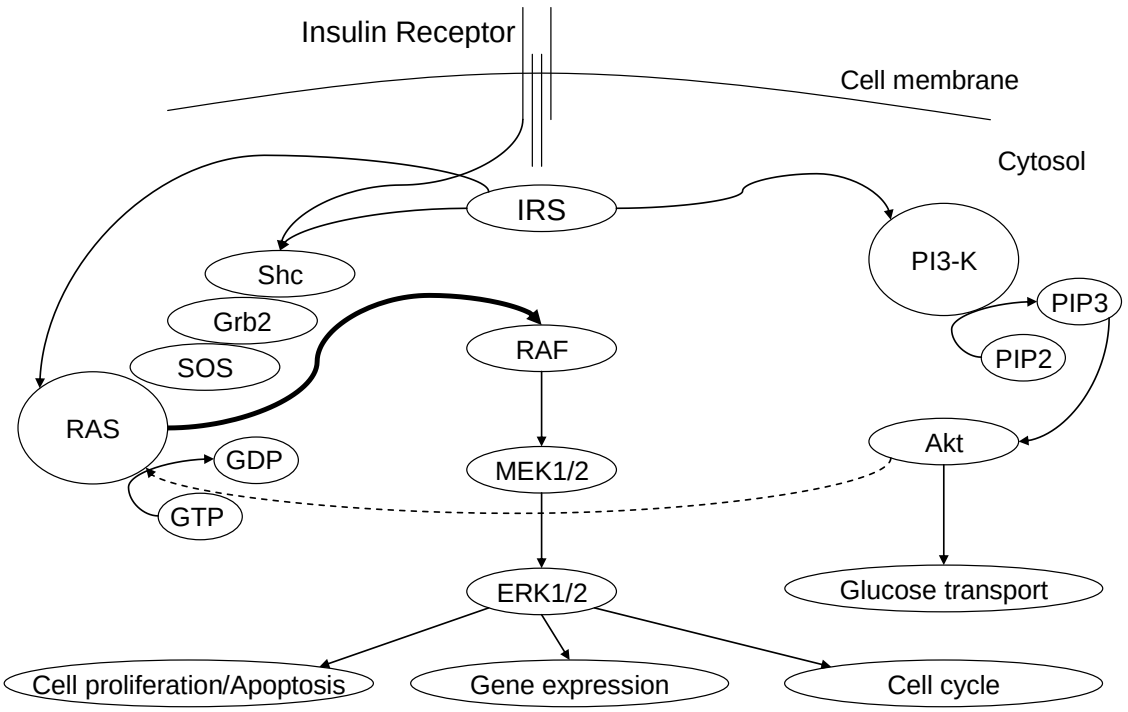

Figure 1-7: Representative overview of the PI3-kinase and MAP-kinase cell signalling pathway, their potential activation by insulin and the physiological effects that are predominantly mediated by these pathways.

[32] See Figure 1-4 for illustration of PTEN's role in regulation of the PI3-kinase cell signalling pathway.

1.6.2 The RAS-mediated MAP-kinase pathway

The MAP-kinase signalling cascade involves three major pathways, which follow the same activation pattern; the RAS[33], the p38 and the JNK-pathway (Santen *et al.*, 2002). While the p38- and the JNK-pathway are generally associated with a decrease in cell proliferation and increased apoptotic rate, the MAP-kinase pathway has been linked to the opposite, an increase in cell proliferation and suppression of apoptosis. The MAP-kinase pathway is activated by the IRS. During insulin resistance, signalling by IRS to PI3-kinase is inhibited. Activation of the MAP-kinase pathway however is not affected in human muscle (Cusi *et al.*, 2000). Thus with normal insulin signalling inhibited, the effects of hyperinsulinaemia may increase MAP-kinase activation in obese insulin resistance individuals. Contrary to this, if RAS activation is mediated by IRS stimulation of the insulin receptor, insulin resistance may also decrease MAP-kinase signalling as insulin resistance is linked to decreased IRS signalling by impaired serine phosphorylation (Qiao *et al.*, 1999; Qiao *et al.*, 2002).

The MAP-kinase pathway comprises of a kinase cascade initiated by a stimulus at the cell surface to stimulate a biological response. Since the main target for this pathway is a transcription factor (ERK1/2), this biological response is usually a change in gene expression. The stimulus can be exerted by growth factors, but might also be triggered by insulin or adipokines. Kolch (2000) examined in detail the protein interactions between each member of this kinase signalling cascade and one may refer to his review for further insights. Receptors on the cell surface are able to pick up the signal and start

[33] This pathway is not consistently named in the literature. Due to its dependence on RAS it has called "RAS-dependent" or "RAS-mediated" pathway. It is also called the ERK1/2 pathway, the RAS/RAF/MEK/ERK pathway or simply the MAPK pathway. Here all future reference to this pathway will be made as "MAP-kinase pathway".

the phosphorylation cascade that is involved in MAP-kinase signalling (Figure 1-7). In case of insulin binding to its receptor, IR undergoes an auto-phosphorylation step followed by activation of kinase domains within the receptor, which phosphorylates non-receptor proteins containing SRC-homology domains, like SHC (Src homology 2 domain-containing). The receptor-SHC complex further binds with another adaptor-protein GRB-2. The final protein complex is completed by acquiring the nucleotide exchange factor SOS. This receptor-SHC-GRB-2-SOS-complex then catalyses the conversion of membrane-bound GDP-RAS to GTP-RAS. The complexion of SHC-GRB-2-SOS is necessary to mediate activation of RAS by *e.g.* the GH receptor, while IR activation of RAS may be achieved in a different way. Since the insulin receptor's primary target is the IRS, additional possibilities for activating RAS may be possible (Figure 1-7). Subsequently, GTP-RAS phosphorylates RAF, the first of the MAP-kinases. RAF then phosphorylates MAPK/ERK-kinase-1 and 2 (MEK1/2). This phosphorylation of MEK1/2 increases its kinase activity 7000-fold (Santen *et al.*, 2002). Additionally, cytoplasm concentrations of MEK1/2 are substantially higher than RAF, which results in an amplification of the signal. Activated MEK1/2 is phosphorylating ERK1/2 at Tyr183 and Thr185 in a TEY-motif (Santen *et al.*, 2002). There is a 1000-fold increase in kinase-activity of ERK1/2 after being phosphorylated, even though no signal amplification takes place at this step, as MEK-1/2 and ERK1/2 concentrations are similar. ERK1/2 then translocate into the nucleus and stimulate downstream events, which are involved in gene regulation, specifically increasing cell proliferation and decreasing apoptosis. The precise downstream mechanism of ERK1/2 in the nucleus is not completely understood. ERK1/2 stimulates activation of other protein kinases from the ribosomal S6 kinase family (RSK). RSK proteins are able to

phosphorylate a number of downstream targets, such as cAMP response element binding protein (CREB), the co-activator CBP, c-Fos, the serum response factor and the oestrogen receptor (Pearson *et al.*, 2001). ERK1/2 may act as a direct transcription factor or activate other transcription factors and as such is a versatile protein. The MAP-kinase pathway may also increase gene expression regulation through interaction with histone-modifying proteins. Changes in chromatin-structure can change gene expression, as deacetylated histone molecules complex with heterochromatin protein 1 to block transcription. Thus activation of histone acetylases (HAT) could increase gene transcription (Dunn *et al.*, 2005). Thus it has been demonstrated that ERK-2 can activate SRC-1 which again activates the HAT protein p300/CBP (Santen *et al.*, 2002). Together the effects of ERK1/2 involve chromatin remodelling and changes in gene expression, by phosphorylation of a number of important factors.

1.6.2.1 MAP-kinase pathway and breast cancer

Several studies indicate a connection between increased signalling in the MAP-kinase pathway and breast cancer (McCubrey *et al.*, 2006; McCubrey *et al.*, 2007; Reddy *et al.*, 2003; Santen *et al.*, 2002). RAS and RAF have both been suggested to act as proto-oncogenes, *i.e.* their normal function is not oncogenic, but changes in expression or mutations may increase their growth promoting potential and thus make them oncogenic. Indeed increased kinase activity, by mutation, in both kinases has been observed in cancer cells. Constitutively active RAS proteins have been found in about 30% of human leukaemia patients (Flotho *et al.*, 1999; Stirewalt *et al.*, 2001). The V600E mutation in the RAF isoform B-RAF has been linked to increased MAP-kinase signalling and carcinogenesis of breast epithelial cells. A mutational analysis of

established breast cancer cell lines found that RAS and RAF isoforms were frequently mutated (Hollestelle *et al.*, 2007). Another significant determinant for this pathway in breast cancer is its cross-talk with the ER (Santen *et al.*, 2002). On the one hand ERK1/2 has been observed to serine phosphorylate the ER and enhance its transcriptional activity (Kato *et al.*, 1995). Similarly, a downstream target of ERK1/2, RSK has similar effects on ER (Joel *et al.*, 1998). It has also been shown that oestrogen stimulation of MCF-7 breast cancer cells increased phosphorylation of ERK1/2 and cell proliferation (Jeng *et al.*, 2000). Similarly, inhibition of MAP-kinase activation by PD98059, a MEK1/2 specific inhibitor, decreased oestrogen induced cell proliferation in MCF-7 breast cancer cells (Lobenhofer *et al.*, 2000). Thus in breast cancer the MAP-kinase cell signalling pathway plays a major role in promoting cell proliferation.

Of additional interest may be that PI3-kinase and MAP-kinase pathways are known to cross-talk. RAS has been observed to activate PI3-kinase (Brader and Eccles, 2004; McCubrey *et al.*, 2006). Conversely there is some indication that AKT can activate the MAP-kinase pathway by phosphorylation of RAF (Zimmermann and Moelling, 1999).

1.6.2.2 MAP-kinase pathway and obesity

Both leptin and insulin have been demonstrated to activate the MAP-kinase pathway in breast cancer cell lines (Alblas *et al.*, 1998; Catalano *et al.*, 2004). In obese insulin resistant mice however a decrease in MAP-kinase activity was observed (Chang *et al.*, 1995). There does not seem to be a direct effect of obesity on MAP-kinase signalling in general. The effect seems to be tissue specific and varied.

1.7 Aim

The main aim of the study presented here is to investigate the molecular mechanisms in an *in vitro* cell system that could explain the observed epidemiological increase in breast cancer incidences and breast cancer mortality in obese postmenopausal women. In particular this study aimed to assess the effect of high concentrations of insulin in a breast cancer and normal breast epithelial cell system (chapter 3 and 7), in addition to the effects of adipokines leptin (chapter 4), TNF-α (chapter 5), adiponectin and IL-6[34] (both in chapter 6). The role played by insulin and these adipokines in cell proliferation, activation of cell signalling pathways, apoptosis and progression of cell cycle has been studied.

[34] For adiponectin and IL-6 only cell proliferation was studied.

Chapter 2
Materials and Methods

2 MATERIALS AND METHODS

2.1 Cell Culture

2.1.1 Cell lines and cell maintenance

Human Caucasian breast adenocarcinoma cells MDA-MB-231 (Cat No: 92020424, passage No: 36) cells were purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK). Cells were harvested from a pleural effusion of a breast cancer patient and subsequently determined not to express ER, PgR (Cailleau *et al.*, 1978) and low levels of the HER2/neu receptor (Kunisue *et al.*, 2000). Additionally these cells express the WNT7B oncogene (Huguet *et al.*, 1994), carry a mutant *tp53* allele and do not express p53 (Negrini *et al.*, 1994). Importantly for this project these cells are reported to have a miss-sense mutation in genes coding for KRAS (G13D) and BRAF (G464V) (Hollestelle *et al.*, 2007). These genes express kinases involved in the MAPK cell signalling pathway. The BRAF G464V increases RAS dependent phosphorylation two-fold (Davies *et al.*, 2002). The G13D mutation in KRAS has been linked to increased microsatellite instability (MSI) in colorectal cancers (Oliveira *et al.*, 2005).

Human Caucasian breast epithelial cells MCF-10A (ATCC No: CRL-10317, passage No: 102) were purchased from the American Type Culture Collection (ATCC, Manassas, USA). This cell line derived from a patient with fibrocystic disease undergoing breast biopsy. Subsequent analysis of the cell lines showed it to be non-tumourigenic in nude mice (Soule *et al.*, 1990). Further investigation led to the conclusion that these cells are characterised as normal breast epithelial cells (Tait *et al.*,

1990). These cells are also ER-negative with wild-type HRAS gene (Debnath *et al.*, 2003).

Human Caucasian breast adenocarcinoma cells SK-BR-3 cells (ATCC No: HTB-30, passage No: 28) were purchased from ATCC. This cell line was established from a pleural effusion of a 43-year old female Caucasian breast cancer patient (Trempe, 1976). This cell line is known to overexpress the product of the HER2/ErbB-2 gene (Hudziak *et al.*, 1997). These cells do not express the ER (Thomas *et al.*, 2005). These cells do not have any of the common mutations in the members of the PI-3 kinase or MAP kinase cell signalling pathways (Hollestelle *et al.*, 2007).

MDA-MB 231 cells and SK-BR-3 cells were routinely cultured in RPMI 1640 Medium (including 25 mM HEPES, 1x Glutamax) (Gibco (Invitrogen), Paisley, UK, Cat No: 72400) supplemented with 10% FCS (Pierce Biosciences, Cramlington, UK, Cat No CHD0413) and 100 U/ml Penicillin and 100 µg/ml Streptomycin (Gibco, Cat No: 15140). MCF-10A cells were cultured in DMEM/F-12 Medium (Bio-Whittaker UK (Lonza Biologics), Slough, UK, Cat No: BE12-7199) supplemented with 5% Horse Serum (Sigma-Aldrich, Gillingham, UK, Cat No: H1138), 10 µg/ml human insulin (Sigma, Cat No: I9278)), 0.5 µg/ml hydrocortisone (Sigma, Cat No; H0888), 20 µg/ml human Epidermal Growth Factor (Invitrogen, Cat No: 13247-051), 100 ng/ml cholera toxin (Sigma, Cat No: 8052), 50 U/ml penicillin and 50 µg/ml Streptomycin (Gibco). All cell lines were cultured adherent in 75 cm^2 tissue culture flasks (Nunc (Fisher

Scientific), Loughborough, UK) at 37ºC with 5% CO_2 supplemented, humidified atmospheric air.

Cells, grown in 75 cm^2 tissue culture flasks, were sub-cultured every 72-96 h for up to 30 passages. To subculture, cells were washed twice with 10 ml sterile 1 x phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10mM H_2PO_4, pH 7.4; Sigma, Cat No: P4417). PBS was then completely removed and 2.5 ml of sterile Trypsin/EDTA solution was added. MDA-MB 231 cells were incubated at 37°C for up to 5 min, SK-BR-3 cells were incubated at 37°C for up to 10 min and MCF-10A cells were incubated for up to 25 min until all cells were detached and suspended in Trypsin/EDTA solution as confirmed by microscopy. Cells were added to 10 ml of their respective growth medium and centrifuged at 1500g for 3 min. Supernatant was discarded and cells were re-suspended in 5 ml of growth medium. Cells were passed several times through a needle to ensure homogeneous single cell suspension. Concentration of cells was determined using a standard counting chamber (Haemocytometer, Neubauer). For routine culture $1x10^6$ cells of MDA-MB 231 cell suspension, $2x10^6$ cells of SK-BR-3 cell suspension and $0.75x10^6$ of MCF-10A cell suspension were transferred to a new 75 cm^2 tissue culture flask containing 15 ml of growth medium. If used for an experiment, cell numbers seeded were dependent on the kind of experiment and are outlined in each experimental description.

2.1.2 Cell cycle synchronisation

Synchronisation of cell cycles for all cell lines was performed by 24 h incubation in serum-free medium before each experiment. Serum-free medium was RPMI 1640

(25 mM HEPES, 1x Glutamax) (Gibco) supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco) for MDA-MB 231 cells and SK-BR-3 cells and DMEM/F12 (Bio-Whittaker) supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin for MCF-10A cells. After an initial incubation period of 24 h to allow cells to attach, growth medium was discarded, cells were washed once in 1 x PBS and subsequently serum-free medium was added and cells were incubated for 24 h. After incubation in serum-free medium, cells were again washed in 1 x PBS. Fresh serum-free medium was given to untreated control cells. Treatment medium was given to treated cells specified for each experiment.

Cells were kept in serum-free medium in an attempt to synchronise the cell cycle within the diverse population of cells. Withdrawal of FCS deprived the cells of hormonal input to keep growing as well as of certain nutrients necessary to maintain cell cycle progression. Cells incubated in serum-free medium should arrest their cell cycle at the G1 stage, essentially going into a quiescent stage known as G0. Thereby a uniform cell population can be obtained. A uniform cell population should react uniformly to any treatment, while a diverse "unsynchronised" cell population will react differently to a treatment.

2.1.3 Cell culture reagents preparations

2.1.3.1 Insulin

Human insulin (Sigma) was supplied as 10 mg/ml stock solution. Before each treatment 58.1 µl 10 mg/ml insulin was mixed with 941.9 µl sterile PBS to create 100 µM insulin stock solution. Insulin was diluted 1:1000 to obtain 100 nM insulin treatment

concentrations. Insulin treatment medium was made by adding 2 µl of 100 µM insulin to 2 ml serum-free medium. Insulin treatment medium was either used directly or further supplemented with cell signalling pathway inhibitors. Fresh 100 µM insulin stock solution was prepared for each experiment.

2.1.3.2 Leptin

Human leptin (Sigma, Cat No: L4146) was supplied as 1 mg lyophilised powder with >97% purity. For reconstitution 10 ml of 15 mM HCl (12.5 µl of 12 M HCl stock in 10 ml ddH_2O) and 10 ml of 7.5 mM NaOH (3 mg solid NaOH in 10 ml ddH_2O) were 0.2 µm filter-sterilised with syringe driven microfilters. According to the manufacturer's instructions leptin was reconstituted in 0.5 ml of 15 mM HCl. After the protein was completely dissolved 0.3 ml of 7.5 mM NaOH was added. The resulting concentration was 1.25 mg/ml or 78.125 µM leptin. The reconstituted leptin was further diluted to 1 µM (128 µl reconstituted leptin solution in 10 ml sterile PBS) and stored at -20°C in 1 ml aliquots. Before treatments aliquots were diluted 1:10 in serum-free medium to obtain 100 nM leptin treatment solution. Further leptin concentrations were obtained by serial dilution.

2.1.3.3 TNF-α

Human tumour necrosis factor-alpha (TNF-α, Sigma, Cat No: T0157) was supplied as 10 µg/ml PBS solution containing 0.1% BSA carrier protein. As recommended by the manufacturer supplied TNF-α solution was further diluted 1:10 resulting in 10 ml of 1 µg/ml TNF-α. Diluent was PBS without further BSA addition. Addition of BSA at 0.1%-1% was recommended by the manufacturer but was omitted as BSA could not be

added aseptically and filter-sterilisation was not recommended by the manufacturer due to possible adsorption of TNF-α onto the filter membrane. The diluted TNF-α solution was stored at -20°C in 1 ml aliquots. Upon commencing treatment, the TNF-α solution was diluted 1:100 in serum-free medium to obtain 10 ng/ml TNF-α treatment solution.

2.1.3.4 Adiponectin

Human adiponectin (R&D systems, Abingdon, UK, Cat No: 1065-AP) was supplied (Lot No: FGJ8608051) in sterile PBS solution containing 50 µg adiponectin. According to the manufacturer's instruction, 0.5 ml sterile PBS was added to make 100 µg/ml solution. Adiponectin stock solution was stored at -20°C in 100 µl aliquots. Treatment medium of 250 ng/ml and 500 ng/ml adiponectin was obtained by diluting reconstituted adiponectin solution 1:400 and 1:200 in serum-free medium, respectively.

2.1.3.5 IL-6

Human IL-6 (Sigma, Cat No: I1395)) was supplied as 10 µg lyophilised powder with >97% purity. IL-6 was reconstituted in 1 ml of sterile ddH_2O and stored at -20°C in 200 µl aliquots. For treatment reconstituted IL-6 solution was diluted 1:1000 in serum-free medium to obtain 10 ng/ml IL-6 treatment solution.

2.1.3.6 Cell signalling pathway inhibitors

Wortmannin is a specific and irreversible inhibitor of the PI3-kinase, which is responsible for mediating normal insulin signalling (see Figure 1-4 and Figure 1-7). It was supplied as 1 mg lyophilised powder (Calbiochem (Merck), Darmstadt, Germany, Cat No: 681675) and reconstituted in 466.9 µl dimethylsulfoxide (DMSO; Sigma) to

create 5 mM wortmannin stock solution. The stock solution was further diluted 1:1000 in PBS and stored in 100 µl aliquots as 5 µM solution at -20°C. Thawed aliquots were diluted 1:50 in serum-free medium to obtain 100 nM wortmannin treatment solution. Wortmannin treatment solution was used alone for 1 h pre-treatment or in combination with 100 nM insulin.

The MAP kinase inhibitor PD98059 (Cell Signalling Technology (New England Biolabs UK), Hitchin, UK, Cat No: 9900) is selective to inhibit RAF-mediated phosphorylation of MEK, the ERK-kinase responsible for activation of ERK1/2, thus inhibiting MAP-kinase signalling. It was supplied as 1.5 mg lyophilised powder and, upon reconstitution, was dissolved in 280 µl DMSO to create 20 mM stock solution. Then 280 µl 20 mM PD98059 was dissolved with 4.2 ml PBS and stored in 100 µl aliquots as 1.25 mM stock solution at -20°C. PD98059 readily dissolved in DMSO but required rigorous vortex for several minutes to completely dissolve in PBS and aliquots needed to be vortexed rigorously after being thawed to dissolve all precipitate. PD98059 stock solution was diluted 1:25 in serum-free medium to create 50 µM treatment medium.

2.2 Assessment of cell proliferation

2.2.1 Assessment of cell proliferation by BrdU incorporation

Cell proliferation was detected using a colorimetric Cell Proliferation ELISA Kit (Roche Diagnostics, Penzberg, Germany, Cat No. 11 647 229 001), which assesses DNA replication by measuring bromodeoxyuridine (BrdU) incorporation (Figure 2-1). This assay can be used as non-radioactive alternative to [^{3}H]-thymidine-incorporation as

a measure of newly synthesised DNA. BrdU is a thymidine analogue and, as such, is incorporated into newly synthesised DNA (Figure 2-2).

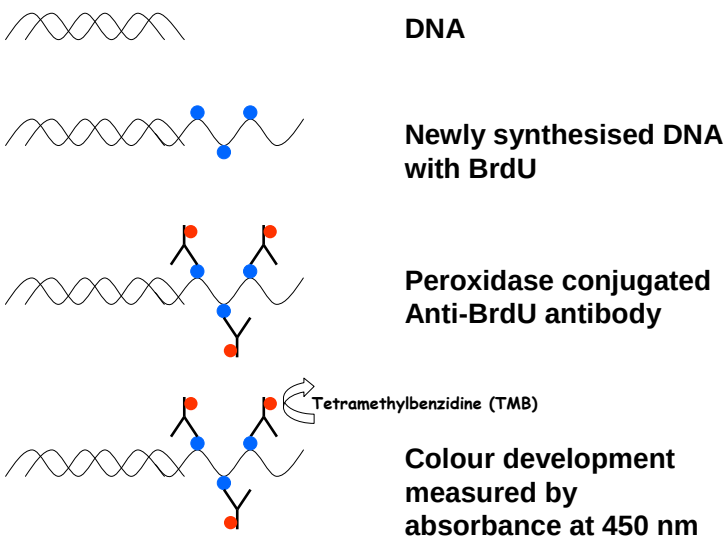

Figure 2-1: Overview of cell proliferation analysis

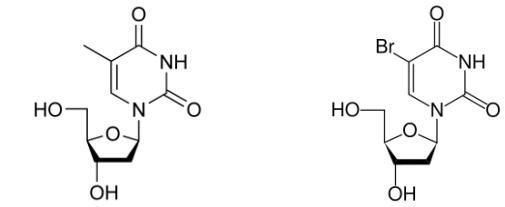

Figure 2-2: Comparison of chemical structure of deoxythymidine and bromodeoxyuridine (BrdU).

The amount of BrdU incorporated into the DNA is proportional to the amount of DNA synthesis during chromosome replication. Chromosome replication is an accurate measurement of cell proliferation as replication only takes place during the S-phase of the cell cycle, indicating a progression from the quiescent G0/G1 stage. After

incorporation BrdU is detected by a specific anti-BrdU antibody coupled with a peroxidase enzyme. The peroxidase enzyme then turns a colourless substrate into a green product. This colour reaction is stopped by adding sulphuric acid after a determined timepoint to allow consistent measurement between each experiment. The amount of colour development is then quantified on a spectrophotometer and is directly proportional to the amount of BrdU antibody bound, to the amount of BrdU incorporated and to the proliferation of the cells. The amount of developed product is measured at 450 nm with a correction wavelength of 690 nm.

2.2.1.1 Detection of incorporated BrdU

BrdU was supplied as 1 ml of 1000x concentrate, which was diluted in 100 ml PBS (10x = 100 µM BrdU), 0.2 µm filter-sterilised and stored at -20°C in 5 ml aliquots. Before treatment 500 µl of 100 µM BrdU was added to 4.5 ml serum-free medium resulting in 10 µM BrdU for each treatment. The BrdU antibody was supplied as an unspecified amount of lyophilised powder, reconstituted in 1.1 ml ddH_2O as recommended and stored at -20°C in 100 µl aliquots. Before analysis, the reconstituted antibody was diluted 1:100 in supplied "antibody dilution solution". No information was given by the supplier on the composition of the "antibody dilution solution" and about the final concentration of the BrdU antibody supplied with the kit.

After discarding the treatment medium, the treated plates could be kept for up to 7 days at 4°C according to the manufacturer's protocol. This did not lead to a break-down of incorporated BrdU. If 48 h and 24 h treatments were performed simultaneously, the 24 h plate was usually kept at 4°C overnight. BrdU detection was started by adding

200 µl/well supplied fixing/denature solution and incubating for 30 min at room temperature. This step makes the cells permeable for BrdU-antibody. No information about the composition of the fixing/denature solution was available. During fixing, 50 µl of reconstituted BrdU-antibody was diluted 1:100 in 5 ml supplied "antibody dilution solution". The fixing/denature solution was discarded and immediately 100 µl of diluted antibody solution was added to each well and incubated for 90 min at room temperature. The antibody solution was completely removed. Supplied concentrated wash solution was diluted 1:10 and wells were washed three times with 250 µl wash solution per well. Wash solution was PBS. After discarding the last wash, 100 µl substrate solution was added to each well. The supplied manual identified substrate solution to contain tetramethylbenzidine. Cells were incubated for 10 min at room temperature. The colour reaction was stopped by adding 25 µl 1 M H_2SO_4 to each well and gentle shaking for 1 min. Absorbance was immediately measured at 450 nm with a reference wavelength of 690 nm. Using this method the effect of several treatments on cell proliferation was analysed (Table 2-1).

Table 2-1: Summary of cell proliferation experiments using BrdU incorporation. NA-Not analysed

Treatment	Treatment Concentration	Treatment time	Inhibitors (incl. 60 min pre-treatment)	BrdU (10 µM) added	Chapter
Insulin	100 nM	24h	100 nM Wortmannin, 50 µM PD98059	immediately	3
Insulin	100 nM	48h	NA	after 24 h	3
Leptin	100 nM	24h/48h	NA	immediately	4
TNF-α	10 ng/ml	24h/48h	NA	immediately	5
IL-6	10 ng/ml	24h/48h	NA	immediately	6
Adiponectin	250 ng/ml 500 ng/ml	24h/48h	NA	immediately	6

2.2.2 Assessment of cell proliferation by MTT-turn-over

In addition to assessment of cell proliferation using BrdU incorporation, cell proliferation in MDA-MB-231 cells was also assessed using the MTT-assay. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), a yellow tetrazole salt can be reduces to purple Formazan by mitochondrial NADH-dependent dehydrogenases (Figure 2-3). Mitochondrial activity is assumed to reflect the rate of cell metabolism and thus the rate of Formazan formation is directly representative of cell viability.

DMSO was used to solubilise hydrophobic Formazan and create a uniform colour solution. Soluble Formazan has its highest absorption value at wavelength between 500 nm and 600 nm, thus Formazan production can be quantified colourimetrically.

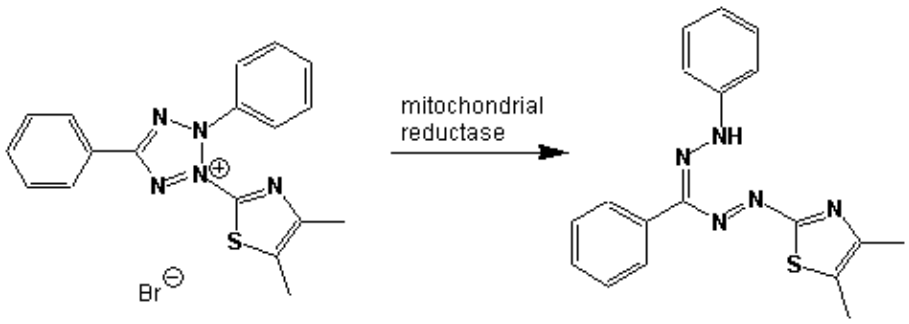

Figure 2-3: Reduction of MTT to Formazan in viable cells

MDA-MB-231 breast cancer cells were plated in 96-well plates at $5x10^3$ cells/well, incubated in serum-free medium and treated as described in section 7.2.2.1. Four hours before the end of the 24 h or 48 h treatment with 100 nM insulin, 1 mg/ml MTT was added to cells. At the end of the total treatment time, all medium was removed and

200 µl DMSO was added to each well. Plates were incubated on an orbital shaker for 15 min in the dark. Then absorption was determined at a wavelength of 560 nm.

2.3 Assessment of AKT-phosphorylation and ERK1/2-phosphorylation after insulin treatment

Cell-based ELISA Phospho-AKT (S473) Immunoassay (Cat No: KCB887) and Phospho-ERK1/ERK2 (T202/Y204) Immunoassay (Cat No: KCB1018) were used and purchased from R&D Systems (Abingdon, UK). Both kits work on the same principle and with the same chemicals except the difference in antibodies. These assays quantify the phosphorylation and expression of AKT and ERK1/2 directly on the cells without the necessity of extracting the protein first (Figure 2-4). Phosphorylation of either protein indicates activation of their respective pathway. The whole assay is performed in a supplied 96-well plate. Specific antibodies binding the total or phosphorylated protein are used and each specific antibody is tagged with a different enzyme. Two substrates produce a fluorescent product specific for each enzyme. Each substrate can only and exclusively be processed by one of the two enzymes. Each reaction creates a product that has a different excitation and emission wavelength, allowing for simultaneous detection of phosphorylated and total protein. Measuring total and phosphorylated protein allows a direct determination of phosphorylation changes independent of variables that change total protein concentrations.

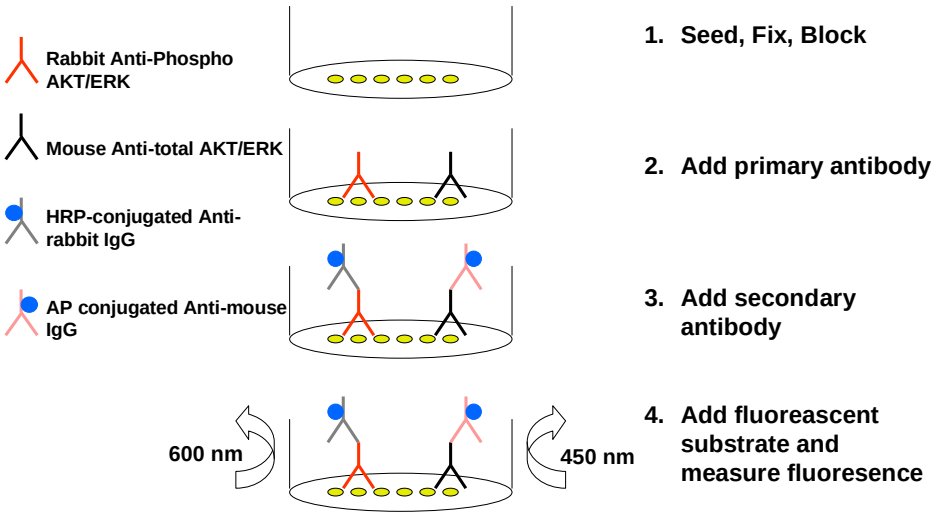

Figure 2-4: Overview of detection assay of phosphorylated and total protein expression of cell signalling kinases AKT and ERK1/2

Each kit required preparations to be carried out before the start of the first experiment. The primary antibodies were supplied as freeze dried powder. The phosphorylated and total antibody were reconstituted in 110 µl sterile PBS each. The reconstituted antibodies were then stored in 10 µl aliquots at -20°C. The supplied 5x wash buffer concentrate (60 ml) was added to 240 ml sterile PBS and stored at 4°C. According to the manufacturer's manual, the wash buffer contained an unspecified amount of "a buffered surfactant with preservatives". The "Substrate F1 concentrate" (50 µl) was transferred to the provided "F1 diluent" (10 ml). The prepared "substrate F1 diluent" was stored at 4°C.

After treatment the cells were fixed in 4% Formaldehyde (1.3 ml 37% formaldehyde (Sigma) and 10.7 ml sterile PBS) solution for 20 min at room temperature. After fixing the cells, the formaldehyde solution was removed and cells were washed three times in 200 µl/well wash buffer. Each washing step was performed for 5 min with gentle

shaking on an orbital shaker. During the washing step, the "quenching buffer" was prepared by adding 200 µl 30% hydrogen peroxide (H_2O_2, Sigma) to 9.8 ml washing buffer. After the last wash was discarded, 100 µl of quenching buffer was added to each well and the plate was incubated for 20 min at room temperature. After this incubation, the quenching buffer was discarded and the cells were washed as before. After discarding the last wash, 100 µl/well supplied blocking buffer was added and the plate was incubated for 1 h at room temperature. The blocking buffer contained 10% FCS. After 1 h incubation, the blocking buffer was discarded and the cells were washed as before. Meanwhile the primary antibody working solution was prepared by diluting each primary antibody (phosphorylated and total protein) 1:1000 in blocking buffer. After discarding the last washing step, 100 µl of primary antibody working solution was added to each well, except in the "no primary antibody"-wells, which were re-supplemented with 100 µl of blocking buffer. The plate was sealed with a plate sealer and incubated overnight at 4°C. After the overnight incubation, the primary antibody solution was discarded and cells were washed as before. Meanwhile the secondary antibody solution was prepared by diluting each secondary antibody (horseradish-peroxidase (HRP)-conjugated secondary antibody to detect phosphorylated protein specific primary antibody and alkaline phosphatase (AP)-conjugated secondary antibody to detect total protein specific primary antibody) 1:1000 in blocking buffer. After discarding the last wash, 100 µl of the secondary antibody solution was added to each well including the "no primary antibody"-wells and incubated for 2 h at room temperature. After this incubation, cells were washed twice with 200 µl/well washing buffer and twice with 200 µl/well sterile PBS. Each washing step was performed for 5 min with gentle shaking on an orbital shaker. After discarding the last wash,

75 µl/well diluted substrate F1 was added to each well and the plate was incubated for 30 min at room temperature. The provided protocol advises to protect the plate from light at this stage, so the plate was wrapped in aluminium foil. After 30 min incubation, 75 µl/well substrate F2 was added and incubated for an additional 30 min. Fluorescence was immediately measured on Fluoroskan Ascent (Labsystem, UK) with excitation at 544 nm and emission at 590 nm, followed by a second read with excitation at 355 nm and emission at 460 nm. The 590 nm read represents the amount of phosphorylated AKT or phosphorylated ERK1/2, while the readings at 460 nm represent the amount of total AKT or total ERK1/2 in the cells. The supplied protocol advises to read the phosphorylated protein with excitation at 540 nm and emission at 600 nm and the total protein with excitation at 360 nm and emission at 450 nm. The fluorescent plate reader that was used does not allow to read at exactly these wavelengths so the above mentioned wavelength were used instead, which were reasonably close to the advised wavelength and should not affect the outcome as relative values were desired.

2.4 Quantification of protein concentrations

Protein concentrations were determined using the *DC* Protein Assay Kit (BioRad, Hertfordshire, UK, Cat No: 500-0116) irrespective of extraction method. This kit is based on measuring protein concentrations using the method first described by Lowry and colleagues (1951). Protein standards (0.5 mg/ml, 0.75 mg/ml, 1 mg/ml, and 1.25 mg/ml) were made from BSA (Sigma) in the same lysis buffer used for protein extraction for each experiment. In a 96-well plate 5 µl of standards in replicates of eight and of 1:2 dilutions of samples in replicates of 4 were plated. Then 25 µl of solution A (containing alkaline copper tartrate) was added to each well, immediately followed by

200 µl solution B (containing folin) in each well. Plates were then incubated for 15 min at room temperature. Absorption was measured at 650 nm.

2.5 Insulin receptor phosphorylation assay

The protein expression of insulin receptor in the cell culture model was assessed by western blotting (see section 2.6), the insulin receptor (IR) phosphorylation however was examined using DuoSet IC human phospho insulin receptor kit (R&D systems, Cat No: DYC2718). The principle of this assay was to isolate the insulin receptor from a whole cell lysate and then probe the isolated insulin receptor protein with a phospho-tyrosine specific antibody to test for phosphorylation. To isolate the insulin receptor from the whole cell lysate, an insulin receptor capture antibody is immobilised on the bottom of 96-well plate well. The cell lysate is then put on top with only the insulin receptor binding to the antibody and all other proteins being washed away in subsequent washing steps. The amount of phosphorylation is then examined by incubating the bound insulin receptor with a phospho-tyrosine antibody, which binds exclusively to phosphorylated tyrosine residues. This secondary antibody is tagged with a peroxidase enzyme and the same colour reaction as described for the BrdU incorporation assay (section 2.2.1.1) can be used to examine the amount of phosphorylated insulin receptor.

This kit required a number of solutions to be prepared and reconstitution of the supplied capture antibody. The manufacturer's instructions suggested using PBS with 8.1 mM H_2PO_4. Instead PBS, containing 137 mM NaCl, 2.7 mM KCl and 10 mM H_2PO_4, was used, which is the same PBS concentration used throughout the project. PBS was

0.2 µm filter sterilised. Wash buffer was prepared containing 0.05% Tween in PBS. Blocking buffer contained 1% BSA and 0.05% NaN_3 in PBS. Buffer #12 contained 1% NP-40, 20 mM Tris, 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM activated Na_3VO_4. Buffer #14 contained 20 mM Tris, 137 mM NaCl, 0.05% Tween, and 0.1% BSA. Lysis Buffer #9 contained 10 µg/ml Aprotinin and 10 µg/ml Leupeptin in Buffer #12. Blocking buffer and buffer #14 were stored at 4°C. Buffer #12 and #9 were made fresh for each new experiment. Stock solutions of 4 M NaCl, 1 M Tris (pH8), 0.5 mM EDTA were stored at room temperature; stock solution of 1 M Na_3VO_4 was kept in 200 µl aliquots at -20°C. Aprotinin (Sigma, Cat No: A6279) and Leupeptin (Sigma, Cat No: L4146) were kept at -20°C in aliquots of 1.4 mg/ml and 2.5 mg/ml, respectively. The supplied insulin receptor capture antibody was reconstituted in 200 µl PBS and 20 µl aliquots were stored at -20°C. The reconstituted antibody concentration was 1440 µg/ml. The supplied phospho-insulin receptor control protein was reconstituted by adding 500 µl buffer #12 to yield 240 ng/ml protein. The control protein was prepared immediately before its use in the assay and reconstituted control protein was not stored.

After treatment of cells, 250 µl Lysis Buffer #9 was added and cells were incubated on ice for 5 min. Cells were then suspended in Lysis buffer #9 using a cell scraper and transferred to sterile microcentrifuge tubes. Cell suspensions were sonicated for 15 sec on ice. Then cells were centrifuged for 5 min at 14000 x g at 4°C. Supernatant was transferred to new microcentrifuge tubes and immersed for 10 sec in liquid nitrogen before being stored at -80°C. Protein concentrations were assessed as described in section 2.4 with protein standard being made in buffer #12.

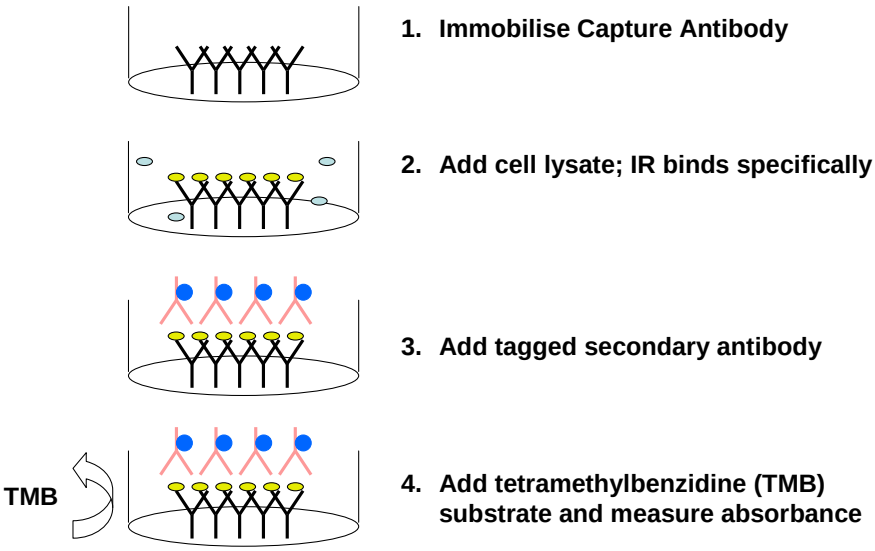

Figure 2-5: Overview of the detection of phosphorylated insulin receptor

Once the total amount of protein was determined for each sample, the 96-well ELISA-microplate was prepared (Figure 2-5). The capture antibody was diluted in PBS to a working concentration of 8 µg/ml. The wells of a 96-well plate were then coated with 100 µl/well of diluted capture antibody. The plate was sealed with a plate sealer and incubated overnight at room temperature. Each well was then washed five times with 400 µl/well wash buffer using a squirt bottle. After removing the last wash, the plate was inverted and blotted against a paper towel to remove residual wash solution. Then 300 µl/well of blocking buffer were added and, after applying a plate sealer, the plate was incubated for 2 h at room temperature. The wells were then washed five times as described before. The cell lysate samples (100 µl) were then added at a concentration of 1 mg/ml resulting in 100 µg protein per well (Figure 2-5). Necessary dilutions of samples were made in buffer #12. Buffer #12 alone was used as negative control. Control protein was diluted to a working concentration of 8 ng/ml and added as positive

control. The plate was then covered with a plate sealer and incubated for 2 h at room temperature. Then the wells were washed five times as described before. The secondary antibody was diluted 1:1000 in Buffer #14 and 100 µl of diluted secondary antibody was added to each well (Figure 2-5). The plate was sealed with a new plate sealer and incubated for 2 h at room temperature. The manufacturer's instructions advise to avoid placing the plate in direct light, so the plate was wrapped in aluminium foil at this point. The wells were washed five times as described previously. Stable peroxide solution and tetramethylbenzidine solutions (R&D systems, Cat No: DY999) were mixed 1:1 and 100 µl of this mixture was added to each well (Figure 2-5). The plate was again wrapped in aluminium foil and incubated for 20 min at room temperature. Directly before measuring absorption 50 µl of 1 M H_2SO_4 was added to each well. Absorption was then read at 450 nm with a correction wavelength of 540 nm.

2.6 Assessment of protein expression and/or phosphorylation by western blotting analysis

Western blotting is a method used to identify phosphorylation and/ or total expression of a specific protein within a whole protein extract. Most proteins are negatively charged and will travel in an electric current towards the anode. This principle is exploited by letting the proteins travel through a polyacrylamide gel on an electric current. The polyacrylamide gel forms a uniform grid acting as an obstacle to the travelling proteins. The smaller the protein, the quicker it will travel through the polyacrylamide grid and *vice versa*, allowing separation of proteins according to their size. The proteins are then immobilized by transferring them on a polyvinylidene difluoride (PVDF) membrane using the same principle of proteins travelling in an electric current. The protein detection is based on a similar principle as the cell

signalling ELISA analyses. A primary antibody is used to detect the protein of interest, and then a secondary antibody tagged to an enzyme recognises the primary antibody. Enzyme detection is then accomplished by adding enzyme substrate to the membrane, which develops a chemiluminescent signal that can be detected by exposing the membrane to an X-ray film or by CCD camera. Quantitative analysis of the intensity of the chemiluminescent signal allows direct measurement of the concentration of protein present in the sample. After the protein of interest was quantified the membranes were re-examined for the expression of β-actin. Expression of this protein is universal and not affected by treatment, thus it can act as control for sample loading and detection differences. Membranes were therefore "stripped" of the original antibodies using a mild stripping buffer (Pierce). If phosphorylated protein was examined, the membranes were not re-examined for β-actin but instead were re-examined for the corresponding total protein, following the same principle as the phosphorylation ELISAs.

2.6.1 Protein extraction

After treatment, cells were washed once with sterile PBS before 250 µl 1x Lysis Buffer (Cell Signalling Technology, Cat No: 9803; 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 µg/ml Leupeptin) supplemented with 1 mM phenylmethanesulfonyl fluoride (PMSF, Sigma, Cat No: P7626) was added to each dish. After incubation on ice for 5 min, cells were then suspended by scraping, transferred to 1.5 ml microcentrifuge tubes and sonicated for 15 sec on ice. Protein extract was centrifuged at 14000 x g for 10 min at 4ºC and supernatant was transferred to new microcentrifuge tubes. If not immediately used, tubes with protein extracts were

immersed in liquid nitrogen for 10 s and stored at -80ºC. Protein concentrations were determined using the *DC* Protein Assay Kit (BioRad) following the method described in section 2.4. BSA protein standards were made in 1% Triton solution.

2.6.2 Protein electrophoresis by SDS-PAGE

Whole protein extracts (25 µg) were subjected to SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE). Resolving gels contained 375 mM Tris (pH 8.8), 0.1% SDS, 0.05% Ammonium Persulfate and 0.05% TEMED and different concentrations of acrylamide. The acrylamide concentrations and electrophoresis times for specific proteins of interest are summarised in Table 2-2. Acrylamide concentrations and electrophoresis times had to be adapted for each protein according to their molecular weight and to obtain clearly defined signals for each protein. Stacking gels contained 125 mM Tris (pH 6.8), 6% acrylamide, 0.1% SDS, 0.05% Ammonium Persulfate, 0.1% TEMED. All electrophoreses were performed at 150 V in 1x Running Buffer (250 mM Tris, 35 mM SDS, 1.92 M Glycine).

Table 2-2: Acrylamide concentrations and electrophoreses times for examined proteins

Protein	Acrylamide concentration in resolving gel [%]	Electrophoresis time [min]
Caspase 3	12	60
Cleaved Caspase 3	17	90
ERK1/2	15	90
Insulin Receptor	9	90
JC1	9	90
MCM2	9	70
PI3 Kinase p85	9	90
PCNA	9	60

2.6.3 Western Blotting

Separated proteins were transferred to PVDF membrane (Amersham Bioscience, Little Chalfont, UK) by lateral electrophoresis. Membranes had to be completely immersed in 100% Methanol and then washed in ddH_2O water before being used. Transfers were executed at 360 mA for 45 min in ice-cold 1 x Transfer Buffer (250 mM Tris, 35 mM SDS, 1.92 M Glycine, 20% Methanol). Membranes were then blocked for up to 3 h in 1 x TBS (20 mM Tris, 137 mM NaCl, pH 7.6) plus 0.01% Tween-20 (TBST) and 5% skimmed milk. Membranes were then washed three times in 25 ml TBST for 5 min with gentle rocking. Then 10 ml primary antibody dilution (5% BSA in TBST, see Table 2-3) was added and membranes were incubated overnight at 4°C on a revolving tray. Membranes were washed as before. Then membranes were incubated in 10 ml secondary antibody dilution (5% skimmed milk in TBST with 1:2500 dilution; Table 2-3) for 1 h at room temperature. Anti-mouse secondary antibodies (sc-2005) and anti-rabbit secondary antibodies (sc-2004) were from Santa Cruz Biotechnology (Santa Cruz, USA). Membranes were washed as before. Horseradish-peroxidase (HRP) conjugated antibodies were detected using ECL Western Blotting Substrate Kit (Perbio Bioscience (Pierce), Cramlington, UK). For each membrane, 2 ml of Substrate A was mixed with 2 ml of Substrate B. The membrane was completely covered in mixed solution and incubated for 5 min at room temperature. Then excessive substrate solution was discarded and the membranes were wrapped in cling film before being analysed by X-ray films and CCD-cameras. Densitometry data was obtained using GelDoc CCD imaging (Bio-Rad) and Quantity One software (Bio-Rad).

Table 2-3: Source, manufacturer and primary and secondary antibody dilutions used for each protein examined.

Protein	Source	Manufacturer of primary antibody (Cat No.)	Primary antibody dilution	Secondary antibody dilution
Caspase 3	rabbit	Cell Signalling Technology (#9662)	1:1000	1:2500
Cleaved Caspase 3	rabbit	Cell Signalling Technology (#9664)	1:200	1:2500
Phosphorylated ERK1/2	rabbit	Cell Signalling Technology (#9102)	1:2000	1:2500
total ERK1/2	rabbit	Cell Signalling Technology (#9101)	1:1000	1:2500
Insulin Receptor	rabbit	Santa Cruz (sc-711)	1:1000	1:2500
JC1	mouse	Santa Cruz (sc-53424)	1:4000	1:2500
MCM2	rabbit	Santa Cruz (sc-10771)	1:1000	1:2500
PI3 Kinase p85	mouse	Santa Cruz (sc-1637)	1:1000	1:2500
PI3 Kinase p85 (Tyr 508)	rabbit	Santa Cruz (sc-12929)	1:1000	1:2500
PCNA	mouse	Sigma (P8825)	1:1000	1:2500
Beta actin	mouse	Sigma (A5441)	1:5000	1:10000

2.7 Gene expression analysis

Two methods were used to examine gene expression changes. Microarray analysis allowed examination of several dozen of genes in a single experiment. Reverse-transcription polymerase chain reaction (RT-PCR) was used to analyse expression of a specific gene.

2.7.1 Microarray analysis

The microarray used was the Oligo GEArray® Human Cancer PathwayFinder™ Microarray (SABiosciences, Frederick, MD, USA, Cat No: OHS-033). It allowed simultaneous examination of the expression of 113 genes, grouped around six cancer specific areas, termed "pathways" by the manufacturer. These six pathways were

1: Cell Cycle Control and DNA Damage Repair

2: Apoptosis and Cell Senescence

3: Signal Transduction Molecules and Transcription Factors

4: Adhesion

5: Angiogenesis

6: Invasion and Metastasis

The genes examined are summarised in Table 2-4. The number and variety of genes represented in the array should provide an initial overview into the areas that are important for cancer growth and progression.

Table 2-4: Gene layout of Oligo GEArray® Human Cancer PathwayFinder™ Microarray, see also Table 9-1 and Table 9-2 in the Appendix, listing the full names and results of this microarray.

RPS27A 1	AKT1 2	ANGPT1 3	ANGPT2 4	APAF1 5	ATM 6	BAD 7	BAI1 8
BAX 9	BCL2 10	BCL2L1 11	BIRC5 12	BRCA1 13	BRCA2 14	CASP8 15	CASP9 16
CCND1 17	CCNE1 18	CD44 19	CDC25A 20	CDH1 21	CDK2 22	CDK4 23	CDKN1A 24
CDKN1B 25	CDKN2A 26	CFLAR 27	CHEK2 28	COL18A1 29	CTNNB1 30	E2F1 31	EGF 32
EGFR 33	ERBB2 34	ETS2 35	FGF2 36	FGFR2 37	FLT1 38	FOS 39	GZMA 40
HGF 41	HTATIP2 42	ICAM1 43	IFNA1 44	IFNB1 45	IGF1 46	IL8 47	ITGA1 48
ITGA2 49	ITGA3 50	ITGA4 51	ITGA5 52	ITGA6 53	ITGAV 54	ITGB1 55	ITGB3 56
ITGB5 57	JUN 58	CD82 59	KISS1 60	MAP2K1 61	MAPK14 62	MCAM 63	MDM2 64
MET 65	MICA 66	MMP1 67	MMP2 68	MMP9 69	MTA1 70	MTA2 71	MTSS1 72
MYC 73	NCAM1 74	NFKB1 75	NFKBIA 76	NME1 77	NME4 78	PDGFA 79	PDGFB 80
PIK3CB 81	PIK3R1 82	PLAU 83	PLAUR 84	PNN 85	PRKDC 86	PTEN 87	RAF1 88
RASA1 89	RB1 90	S100A4 91	SERPINB2 92	SERPINB5 93	SERPINE1 94	SNCG 95	SRC 96
SYK 97	TEK 98	TERT 99	TGFB1 100	TGFBR1 101	THBS1 102	THBS2 103	TIMP1 104
TIMP3 105	TNF 106	TNFRSF10B 107	TNFRSF1A 108	TNFRSF25 109	FAS 110	TP53 111	TWIST1 112
EPDR1 113	VEGFA 114	PUC18 115	Blank 116	Blank 117	AS1R2 118	AS1R1 119	AS1 120
GAPDH 121	B2M 122	HSP90AB1 123	HSP90AB1 124	ACTB 125	ACTB 126	BAS2C 127	BAS2C 128

2.7.1.1 Principle of the analysis

Complementary DNA-oligomers (60 nucleotides in length) specific for each gene are printed in quadruplicates on a nylon membrane of 3.8 cm length and 2.5 cm width.

2.7.1.2 Cell preparation and treatment

In 75 cm^2 tissue culture flasks, 2.8 x 10^6 cells (MDA-MB-231 or MCF-10A) were plated and grown in 8.5 ml of medium. This number of cells was proportional to the number of cells (1 x 10^6 cells) growing in 60 mm^2 dishes in 3 ml medium, which was used to extract RNA and protein in other experiment. After incubation in serum-free medium, cells were treated with 100 nM insulin for 60 min. Cells were trypsinised and collected in 1.5 ml RNase-free microcentrifuge tubes.

2.7.1.3 RNA extraction

Array Grade total RNA Isolation Kit (SABiosciences) was used to extract total RNA, following the manufacturer's instructions. No information was given on the supplied reagents. The cell pellet was re-suspended in 350 µl supplied Lysis Buffer (G6) and loaded onto a purple-ringed filter column. The column was placed in a collection tube and centrifuged for 1 min at 11000 x g. Collection tubes did not have their own lid. Thus before the filter columns were discarded, their lids were snapped off and used to seal the collection tubes during the next step, in which 350 µl 70% ethanol was added to each sample and the tubes were vortexed for 10 sec. The sample was loaded on a light-blue-ringed Spin Column, put back into the collection tube and centrifuged for 30 sec at 8000 x g. Then 350 µl of Desalting Buffer (G15) was added to the Spin Column and centrifuged for 1 min at 11000 x g. After discarding the flow-through, 200 µl of Pre-Wash Buffer (G 16) was added to the column and centrifuged for 30 sec

at 8000 x g. Four volumes of 100% Ethanol were added to the concentrated wash Buffer (G17), of which 350 ml was added to the Spin Column and centrifuged for 30 sec at 8000 x g. An additional 200 µl diluted Wash Buffer was added to the Spin Column and centrifuged for 3 min at 11000 x g. Spin Column was removed from the collection tube without discarding the flow through first, in order to avoid contaminating the Spin column with the flow through, which was highly emphasised in the manufacturer's instructions. Extracted RNA was eluted by adding 50 µl RNase-free water to the Spin Column and centrifuge for 1 min at 11000 x g.

2.7.1.4 RNA Quality Control

The following step of the protocol and the hybridisation onto the array membrane required high yield and high quality extracted RNA. Therefore the manufacturer recommended several quality control measures. First a 1:50 dilution of each sample was analysed spectrophotometrically at wavelengths of 230 nm, 260 nm and 280 nm. The 260 nm reading is directly related to the amount of RNA in the sample by the following equation, whereby 50 is the dilution factor:

Concentration [µg/ml] = Absorbance 260nm x 40 x 50 µg/ml

Proteins show high absorbance at 280 nm. The ratio of readings at 260 nm and at 280 nm provides an indication of a possible protein contamination. Ratio values of or above 2 were seen as not contaminated. Likewise a ratio of readings at 260 nm and at 230 nm provides an indication of possible guanidine contamination, whose salt was used during RNA extraction. A ratio of or above 1.7 was seen as not contaminated.

Additionally, 5 µl of each RNA extraction was subjected to agarose electrophoresis. To obtain a 2% agarose gel, 1 mg of agarose was dissolved in 50 ml of 1 x Tris-Borat-EDTA (TBE) buffer. Concentrated 10 x TBE buffer was purchased from Sigma (CatNo: T4415). 1 x TBE buffer contained 89 mM Tris-Borate and 2 mM EDTA at pH 8.3. The agarose solution heated to the boil to melt the agarose. After the agarose solution had cooled, 1 µl of 10 µg/ml ethidium bromide solution (Sigma, CatNo: E1510) was added to obtain 0.2 ng/ml ethidium bromide stained agarose gel. After addition of ethidium bromide, the gel was poured in a prepared tray and left to cool. Meanwhile 1 µl 6 x Blue/Orange loading dye (Promega, Madison, WI, USA, Cat No: G190A) was added to each RNA sample. This dye contained 15% Ficoll® 400, 0.03% bromophenol blue, 0.03% xylene cyanol FF, 0.4% orange G, 10 mM Tris (pH 7.5) and 50 mM EDTA in its concentrated form. After the gel had completely polymerised, the prepared samples were loaded to the wells and subjected to electrophoresis for 45 min at 80 V constant in 1 x TBE solution. Gels were visualised by exposure to UV light and images were captured after exposure between 0.04 sec and 0.08 sec. Images were obtained by GelDoc CCD imaging (Bio-Rad) and Quantity One software (Bio-Rad). The samples were thought to be of good quality, when a band for both 28S and 18S rRNA were clearly distinguishable, showed minimal smearing and an intensity ratio of about 2:1, respectively (Figure 2-6).

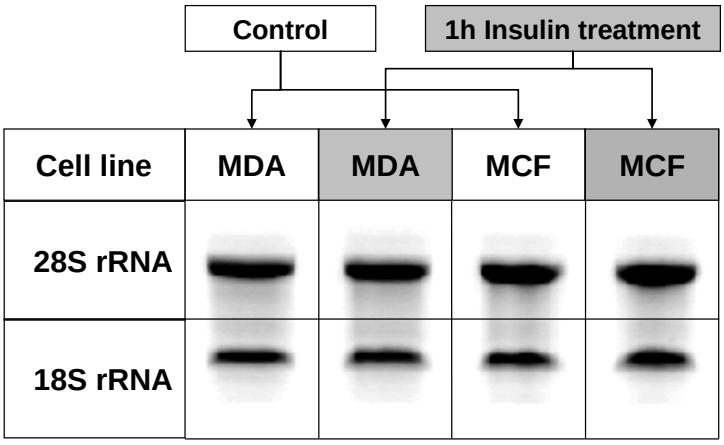

Figure 2-6: Agarose gel electrophoresis results of rRNA extractions for microarray analysis.

2.7.1.5 Sample preparation

After RNA integrity had been established, mRNA was reverse transcribed using "TrueLabeling-AMP 2.0" Kit (SABiosciences, Cat No: GA-030). The procedure is summarised in Figure 2-7. Each sample was diluted to contain 3 µg of total RNA in a 9 µl volume in a 0.2 µl PCR tube. Then 1 µl of "Component G1" was added and sample was heated to 70°C for 10 min. Meanwhile, "cDNA Synthesis Master Mix" was prepared. In a 0.5 ml microcentrifuge tube, 4 µl RNase-free water, 4 µl 5 x cDNA Synthesis Buffer (G3), 1 µl RNase Inhibitor and 1 µl cDNA Synthesis Enzyme Mix (G2) were mixed by pipetting. After 10 min heating, the RNA sample was cooled on ice and 10 µl of "cDNA Synthesis Master Mix" was added to each sample. Samples were placed in a thermocycler and incubated at 42°C for 50 min followed by 75°C incubation for 5 min. Then samples were cooled to 37°C. Potential condensation was collected by centrifugation and samples were kept at 37°C. During cDNA synthesis the "Amplification Master Mix" was prepared. In a 0.5 ml microcentrifuge tube, 16 µl 2.5 x RNA Polymerase Buffer (G24), 2 µl 10 mM Biotinylated-UTP (Roche Applied Sciences, Burgess Hill, UK, Cat No: 11388908910), 2 µl RNA Polymerase Enzyme

(G25) were mixed by pipetting. After the cDNA synthesis had finished, 20 µl of the "Amplification Master Mix" was added to each sample, which were mixed and incubated overnight at 37°C.

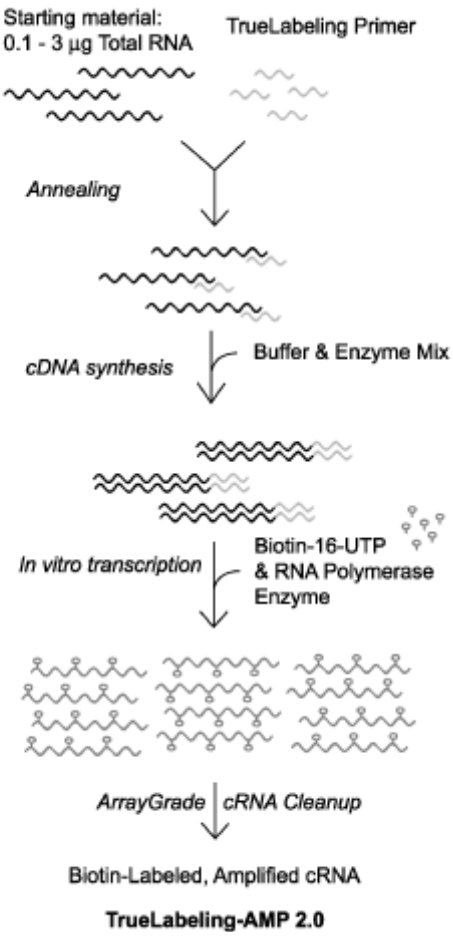

Figure 2-7: Overview of the labelling procedure (adapted from "TrueLabeling-AMP 2.0" Kit Manual, v.1.3, p.4)

2.7.1.6 cRNA purification

Before the labelled cRNA could be hybridised to the membrane, it needed to be cleaned of excess components of the labelling and amplification process, using "ArrayGrade cRNA cleanup Kit" (SABiosciences, Cat No: GA-012). To each sample from overnight incubation, 50 µl RNase-free water was added and the total volume of 90 µl was

transferred to a 1.5 ml RNase-free microcentrifuge tube. Then 315 µl Lysis and Binding Buffer was added (G6) and mixed by pipetting. A 315 µl 100% ethanol volume was added, mixed and the entire sample was loaded to a pre-assembled Spin Column. Sample was centrifuged for 30 sec at 8000 x g. The flow through was discarded and 600 µl of diluted Wash Buffer (G17 with 100% Ethanol) was added. The sample was centrifuged again for 30 sec at 8000 x g. After the flow-through was discarded, 200 µl of diluted wash buffer was added to the sample and the tube was centrifuged first for 1 min at 11000 x g and after removal of the flow-through an additional centrifugation for 2 min at 11000 x g was preformed. The Spin Column was then transferred to an empty 1.5 RNA-free "Elution Tube" (supplied). To elute RNA, 50 µl RNase-free 10 mM Tris buffer pH 8.0 (G26) was added to each column and incubated for 2 min at room temperature. Then samples were centrifuged for 1 min at 8000 x g. cRNA amount was determined spectrophotometrically as described in section 2.7.1.4. The supplied 10 mM Tris buffer (G26) was used to "zero" the photometer.

2.7.1.7 Hybridisation of cRNA to microarray membrane

The clean cRNA could be hybridised to the array membrane. The "Oligo GEArray HybTube Protocol" (SABiosciences, Version 3.2) was followed for this analysis. An overview of the procedure is given in Figure 2-8.

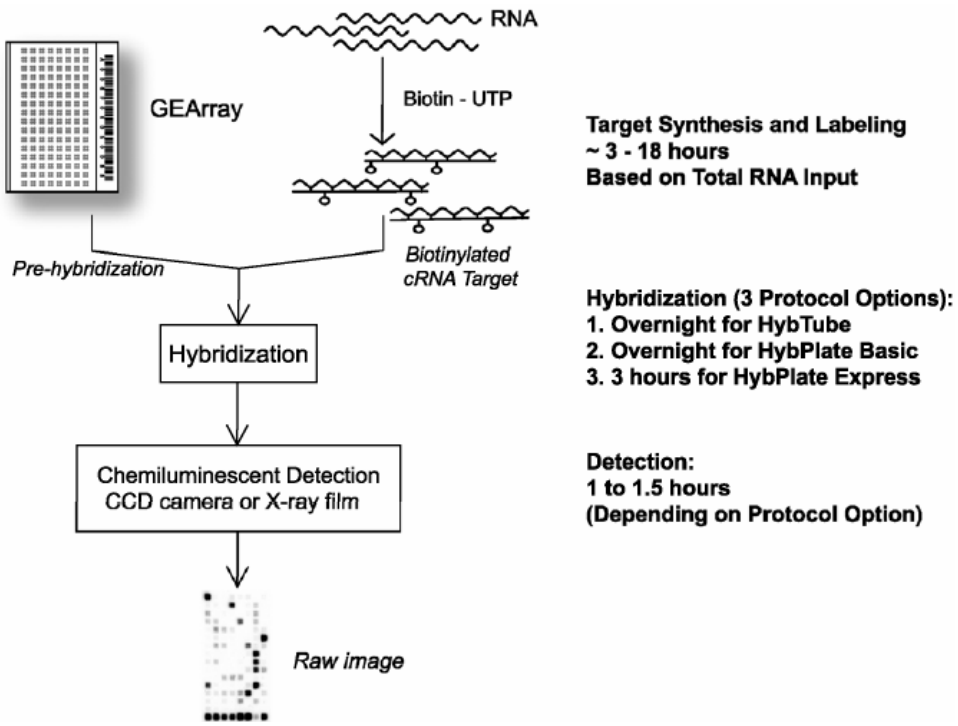

Figure 2-8: Overview of the hybridisation procedure (adapted from "Oligo GEArray System Manual, v.3.2, p.5")

To prepare the membrane array, a Hybridisation Oven (Techne Hybridiser, Model HB-1D without speed control) and the GEAhyb Hybridisation Solution were heated to 60°C. To start the hybridisation procedure, 5 ml single distilled water was added to the array tube, and incubated inverted for 5 min at room temperature. After discarding the water, 2 ml of pre-warmed GEAhyb Hybridisation solution was added to the array tube. The tube was placed inside a hybridisation cylinder and incubated with rotation for 90 min at 60°C. Meanwhile the "Target Hybridisation Mix" was prepared. To 0.75 ml pre-warmed GEAhyb Hybridisation Solution, 4 µg of labelled cRNA was added and kept at 60°C. The pre-hybridisation solution was discarded and the "Target Hybridisation Mix" was added to the array tube. Labelled cRNA was hybridised overnight at 60°C with slow rotation. On the same day wash solutions for the next day were prepared. Stock solutions were 100 ml 20 x SSC buffer (3 M NaCl, 340 mM

$Na_3C_6H_5O_7$, pH 7.0) and 50 ml 20% SDS. From these 100 ml wash solution 1 (2 x SSC, 1% SDS) and 100 ml wash solution 2 (0.1 x SSC, 0.5% SDS) were prepared and kept overnight at 60°C.

After overnight hybridisation, the "Target Hybridisation Mix" was transferred to an empty 7 ml "bijou" tube and frozen at -20°C. The membrane was washed once in wash solution 1 and once in wash solution 2 for 15 min each at 60°C with rotation. After the second wash was discarded the array tube was left to cool to room temperature. Then 2 ml GEAblocking Solution Q was added to the tube, vortexed and incubated for 40 min at room temperature with rotation. Meanwhile 40 ml 1 x Buffer F was prepared. Alkaline phosphatase-conjugated streptavidin (AP, supplied), was diluted 1:8000 in 16 ml 1 x Buffer F. After blocking the array membrane, 2 ml diluted AP-buffer was added to the membrane and incubated for 10 min at room temperature. The membrane was washed four times with 1 x Buffer F and vortexed briefly after each addition of fresh buffer. After the last wash 3 ml Buffer G was added, the tube was inverted three times and repeated once for a total of two rinses. Then 1 ml of CDP-Star chemiluminescent substrate (supplied) was added to the tube, which was incubated for 5 min at room temperature with rotation. The membrane was removed from the tube and blotted between two sheets of cling film. The membrane was exposed to X-ray films and pictures were captured using GelDoc CCD imaging (Bio-Rad) and Quantity One software (Bio-Rad).

2.7.2 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Expression of several genes, summarised in Table 2-5, was examined by RT-PCR.

Table 2-5: Genes examined for expression changes and primers used for each gene. All primers were purchased from Invitrogen.

Gene name	Gene product	Forward primer sequence (5'-3')	Reverse primer sequence(5'-3')
BCL-2	Bcl-2	ACT TGT GGC CCA GAT AGG CAC CCA G	CGA CTT CGC CGA GAT GTC CAG CCA G
CCND1	cyclin D	GCT CGA GCC CGTGAA AAA GA	CTC CGC CTC TGG CAT TTT G
CCNE1	cyclin E	TTA CCC AAA CTC AAC GTG CAA	GCT CAA AGT GCT GAT CCC
ACTB	beta actin	CAT GTA CGT TGC TAT CCA GGC	CTC CTT AAT GTC ACG CAC GAT

2.7.2.1 RNA extraction using TRIzol

After cells were treated, total RNA was extracted using TRIzol reagent (containing Phenol and guanidine isothiocyanate; Invitrogen (Cat No: 15596)). Cells were grown and treated in 60 mm^2 dishes. After treatment cells were washed with 1 x PBS and 1 ml TRIzol reagent was added. Cells were incubated for 5 min at room temperature before the lysed cells were transferred to 1.5 ml RNase-free microcentrifuge tubes. Cell lysates were frozen at -80°C before extraction of total RNA.

Samples were thawed and 200 µl chloroform were added to each tube. The contents were mixed by shaking up and down for 15 sec. Tubes were incubated for 3 min at room temperature, before being centrifuged for 15 min at 11000 x g at 4°C. The aqueous, colourless, top-layer containing RNA was carefully transferred to a new 1.5 ml RNase-free microcentrifuge tube and 500 µl propan-2-ol were added. Tubes were inverted several times to mix contents and incubated at room temperature for 10 min. Tubes were centrifuged for 10 min at 11000 x g at 4°C. The supernatant was removed and pellets were washed with 500 µl 75% DEPC-treated Ethanol. Tubes were centrifuged for 5 min at 11000 x g at 4°C and Ethanol was removed. Tubes left for up to 20 min to "air-dry". Then 20 µl RNase-free H_2O was added to each sample. Samples were frozen overnight at -20°C, then heated to 65°C for 10 min and RNA concentration

was determined as described in section 2.7.1.4, with the exception that samples were only measured at 260 nm and 280 nm wavelength.

2.7.2.2 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

RNA samples were diluted to100 ng/µl, of which 1 µl was reverse transcribed in a 20 µl volume containing 50 mM Tris-HCL (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM Dithiothreitol (all from Invitrogen, Cat No: 18080-044), 1 mM of each dNTP (Roche, Cat No: 11969064001), 100 µg/ml BSA (New England Biolabs, Cat No: B9001S), 25 µg/ml Random Primers (Promega, Cat No: C1181), 40 units RNase OUT (Invitrogen, Cat No: 10777-019), 80 units SuperScript® III Reverse Transcriptase (Invitrogen, Cat No: 18080-044). Samples were then incubated for 10 min at 25°C, followed by 52 min at 42°C and 15 min at 72°C. Samples were cooled to 4°C after reverse transcription.

The resulting cDNA (4 µl of RT mix) was amplified for a specific gene (Table 2-6) in a 20 µl volume containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.75 mM $MgCl_2$ (all from Sigma, Cat No: D4545–250UN), 1 µM of each primer (Table 2-5) and 1 unit of *Taq* DNA polymerase. On a Thermocycler (Biorad) a hot-start (95°C) was performed followed by 4 min at 94°C, then a gene-specific number of cycles (Table 2-6) with 1 min at 94 C, 2 min at gene-specific annealing temperature (Table 2-6) and 2 min at 72°C. This was followed by a 10 min final extension step at 72°C. Samples were cooled to 4°C for up-to overnight.

Table 2-6: PCR-step properties for each gene examined

Gene name	Gene product	Annealing temperature (°C)	Cycle number	Product size (bp)
BCL-2	Bcl-2	68	35	389
CCND1	cyclin D	60	25	247
CCNE1	cyclin E	59	33	149
ACTB	beta actin	68	25-35[1]	250

2.7.2.3 Analysis of PCR product by agarose gel electrophoresis

PCR products were examined by agarose gel electrophoresis as performed for RNA quality control (section 2.7.1.4) for microarray analysis. According to theoretical size of the PCR-product (see Table 2-6) a 1.5%-2% agarose gel containing 0.2 ng/ml Ethidium Bromide (Sigma, CatNo: E1510) was prepared with 10 or 20 wells. A standard 100 bp DNA ladder (Promega, Cat No: G2101) was added for size references. This standard contained 11 double-stranded DNA fragments with sizes at 100 bp, 200 bp, 300 bp, 400 bp, 500 bp, 600 bp, 700 bp, 800 bp, 900 bp, 1000 bp and 1500 bp, with all fragments in same concentration except 500 bp, which is present in triple the concentration than the other fragments, *e.g.* a 10 µl volume of ladder, which was routinely used, contained 300 ng of the 500 bp fragment and 100 ng of every other fragment (Figure 2-9). Samples and standard were diluted in 6 x Blue/Orange loading dye (Promega, Cat No: G190A) as described in section 2.7.1.4. The loaded gel was subjected to electrophoresis for 45 min at 80 V constant in 1 x TBE solution. Gels were visualised by exposure to UV light and images were captured after exposure between 0.04 sec and 0.08 sec. Images were obtained by GelDoc CCD imaging (Bio-Rad) and Quantity One software (Bio-Rad).

[1] Cycle number for beta actin varied according to the analysed gene, as it acted as control for all examined genes.

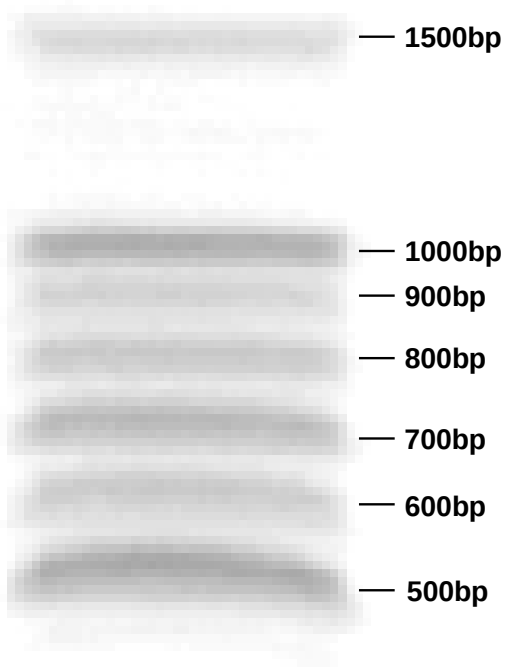

Figure 2-9: Overview of the 100 bp DNA ladder used for size control of RT-PCR product. DNA ladder run on 1% agarose gel. Only higher molecular weight bands are visible.

2.8 Flow cytometry

Flow cytometry was used to detect apoptosis and changes in cell distribution across cell cycle stages. Flow cytometry exploits the light scattering properties of particles and the light excitation and emission of fluorochromes. An optimally focused laser beam is projected onto a stream of single particles, *i.e.* human cells for this project. The cells are hydro-dynamically focused in a sheath of PBS (Figure 2-10).

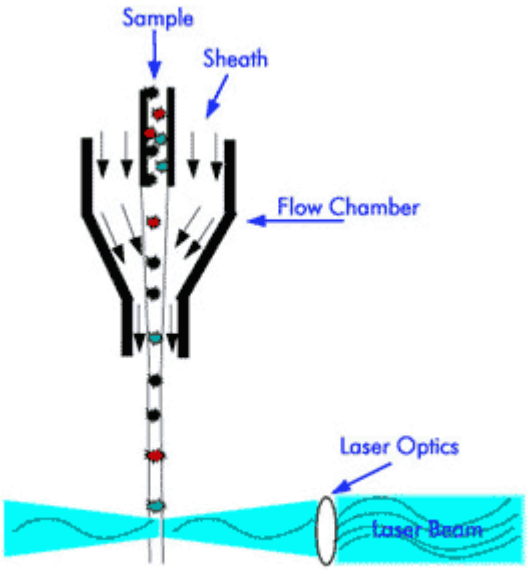

Figure 2-10: Representation of hydrodynamic focusing to create flow of single cell stream (adapted from the University of Berkeley, http://biology.berkeley.edu/crl/flow_images/fig1.gif)

As the cells pass the laser beam, they scatter light in two ways. The Forward Scatter (FS) detector provides information on the size of the cells. The Side Scatter (SS) detector provides information of the granularity of internal components of the cell (Figure 2-11).

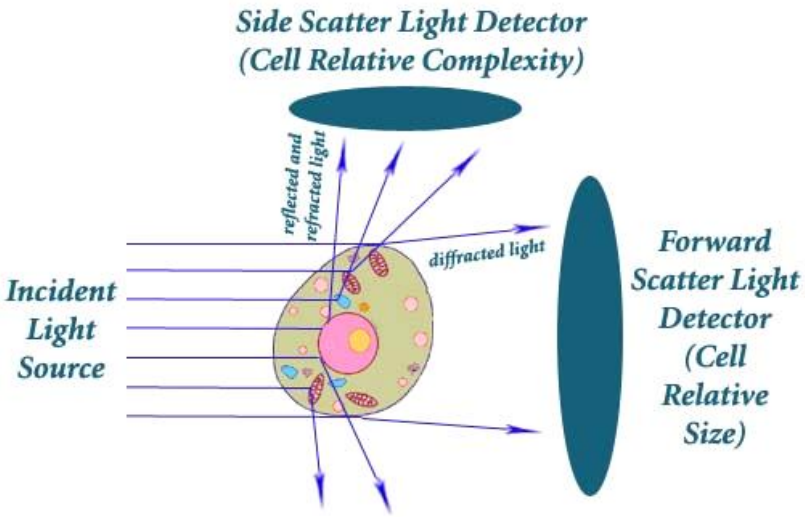

Figure 2-11: Measurement of Forward and Side Scatter in a Flow Cytometry experiment (adapted from University of Alberta, http://flowcytometry.ualberta.ca/images/scatter.jpg)

Additionally tagging a specific property of the cell with a fluorochrome detects quantifiable changes in the cell population, *e.g.* a tagged antibody for Annexin-V gives information about early apoptosis. The flow cytometer used (Beckman Coulter, Model: EPICS XL-MCL, High Wycombe, UK) had four fluorochrome detectors (FL1-FL4) besides FS and SS detectors providing up-to six parameters in one experiment. The two protocols used examined apoptosis and cell cycle changes after treatment of cells.

2.8.1 Cell cycle analysis using propidium iodide

The first analysis using the flow cytometer was examining changes in cell cycle after treatment with insulin, leptin and TNF-α. Cell cycle stages can be distinguished according to DNA content (Figure 2-12). Thus measuring DNA content using fluorescent DNA specific dye provides information about the cell cycle. Examining cell populations (usually 1 x 10^4 cells) using flow cytometry allows quantification of cells within each stage with different treatments and determination of shifts in distribution between cell cycle stages. The DNA specific dye was propidium iodide (PI) (Sigma, Cat No: P4170).

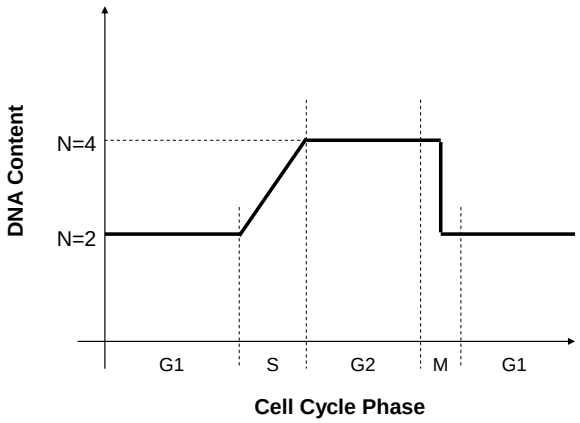

Figure 2-12: Changes of DNA content in dependence of cell cycle stage

After cells had been treated, medium, PBS-wash and trypsinised cells were collected in 15 ml centrifuge tubes and centrifuged for 5 min at 500 x g. Cell pellet was re-suspended in 1 ml PBS and transferred into a sterile 1.5 ml microcentrifuge tube. Tubes were centrifuged for 5 min at 500 x g. Cell pellet was re-suspended in 100 µl PBS, vortexed and 900 µl 70% ethanol (4°C) was added. Cells were incubated at 4°C for 30 min. Cell were centrifuged for 5 min at 750 x g. Excessive ethanol was removed by adding 1 ml PBS and centrifugation for 5 min at 750 x g. Cell pellet was re-suspended in 500 µl PBS, vortexed and 500 µl "DNA Extraction Buffer" (192 mM Na_2HPO_4, 4 mM $C_6H_8O_7$ (citric acid)) was added. Cells were incubated for 5 min at room temperature and then centrifuged for 5 min at 750 x g. Cells were re-suspended in 500 µl DNA staining solution (20 µg/ml PI (Sigma, Cat No: P4170), 200 µg/ml DNase-free RNase (Sigma, Cat No: R6513)). Cells were incubated for 30 min in the dark at room temperature. Then cells were analysed by flow cytometry without further dilution. The parameter settings for the cell cycle protocol are summarised in Table 2-7. All living cells should have a DNA-content between N2 and N4, *i.e.* are detected in G1, S or G2-phase. Cells that have however a DNA-content lower than N2, *i.e.* less than in G1-phase (subG1), could be considered late apoptotic with break-down of DNA. This analysis also identified those cells. The results from the subG1-group could provide a more complete picture of cell re-distribution across cell cycle stages than the mere three stages would have. Data was captured and analysed using EXPO-32 Software (Applied Cytometry Systems). In order to obtain cell amount values with this software, DNA content had to subjectively be defined to correspond to a particular cell cycle stage (see Appendix section 9.3.1). For each experiment this determination was performed as follows: following the increase in DNA content, *i.e.* the x-axis of the graph in Figure

9-4, the "subG1"-phase extended from the detection minimum to the base of the G1-peak, the "G1"-phase extended from the base at the lower DNA content of the G1-peak to the base at the higher DNA content, *i.e.* from left to right on the x-axis in Figure 9-4. The "S"-phase extended from the base of the G1-peak at the higher DNA content to the base of the G2-peak at the lower DNA content. The "G2"-phase extended from the base at the lower DNA content of the G2-peak to the base at the higher DNA content. This determination was performed for each experiment and each cell line separately.

Table 2-7: Settings of cytometer parameters for cell cycle analysis. Detector FL2 detects PI-emission. Protocol was equal for all cell lines examined.

Detector	identifies	Volts	Gain
FS	Size	55	5.0
SS	Granularity	400	20.0
FL2	PI	764	1.0
AUX	AUX	500	10.0

2.8.2 Flow cytometer analysis of apoptosis

The Annexin-V-FITC/7-AAD apoptosis kit (Beckman Coulter, Cat No: IM3614) was the second flow cytometry application and used to examine apoptosis after insulin treatment. Several morphological features occur during initiation of apoptosis within the cell, including cell shrinkage, nuclear condensation and DNA cleavage. During early apoptosis the integrity of the cell membrane is kept intact, membrane-bound intracellular phospholipids however are exposed on the cell surface. The protein Annexin-V binds specifically to phosphatidylserine (PS), with high affinity. In the present kit, Annexin-V is complexed 1:1 stoichiometrically with fluorescein isothiocyanate (FITC), which can be detected fluoroscopically. Additionally this kit

contains fluorescent 7-Aminoactinomycin D dye (7-AAD), which binds to and thus stains DNA. This dye can only diffuse across nuclear membranes, but not across cell membranes. In normal cells, the cell membrane is intact and no dye can enter to stain the DNA. In necrotic cells, the cell membrane is compromised and 7-AAD can enter the nucleus and bind to DNA. Bound 7-AAD can therefore be used to estimate late apoptosis and necrosis within the cell population. With excitation wavelengths distinct between the two compounds, they can be used in combination to examine for early apoptosis and necrotic cells.

For this analysis, the kit manufacturer's instructions were followed. After cells had been treated, treatment medium, PBS-wash and trypsinised cells were collected in 15 ml centrifuge tubes and centrifuged for 5 min at 500 x g at 4°C. Cells were washed in 1 ml PBS, transferred into sterile 1.5 ml microcentrifuge tubes and collected by centrifugation for 5 min at 500 x g at 4°C. After PBS removal, 130 µl of Annexin-V and 7-AAD master mix was added to each sample. Master Mix contained 100 µl of 1 x Binding Buffer (supplied), 10 µl Annexin V-FITC (supplied) and 20 µl 7-AAD dye (supplied). No information on composition of binding buffer or Annexin-V and 7-AAD concentrations was available. Samples were incubated on ice in the dark for 15 min. Then an additional 400 µl of 1 x Binding Buffer was added to each sample, before the samples were analysed. Since each cell line had different properties in size and fluorescence background the acquisition parameters for the cytometer were adapted once a new cell line was examined (Table 2-8). Data was captured and analysed using EXPO-32 Software (Applied Cytometry Systems, Sheffield, UK). See Appendix section 9.3.2 for a raw data histogram, produced by this software.

Table 2-8: Settings of cytometer parameters for Annexin V-FITC/7-AAD apoptosis analysis for each cell line examined. Detectors FL-2 and FL-3 were not used.

		MDA-MB-231		MCF-10A		SK-BR-3	
Detector	identifies	Volts	Gain	Volts	Gain	Volts	Gain
FS	Size	89	1.0	80	1.0	30	1.0
SS	Granularity	714	2.0	620	2.0	502	2.0
FL1	FITC	563	1.0	680	1.0	600	1.0
FL4	7-AAD	651	1.0	850	1.0	670	1.0
AUX	AUX	500	10.0	500	10.0	500	10.0

2.9 Statistical Analysis

Except for microarray analysis and where otherwise described, at least three independent experiments were performed for each of the analyses described above. Each experiment was performed with at least two replicates per control and treatment. Number of replicates is described in each of Materials and Methods sections in the following chapters. After consultation (Dr. Alex Wilson, School of Computing, Robert Gordon University) two statistical analyses were used. One-way analysis of variance was performed on experiments involving comparing one control to one treatment, *i.e.* cell proliferation, insulin receptor phosphorylation. Experiments for which data from several time points or different treatments was available (ERK1/2 and AKT ELISA, cyclin expression, flow cytometry, western analysis) univariate analysis of variance was performed, followed by post-hoc Tukey HSD (Honestly Significant Difference) or Dunnett's t-tests, comparing each treatment to another or each treatment to the control, respectively. When probability values are given, they were generally obtained using Dunnett's t-test, thus reflecting a comparison of the treatment in question to the control. Data from Tukey's HSD, which compares treatment times to each other, was used when unexpected or interesting findings between treatments were obtained. For clarity however not all significantly different changes between treatments are mentioned. All

tests assumed normal distribution of data. Significant differences were observed when comparison probability values were ≤0.05. All results will have a short indication as to which statistical analysis was used to obtain any indicated significant differences. All tests were performed after transformation of data.

All data was transformed to obtain a percentage value of the control. Controls were incubated in serum-free medium, in the same way as the treatment samples. After incubation in serum-free medium, treatment medium was added to the treatment samples, while fresh serum-free medium was added to the controls, unless otherwise described. Control samples were run for each experiment and in replicates of at least two. After data acquisition, raw control values were averaged and each control value was transformed as percentage of the average control value. The average of the control would then comprise 100%. Every single treatment sample value (at least two for each treatment) was then transformed as a percentage of the averaged raw control data. After repeating each experiment at least three times, all single transformed values were averaged (at least six values for each treatment). Standard error of the mean of this average was calculated and added as error bar to each graph. Statistical analysis, as described above, was performed on these transformed values. Statistical differences from the control are indicated with asterisk (*) in graphs and p-value results of the statistical tests are described in the text.

Chapter 3
Insulin part 1

3 INSULIN PART 1

In this chapter the effect of insulin as a possible mediator between obesity and postmenopausal breast cancer is addressed. It is aimed to determine the effect of insulin on cell proliferation, IR-activation, cell signalling pathway activation and cell cycle in MDA-MB-231 breast cancer cells, MCF-10A breast epithelial cells and SK-BR-3 breast cancer cells.

3.1 Introduction

Insulin is a peptide hormone of 51 amino acids length and approximately 58 kDa of weight. It is exclusively produced by β-islet cells (also known as Langerhans cells) in the pancreas. Its secretion is stimulated in response to increased blood sugar concentrations, usually following a carbohydrate containing meal. In its presence muscle, liver and adipose cells increase their uptake of glucose from the blood, resulting in decreased blood glucose, which in turn decreases pancreatic insulin secretion. The insulin receptor however is not only expressed in myocytes, hepatocytes and adipocytes but also in other cell types, including breast epithelial cells and breast tumour cells (Belfiore *et al.*, 1996a; Milazzo *et al.*, 1992; Papa *et al.*, 1990). In non-glucose transporting cells insulin is thought to have additional functions, *e.g.* increasing amino acid uptake and synthesis (Malmberg and Adams, 2008). Fasting insulin is usually not detectable. If blood glucose concentrations are high, insulin concentrations oscillate between 0.1 nM and 0.8 nM (Kennedy *et al.*, 2002). Oscillation of insulin concentration is important for maintaining IR excitability, but its exact function is unknown (Lefebvre *et al.*, 1987). Insulin, via its membrane bound receptor, exerts an increase in glucose transport by activation of the PI3-kinase cell signalling cascade (see section 1.6.1).

Additionally insulin signalling may activate the MAP-kinase cell signalling pathway either through its own receptor, through the hybrid IR-IGF-1R or through AKT-RAS cross-talk (see section 1.6.2). Increased activity of both pathways has been found in breast cancer cells, where activation of these pathways is thought to increase survival (see section 1.6.1.1 and 1.6.2.1). Thus insulin, which is increased in insulin resistant-obese patients, may increase breast cancer aetiology or progression.

High insulin concentrations act growth promoting in a number of cells (Strauss, 1984; Whittaker *et al.*, 1987). Obese individuals, through the obesity-insulin resistance connection, have increased insulin circulatory concentrations[1] (see section 1.2). With obesity rates now affecting about a third of the population in westernised societies and increasing in developing nations, an ever increasing amount of individuals will be exposed to elevated insulin concentrations (see section 1.1.2). With obesity also linked to increased risk of developing and dying of breast cancer (Calle *et al.*, 2003), insulin (see section 1.5.2) has been proposed as a possible affector of breast cancer risk (Boyd, 2003). Supportingly, an epidemiological study demonstrated a positive correlation between increased insulin resistance and increased risk of all cancers, even in non-obese individuals (Facchini *et al.*, 2001).

The idea that insulin may have an effect on breast cancer cells is not new and the first experiments were conducted in the 1970s. In the oestrogen responsive breast cancer cell

[1] Physiological insulin concentrations are subject to high variation. Postprandial insulin concentrations may be as high as 0.8 nM (Kennedy *et al.*, 2002; Maki *et al.*, 2009). Dick and Sturek (1996) defined 0.3 nM insulin as corresponding to physiological insulin concentration in their study, suggesting that physiological insulin concentrations may vary between 0.1-0.8 nM. Thus identifying a specific insulin concentration as cut-off point for insulin resistance is not possible. Insulin resistance is rather measured by impairments in glucose clearance.

line MCF-7, insulin stimulated thymidine, uridine and leucine incorporation after 24 h treatment with different concentrations of insulin. The incorporation of nucleotides increased linearly with increasing insulin concentration, reaching its maximum at 100 nM insulin. A significant increase was already observed at an insulin concentration of 0.1 nM. Additionally, the number of cells was significantly increased after three days of 100 nM insulin treatment compared to no treatment control cells, suggesting increased cell proliferation with insulin treatment (Osborne *et al.*, 1976). Similarly 10 μg/ml (172 nM) insulin increased cell proliferation in MCF-7 cells more than six-fold after four days of continued treatment. Other growth factors, including oestrogen, did not achieve a similar increase in cell proliferation. Oestrogen and insulin (10 ng/ml (0.172 nM)) however exerted a strong synergistic increase in cell proliferation in MCF-7 cells (van der Burg *et al.*, 1988).

Oestrogen receptor negative MDA-MB-231 breast cancer cells in contrast did not show an increase in cell proliferation at 100 nM insulin treatment for up to six days. This is surprising as it was observed that these cells had a five-fold increase in IR protein expression compared to normal breast epithelial cells (Costantino *et al.*, 1993). It was suggested that an IR co-precipitating peptide which had tyrosine kinase inhibiting properties was present in these cells (Costantino *et al.*, 1993). A subsequent study identified the co-precipitating peptide as membrane glycoprotein PC-1 (Belfiore *et al.*, 1996b). Godden and colleagues (1992) also observed that MDA-MB-231 cell proliferation does not increase in response to a number of steroids including 100 nM insulin. This suggests that IR phosphorylation and intra-cellular insulin signalling are not present in this cell line.

The mature IR is a transmembrane heterotetramer of two extracellular α-subunits and two transmembrane β-subunits. The β-subunits contain three autophosphorylation sites at tyrosine residues. Its autophosphorylation is inhibited by the α-subunits, which lose their inhibiting function once insulin is bound (White and Kahn, 1994). Autophosphorylation increases kinase activity 10-20-fold (White *et al.*, 1988). The IR target is IRS1, from which the signals may emanate into three major cell signalling pathways, *i.e.* the PI3-kinase, the MAP-kinase and the CAP/Cbl/Tc10[2] pathway (Pirola *et al.*, 2004; Saltiel and Kahn, 2001).

Phosphoinositide-3 kinase consists of two subunits, the 110 kDa catalytic (p110) and the 85 kDa regulatory subunit (p85). The p85-subunit contains two SH2 domains, which specifically associate with phosphorylated IRS1 (Backer *et al.*, 1992). Through phosphorylation of the second messenger phosphatidylinositol, PI3-kinase activates PDK-1 and AKT, which in turn activates the glucose transporter GLUT4 (Kohn *et al.*, 1996). GRB-2 is a small cytoplasmic protein that contains an SH-2 domain specifically binding phosphorylated Tyr^{895} in IRS1 (White and Kahn, 1994). It complexes with the guanine exchange factor for RAS mSOS (homologous to the Drosophila protein, son-of-sevenless (sos); Li *et al.*, 1993; Simon *et al.*, 1993). The GRB2/mSOS complex then phosphorylates RAS and thus activates the MAP-kinase cell signalling pathway (Kolch, 2000). There may be several ways of RAS activation by insulin. The IR and/or IRS may activate RAS through activation of the Shc/GRB2/mSOS, in a similar way as growth factor receptor would (Skolnik *et al.*, 1993). Other studies suggested that RAS may

[2] Similar to PI3-kinase, CAP pathway has been described as necessary for normal functioning of glucose insulin induced glucose transport (Baumann *et al.*, 2000; Chiang *et al.*, 2001; Chiang *et al.*, 2003).

directly bind to IR[3] (Pronk *et al.*, 1992). ERK1/2 is subsequently activated through the RAS/RAF/MEK/ERK phosphorylation cascade (see Figure 1-7 for an overview of PI3-kinase and MAP-kinase cell signalling pathways).

In combination with increased cell proliferation, enhanced cell cycle progression is an additional feature of cancer aetiology. The cell cycle is divided into four stages, two "gap"-stages (G1, G2), a DNA synthesis stage (S), and mitosis (M). The order of the cell cycle is M-G1-S-G2-M (Figure 3-1). An increase in cell cycle progression would be observed by finding fewer cells in the G1-stage, and more in S-phase or G2-phase. During sub-optimal growth conditions, cells may arrest their cell cycle progression at the G1/S cell cycle checkpoint, with all cells remaining in G1-phase beyond their normal time. This quiescent phase is often referred to as G0-stage. If insulin has cell cycle promoting properties a change in cell cycle profile, *i.e.* the distribution of a cell population across the cell cycle stages, will be observed, especially a decrease in G0/G1-stage and an increase in S-phase.

[3] RAS activation by growth hormone receptors (*e.g.* epidermal growth factor receptor EGFR or platelet-derived growth factor receptor PDGFR) takes place through SH2 binding sites on the receptor. However IR does not contain an SH2 binding site, thus any possible direct interaction between RAS and IR is likely to be SH2 independent.

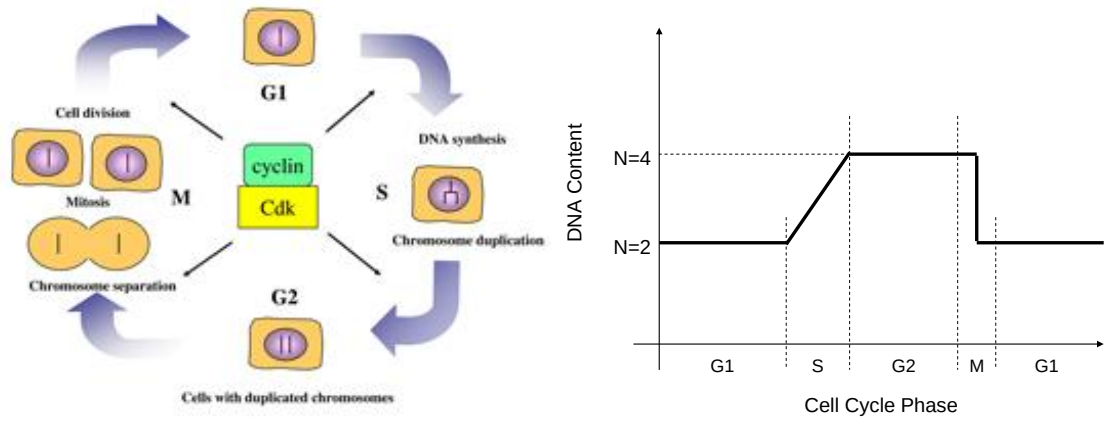

Figure 3-1: Schematic overview of the cell cycle and DNA content in each stage. N: chromatide number

In this chapter the influence of high insulin concentrations (100 nM) on an *in vitro* breast cancer model was examined. From studies mentioned above, it was determined that 100 nM was the concentration at which molecular changes reached their maximum. Importance was put on elucidating the primary effect of high insulin concentrations on ER-negative MDA-MB-231 breast cancer cells and ER-negative MCF-10A normal breast epithelial cells. After specific treatment times (see section 3.2), changes in insulin receptor phosphorylation, cell proliferation, activation of cell signalling pathways and changes in cell cycle were measured. Additionally cell cycle pathway inhibitors were used to estimate the influence of activation of these pathways on any potential insulin-mediated change in cell proliferation. As MDA-MB-231 breast cancer cells are supposed to possess increased MAP-kinase signalling through mutational increased kinase activity of KRAS and BRAF (Hollestelle *et al.*, 2007), several experiments were also executed using the ER-negative breast cancer cell line SK-BR-3, which possesses wild-type MAP-kinases (Hollestelle *et al.*, 2007).

3.2 Materials and Methods

3.2.1 Cell lines and insulin treatment

MDA-MB-231, MCF-10A and SK-BR-3 cells were cultured and treated as described in Materials and Methods sections 2.1.1 and 2.1.2. Formulation of insulin treatment medium and final insulin treatment concentration is described in Materials and Methods section 2.1.3.1.

3.2.2 Cell proliferation assay

Cells were plated into 96-well plates (Fisher Scientific, Cat No: 167008) at a density of 5000 cells/well with 100 µl/well growth medium and incubated for 24 h at 37°C. After 24 h cells were washed once in 100 µl/well sterile PBS and incubated in 100 µl serum-free medium for an additional 24 h at 37°C. If the experiment included the analysis of inhibitors, cells were pre-treated with inhibitors (2 µl/well 5 µM Wortmannin stock solution and/or 4 µl/well 1.25 mM PD98059 stock solution) 60 min before treatment commenced.

Treatment medium was prepared in a small volume (usually 2 ml) of serum-free medium in advance. For a 24 h insulin treatment the composition of the treatment media is described below and treatment was carried out as summarised in Figure 3-2. The treatment media were: 10 µM BrdU (Control), 100 nM wortmannin and 10 µM BrdU, 50 µM PD98059 and 10 µM BrdU, 100 nM insulin and 10 µM BrdU, 100 nM insulin and 100 nM wortmannin and 10 µM BrdU, 100 nM insulin and 50 µM PD98059 and 10 µM BrdU, 100 nM insulin and 100 nM wortmannin and 50 µM PD98059 and 10 µM BrdU. The "Blank"-wells acted as negative control as they did not contain any cells, but also received 10 µM BrdU. Cells were then incubated for 24 h at 37°C. "Blank" values

were necessary to assess unspecific binding of BrdU and BrdU-antibody to the 96-well plate. These values were subtracted from all other values. The corrected absorbance values were below 0.1 in all experiments as recommended by the manufacturer's instructions. In addition for the first experiment of each cell line a "Background" sestuplicate was included. This control contained cells but no BrdU was added. During the analysis the BrdU-antibody was added to these wells and analysed. These controls assessed the unspecific binding of the BrdU-antibody to the cells. Absorbance values were below 0.1 for each cell line as recommended by the manufacturer.

For a 48 h experiment cells were plated and incubated in serum-free medium for 24 h. After incubation in serum-free medium, cells were washed once in PBS and fresh serum-free medium or treatment medium without 10 µM BrdU was added to control wells and treatment wells, respectively. Cells were incubated for an initial 24 h, and then 10 µM BrdU was added. Cells were then incubated for an additional 24 h before BrdU incorporation was detected.

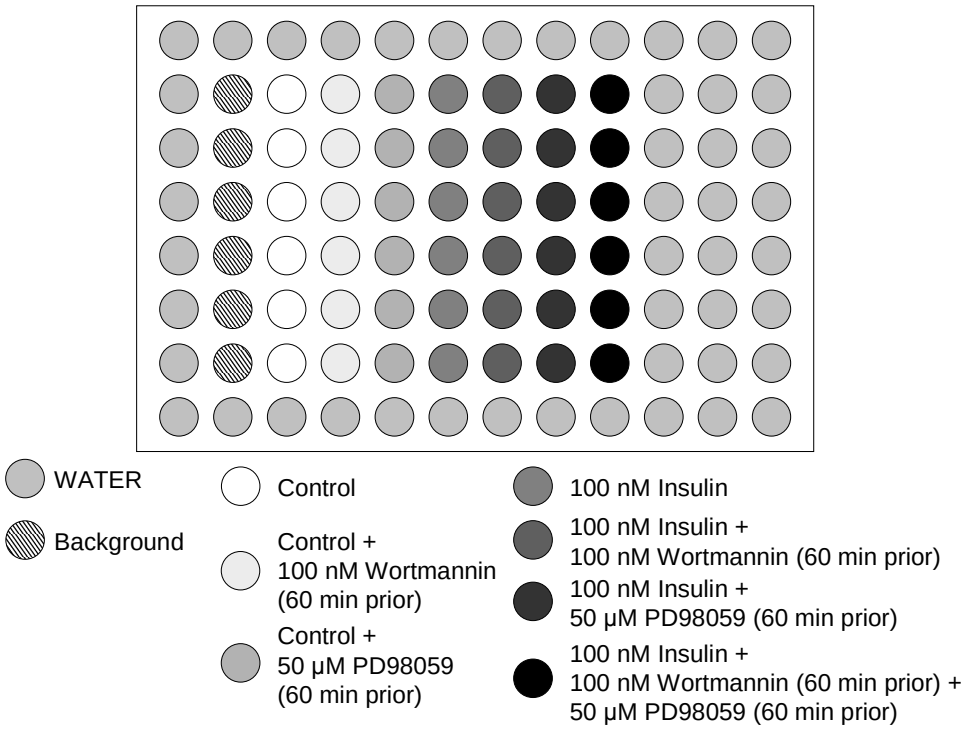

Figure 3-2: Experimental setup of a 96-well plate to measure cell proliferation changes using the BrdU-incorporation assay, 24 h cell proliferation assay only.

Four experiments were performed for MDA-MB-231 cells including all inhibitors. Four experiments were performed for MCF-10A cells, including three with inhibitors. For SK-BR-3 cells, three experiments were performed with insulin alone. Influence of inhibitors was not assessed. After the treatment period, medium from all wells was removed and BrdU-incorporation was analysed as described in Materials and Methods section 2.2.1.

3.2.3 Insulin receptor phosphorylation assay

In 60 mm^2 tissue culture dishes (Fisher Scientific, Cat No: 150288), 1 x 10^6 cells of each of the three cell lines were plated and incubated in 3 ml growth medium for 24 h at 37°C. Dishes were washed once in PBS, 3 ml serum-free medium was added and dishes were incubated for an additional 24 h at 37°C. For each experiment, four dishes per cell

line were prepared in this way. Then two dishes were kept unaltered (Control) and two dishes were washed with PBS, re-supplemented with insulin treatment medium and incubated for 2 min at 37°C. Then all medium was removed, dishes were washed with PBS and protein was extracted. The analysis of insulin receptor phosphorylation is detailed in the Materials and Methods section 2.5. Three experiments with two replicates for each treatment (Control and 100 nM insulin) were performed for all three cell lines. Each experiment for MDA-MB-231 and MCF-10A was performed simultaneously and thus analysed on the same ELISA-plate. All three experiments for SK-BR-3 cells were analysed on a single ELISA-plate.

3.2.4 Assessment of total insulin receptor protein using western blotting

MDA-MB-231 cells and MCF-10A cells were plated in 60 mm^2 dishes (Fisher) at $0.5x10^6$ cells/dish, with 3 ml growth medium. A total of four dishes were plated per cell line per experiments. Cells were incubated for 24 h at 37°C, then washed in sterile PBS and re-supplemented with 3 ml serum-free medium. Cells were incubated in serum-free medium for 24 h at 37°C and two dishes were washed in sterile PBS. In the remaining two dishes serum-free medium was retained until protein extraction. The washed dishes were re-supplemented with 3 ml 100 nM insulin treatment medium and incubated for 2 min at 37°C. Then all four dishes were washed in PBS and protein was extracted as described in Materials and Methods section 2.6.1. These experiments were set-up simultaneously to the IR-phosphorylation experiments described in section 3.2.3.

3.2.5 Cell signalling pathway assays for determination of AKT or ERK1/2-phosphorylation

Cells from all three cell lines were plated into a supplied clear bottom black 96-well plate at a density of 5000 cells/well with 100 µl/well growth medium and incubated for 24 h at 37°C. After 24 h, cells were washed once in 100 µl/well sterile PBS and incubated in 100 µl serum-free medium for an additional 24 h at 37°C. Cells were pre-treated with 100 nM wortmannin or 50 µM PD98059 for 1 h at 37°C as described in section 3.2.2 and indicated in Figure 3-3. During pre-treatment, 2 ml of each of the treatment media indicated in Figure 3-3 were prepared. Dilution factors for insulin and the inhibitors were described in Material and Methods section 2.1.3. All dilutions were made in serum-free medium respective for the examined cell line. After pre-treatment all medium was removed, wells were washed once in PBS and 100 µl of prepared treatment media were added to the wells. The plate was then incubated at 37°C. Medium was replaced by 4% Formaldehyde Solution after the indicated time (5-20 min). After the medium in the last wells was changed to 4% Formaldehyde Solution, the plate was incubated for 20 min before analysis continued as described in Materials and Methods section 2.3. Number of experiments performed for each cell line, treatment time and inhibitors used is summarised in Table 3-1.

Table 3-1: Number of experiments performed for cell signalling pathway phosphorylation analysis, each experiment reflects two treatment replicates. NA: Not analysed.

		MDA-MB-231		MCF-10A		SK-BR-3	
		AKT	**ERK1/2**	**AKT**	**ERK1/2**	**AKT**	**ERK1/2**
Insulin only	control	4	13	4	12	3	3
	5 min	4	9	4	9	3	3
	10 min	4	9	4	9	3	3
	15 min	NA	5	NA	4	3	3
	20 min	NA	6	NA	5	3	3
Insulin + Wortmannin	control	5	3	5	4	3	3
	5 min	5	2	5	3	3	3
	10 min	5	2	5	3	3	3
	15 min	NA	NA	NA	NA	3	3
	20 min	NA	1	NA	1	3	3
Insulin + PD98059	control	3	6	3	5	3	3
	5 min	3	4	3	4	3	3
	10 min	3	4	3	3	3	3
	15 min	NA	NA	NA	NA	3	3
	20 min	NA	1	NA	1	3	3

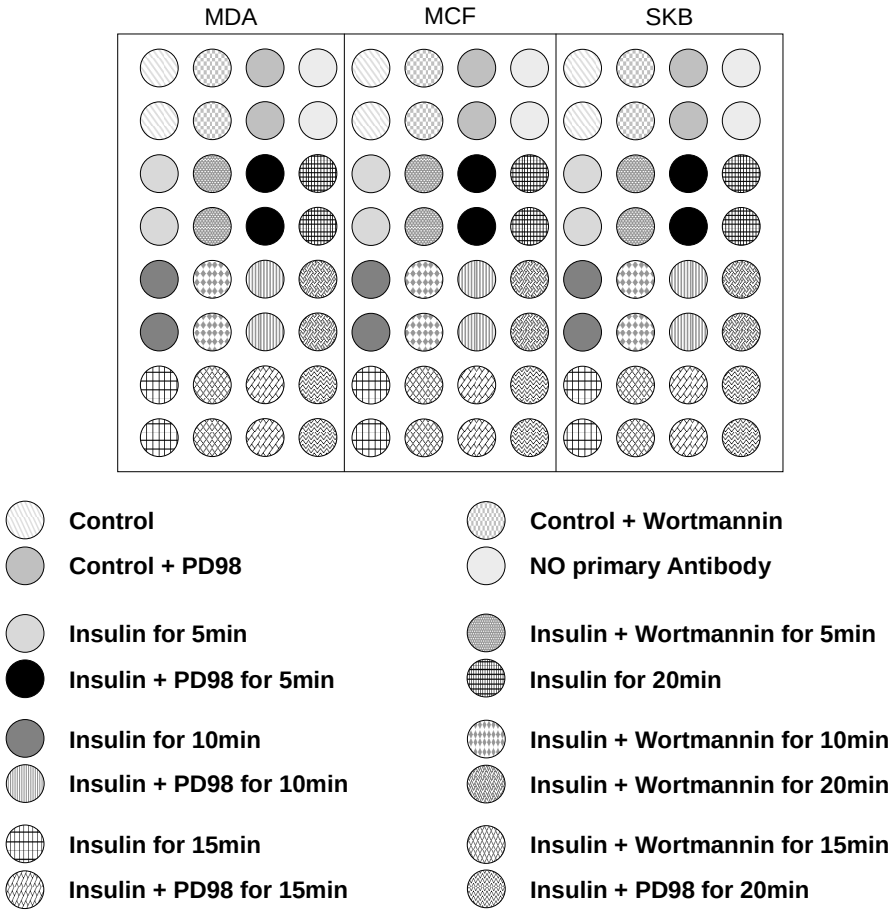

Figure 3-3: Representative layout of a 96-well plate used for the assessment of AKT or ERK1/2-phosphorylation. Individual experiments were adapted.

3.2.6 Cell cycle analysis by flow cytometry

Cells were plated in 6-well plates (Corning Life Sciences, Amsterdam, The Netherlands, Cat No: 3516) at 0.75×10^6 cells/well in 3 ml growth medium and incubated for 24 h at 37°C. In each 6-well plate two wells were used for each treatment, creating two treatment replicates in each experiment. Two wells contained growth medium throughout the experiment (+FCS). After the initial 24 h incubation period, cells were washed in PBS, re-supplemented with 3 ml serum-free medium and incubated for 24 h at 37°C. After 24 h incubation in serum-free medium, cells were washed in PBS and 3 ml of treatment medium was added. In two wells, the serum-free

medium was changed to fresh serum-free medium for an additional 24 h (Control, NO FCS) whilst the medium in the two remaining wells was changed to insulin treatment medium and incubated for additional 24 h. After treatment, cell cycle stage was analysed as described in Materials and Methods section 2.8.1. The flow cytometry raw data was obtained as percentage of cells within each cell cycle stage. Three experiments as described above were performed for each cell line.

3.3 Results

3.3.1 Effect of insulin treatment on cell proliferation

Cell proliferation of all cell lines was assessed after 100 nM insulin treatment for 24 h or 48 h (Figure 3-4). Untreated control cells were incubated in serum-free medium (Control) at all times. All results are presented as percentage change from this control. After 24 h treatment cell proliferation did not change in MDA-MB-231 cells compared to untreated control. In MCF-10A cells, proliferation increased by 184% ($p<0.001$) after 24 h compared to its untreated control (Figure 3-4, B). Cell proliferation did not change in SK-BR-3 cells after 24 h insulin treatment (Figure 3-4, C). Cell proliferation decreased by 18% ($p<0.001$) after 48 h insulin treatment in MDA-MB-231 cells and increased by 34% ($p=0.001$) in MCF-10A cells compared to their respective untreated control. Cell proliferation in SK-BR-3 cells did not change after 48 h (Figure 3-4, C).

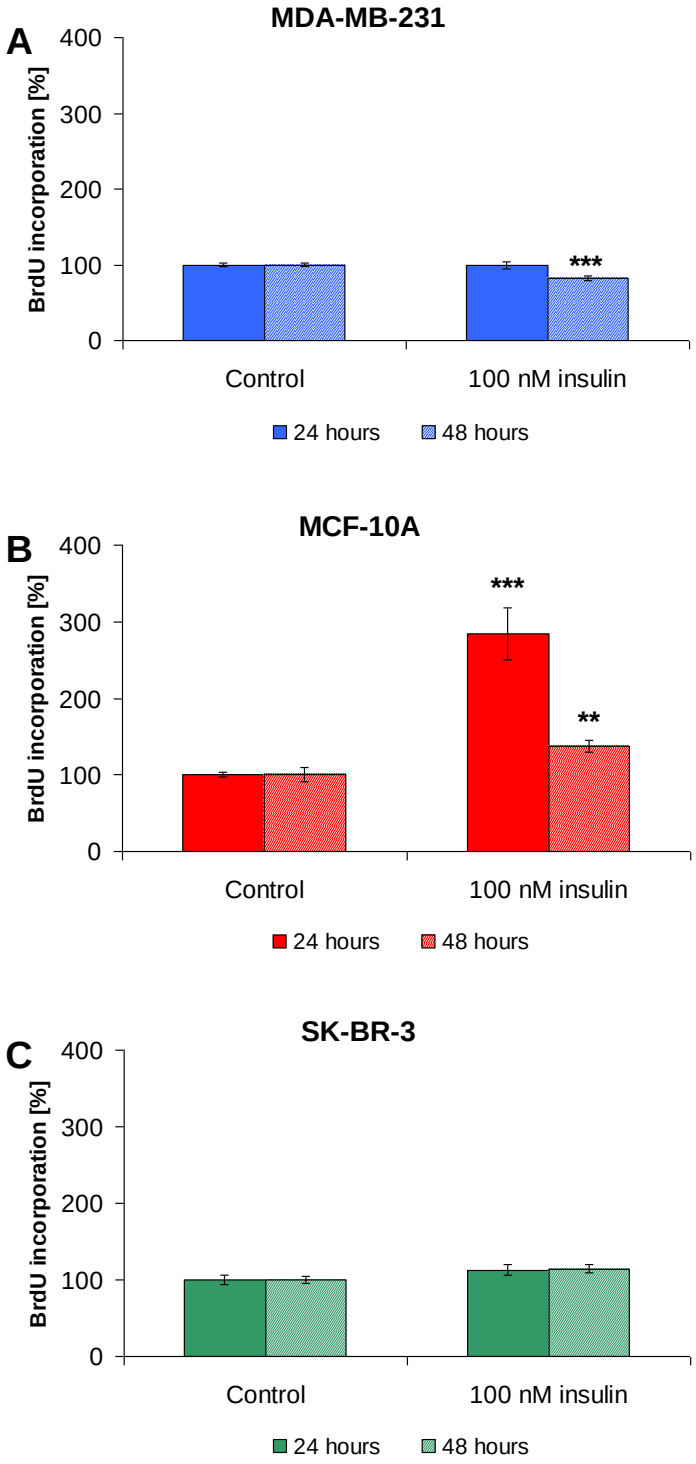

Figure 3-4: Changes in cell proliferation after treatment with 100 nM insulin alone for 24 h or 48 h in A) MDA-MB-231breast cancer cells (blue), B) MCF-10A breast epithelial cells (red) and C) SK BR-3 breast cancer cells (green). Bars represent BrdU-incorporation in relation to the respective control within each graph and are expressed as a percentage thereof. Error bars represent ± SEM of three experiments, each consisting of six or twelve replicates, *i.e.* 18 or 36 data points for each bar.
* Significance value compared to control, obtained using Dunnett's post-hoc t-test following ANOVA analysis. (** 0.01>p>0.001; *** p<0.001)

3.3.2 Effect of insulin treatment in insulin receptor phosphorylation

Phosphorylation and expression of the insulin receptor were measured after 2 min of 100 nM insulin treatment and compared to untreated cells. Untreated control cells were incubated in serum-free medium (Control) at all times. All results are presented as percentage change from this control. In MDA-MB-231 cells insulin receptor phosphorylation increased by 380% ($p<0.001$) compared to control. In MCF-10A and SK-BR-3 cells the increase of phosphorylation was similar with 38% ($p<0.001$) and 35% ($p<0.001$) increase, respectively compared to control (Figure 3-5, A). Total insulin receptor content was assessed for MDA-MB-231 and MCF-10A cells only and showed no change between Control and insulin treatment (Figure 3-5, B). Assessment of total insulin receptor expression allowed estimation of IR protein expression changes, which could have masked relative IR phosphorylation. The non-significant decrease in total IR content may be caused by ubiquitination and lysosomal degradation of the insulin-IR complex. Interestingly total insulin receptor content was higher in MDA-MB-231 cells than in MCF-10A cells[4], supporting previous findings by other groups that MDA-MB-231 cells overexpress the IR compared to breast epithelial cells (Milazzo *et al*., 1992).

[4] As only ratios between control and treated cell extracts was needed, no standard curve to estimate total protein content was included. Of 25 µg total protein loaded during Western Blotting, MDA-MB-231 untreated control reached an average of 53 Int/mm^2 (SEM = 7.53) and untreated control of MCF-10A cells 18 Int/mm^2 (SEM = 3.27). These findings are also supported by Papa and colleague's findings (1997), where overexpression of the insulin receptor compared to breast epithelial cells was observed in MDA-MB-231.

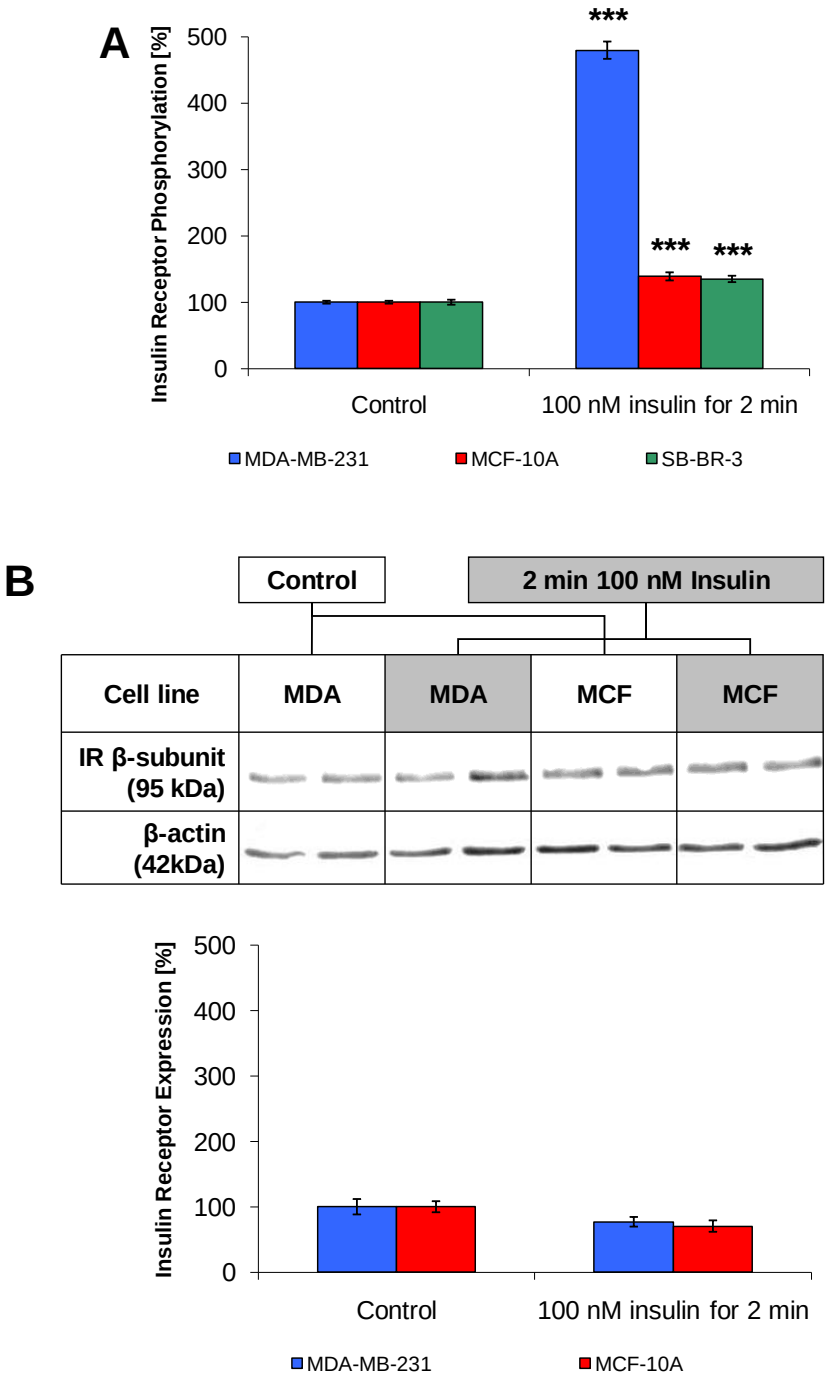

Figure 3-5: Changes in A) insulin receptor phosphorylation detected by DuoSet IC human phosphor-insulin receptor ELISA kit and B) Insulin receptor protein expression detected by Western Blotting after treatment for 2 min with 100 nM insulin in MDA-MB-231 breast cancer cells (blue), MCF-10A breast epithelial cells (red) and SK BR-3 breast cancer cells (green, IR-phosphorylation only). Bars represent A) insulin receptor phosphorylation and B) insulin receptor protein expression in relation to the respective control within each graph and are expressed as a percentage thereof. Error bars represent ± SEM of three experiments, each consisting of two replicates, *i.e.* six data points for each bar.
* Significance value compared to control, obtained using One-way ANOVA testing (*** $p<0.001$)

3.3.3 Effect of insulin treatment on activation of downstream cell signalling pathways

3.3.3.1 Activation with insulin alone

Phosphorylation of AKT or ERK1/2, representative of activation of the PI3-kinase or MAP-kinase cell signalling pathway, respectively, was measured after treatment with 100 nM insulin for different lengths of time ranging from 5 min to 20 min (Figure 3-6). Untreated control cells were incubated in serum-free medium (Control) at all times. All results are presented as percentage change from this control.

In MDA-MB-231 cells AKT-phosphorylation increased by 294% after 5 min ($p<0.001$) and by 141% after 10 min ($p<0.001$) treatment with 100 nM insulin compared to control (Figure 3-6, A). Phosphorylation of ERK1/2 however increased by 95% after 15 min ($p<0.001$) and by 174% after 20 min ($p<0.001$) treatment with 100 nM insulin (Figure 3-6, B).

In MCF-10A cells AKT-phosphorylation increased by 75% after 5 min ($p=0.022$) treatment with 100 nM insulin and no statistically significant change was observed after 10 min insulin treatment (Figure 3-6, C). Phosphorylation of ERK1/2 increased by 29% after 10 min ($p=0.002$) treatment with 100 nM insulin. No statistically significant change was observed at any other time point (Figure 3-6, D).

In SK-BR-3 cells AKT-phosphorylation increased by 67% after 5 min ($p<0.001$) treatment with 100 nM insulin and no significant change in AKT-phosphorylation was observed at the other treatment times (Figure 3-6, E). Phosphorylation of ERK1/2 decreased by 15%, 20% and 24% after treatment with 100 nM insulin for 5 min

($p=0.016$), 10 min ($p=0.002$) and 15 min ($p<0.001$), respectively, but did not significantly change after 20 min of insulin treatment compared to control (Figure 3-6, F).

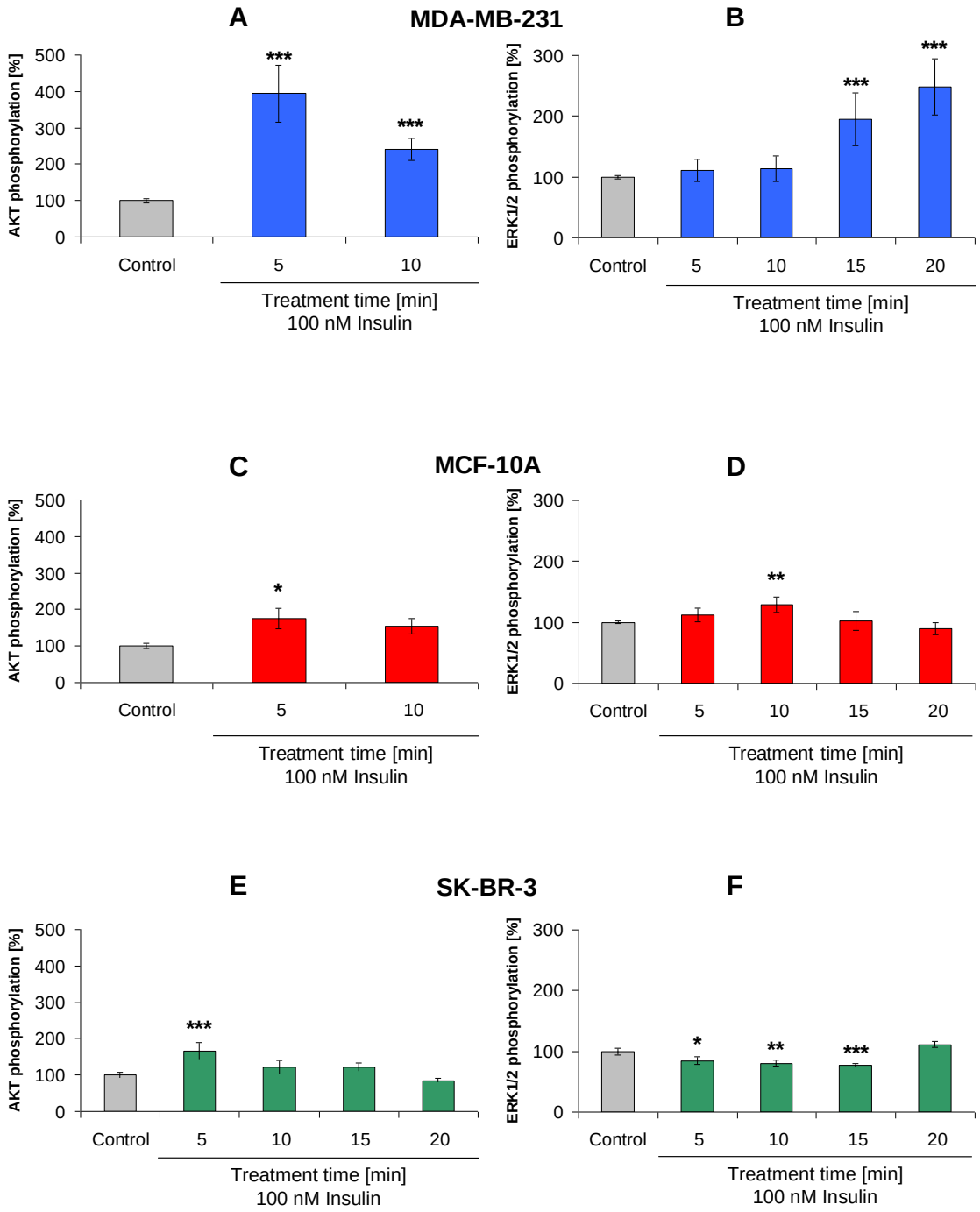

Figure 3-6: Changes in cell signalling pathway activation (PI3-kinase (A, C, E), MAP-kinase (B, D, F)) with 100 nM insulin treatment in A, B) MDA-MB-231 breast cancer cells, C, D) MCF-10A breast epithelial cells and E, F) SK-BR-3 breast cancer cells. Bars represent AKT-phosphorylation or ERK1/2-phosphorylation in relation to the respective control within each graph and are expressed as a percentage thereof. Error bars represent ± SEM of three experiments, each consisting of two replicates, *i.e.* six data points for each bar.
* Significance value compared to control, obtained using Dunnett's post-hoc t-test following univariate analysis of variance. (* 0.05>p>0.01; ** 0.01>p>0.001; *** p<0.001)

3.3.3.2 Effect of cell signalling pathway inhibitors on activation of PI3-kinase and MAP-kinase pathways after insulin treatment

Cross-reactivity and specificity of the insulin action on PI3-kinase and MAP-kinase cell signalling pathway was examined by treating cells with wortmannin, a PI3-kinase cell signalling pathway inhibitor and/or PD98059, a MAP-kinase cell signalling pathway inhibitor and insulin (Figure 3-7, Figure 3-8 and Figure 3-9). Untreated control cells were incubated in serum-free medium (Control, solid grey) at all times. All results are presented as percentage change from this control.

In MDA-MB-231 cells, treatment with 100 nM wortmannin or 50 μM PD98059 alone did not change basal AKT-phosphorylation. AKT-phosphorylation did not change compared to control for the "insulin only"-series or the control for the "wortmannin"-series after 5 min or 10 min treatment with 100 nM insulin in the presence of 100 nM wortmannin. When MEK1/2 was inhibited with 50 μM PD98059, treatment with 100 nM insulin increased AKT-phosphorylation by 327% and by 196% after 5 min ($p<0.001$) and 10 min ($p<0.001$) treatment, respectively, compared to control for the "insulin only"-series. Combined treatment with 50 μM PD98059 and 100 nM insulin increased AKT-phosphorylation by 344% and by 213% after 5 min ($p<0.001$) and 10 min ($p<0.001$) respectively, compared to the control for the "PD98059"-series (Figure 3-7, A). Thus insulin-mediated AKT activation is independent of MEK1/2 phosphorylation, but dependant of normal PI3-kinase function.

In MDA-MB-231 cells no significant change in ERK1/2-phosphorylation after treatment with 50 μM PD98059 alone or in combination with 100 nM insulin was

observed (Figure 3-7, B). ERK1/2-phosphorylation did also not increase after 20 min combined treatment with 100 nM wortmannin and 100 nM insulin (Figure 3-7, B).

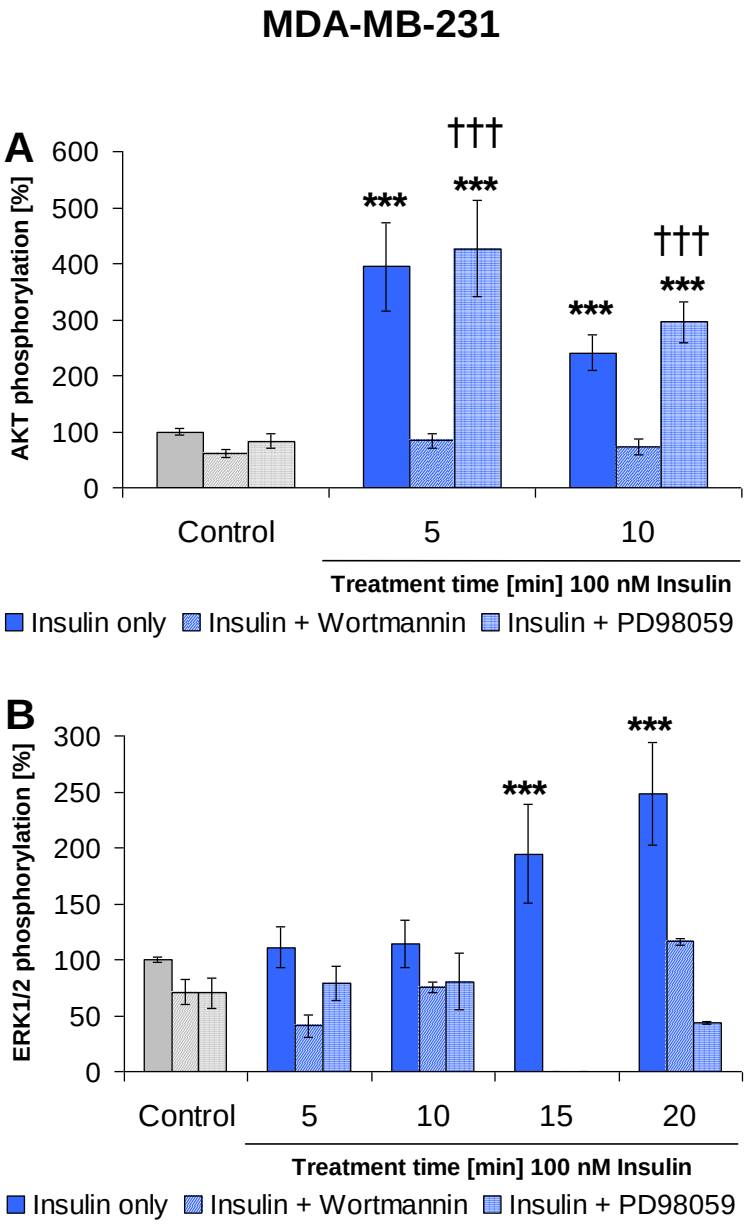

Figure 3-7: Changes in insulin induced cell signalling activity of A) PI3-kinase and B) MAP-kinase cell signalling pathways in MDA-MB-231 breast cancer cells. Bars represent AKT-phosphorylation or ERK1/2-phosphorylation in relation to the respective control within each graph and are expressed as a percentage thereof. Error bars represent ± SEM of three experiments, each consisting of two replicates, *i.e.* six data points for each bar.
* Significance value compared to control, obtained using Dunnett's post-hoc t-test following univariate analysis of variance. (*** $p<0.001$)
†significance value compared to its own control obtained using Tukey's HSD post-hoc test following univariate analysis of variance. (††† $p<0.001$)

In MCF-10A cells treatment with 100 nM wortmannin or 50 μM PD98059 alone did not change basal phosphorylation of AKT (Figure 3-8, A). Combined treatment with 100 nM wortmannin and 100 nM insulin did not increase AKT-phosphorylation compared to control for "insulin only" or "wortmannin" control after 5 min and 10 min treatment. Combined treatment of 50 μM PD98059 and 100 nM insulin increased AKT-phosphorylation by 101% after 5 min ($p=0.007$) treatment compared to control for "insulin only" and by 97% ($p=0.002$) compared to "PD98059" control (Figure 3-8, A).

Treatment with 100 nM wortmannin did not change basal ERK1/2-phosphorylation (Figure 3-8, B). Additionally in the presence of 100 nM wortmannin treatment with 100 nM insulin did not change ERK1/2-phosphorylation between 5-20 min of treatment. In MCF-10A cells ERK1/2-phosphorylation decreased by 30% ($p=0.001$) with treatment with 50 μM PD98059 alone compared to control for "insulin only". Combined treatment of 50 μM PD98059 and 100 nM insulin decreased ERK1/2-phosphorylation by 20% and by 21% after 5 min ($p=0.001$) and 10 min ($p=0.012$) treatment, respectively compared to control for "insulin only". Compared to "PD98050" control however ERK phosphorylation of these incubation times did not show a significant change (Figure 3-8, B).

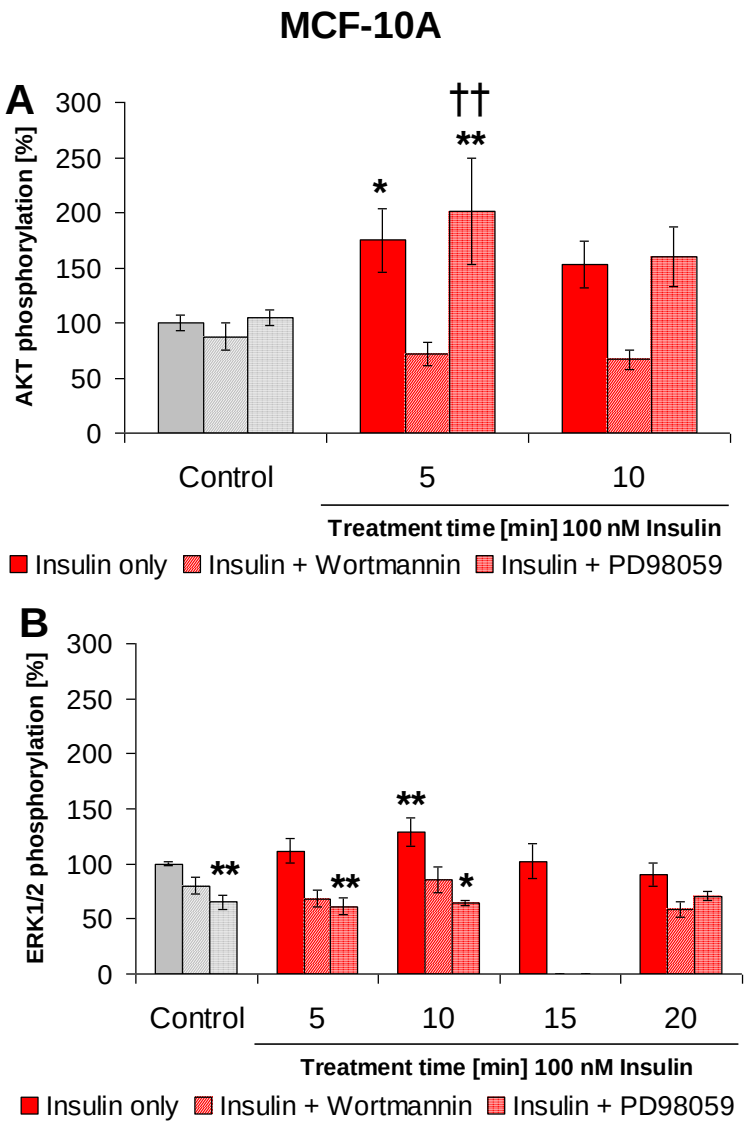

Figure 3-8: Changes in insulin induced cell signalling activity of A) PI3-kinase and B) MAP-kinase cell signalling pathways in MCF-10A breast epithelial cells. Bars represent AKT-phosphorylation or ERK1/2-phosphorylation in relation to the respective control within each graph and are expressed as a percentage thereof. Error bars represent ± SEM of three experiments, each consisting of two replicates, *i.e.* six data points for each bar.
* Significance value compared to control, obtained using Dunnett's post-hoc t-test following univariate analysis of variance. (* 0.05>p>0.01; ** 0.01>p>0.001),
†significance value compared to its own control obtained using Tukey's HSD post-hoc test following univariate analysis of variance. (†† 0.01>p>0.001)

In SK-BR-3 cells basal AKT-phosphorylation was decreased by 85% with 100 nM wortmannin alone ($p<0.001$) compared to control for “insulin only”. In the presence of 100 nM wortmannin, AKT-phosphorylation decreased by 81%, 84%, 86% and 92% after 5 min, 10 min, 15 min and 20 min (all $p<0.001$) treatment with 100 nM insulin, respectively compared to control for “insulin only” (Figure 3-9, A). Combined treatment of 100 nM wortmannin and 100 nM insulin did not significantly change AKT-phosphorylation compared to “wortmannin” control. Basal AKT-phosphorylation did not change in the presence of 50 μM PD98058, compared to control for “insulin only”. In the presence of 50 μM PD9859, AKT-phosphorylation increased by 57%, 59%, 60% and 52% after 5 min, 10 min, 15 min and 20 min (all $p<0.001$) treatment with 100 nM insulin, respectively compared to control for “insulin only”. No significant change in AKT-phosphorylation at any treatment timepoint compared to “PD98059” control was observed (Figure 3-9, A).

In SK-BR-3 cells ERK1/2-phosphorylation was decreased by 56% with wortmannin treatment ($p<0.001$) alone compared to control for “insulin only” (Figure 3-9, B). In the presence of 100 nM wortmannin, ERK1/2-phosphorylation was decreased by 36%, 41%, 54% and 51% after 5 min, 10 min, 15 min and 20 min (all $p<0.001$) treatment with 100 nm insulin, respectively compared to control for “insulin only” (Figure 3-9, B). Compared to “wortmannin” control ERK1/2-phosphorylation did not change with combined treatment of 100 nM wortmannin and 100 nM insulin at any timepoint. In SK-BR-3 cells ERK1/2-phosphorylation decreased by 25% with 50 μM PD98059 treatment alone ($p<0.001$) compared to control for “insulin only”. In the presence of 50 μM PD98059 ERK1/2-phosphorylation decreased by 47%, 38%, 42% and 39% after

5 min, 10 min, 15 min and 20 min (all $p<0.001$) treatment with 100 nM insulin, respectively compared control for "insulin only". Compared to "PD98059" control ERK1/2-phosphorylation decreased by 22% after 5 min ($p=0.02$) treatment with 100 nM insulin (Figure 3-9). It may be concluded that ERK1/2 is not a target for insulin in SK-BR-3 cells. Insulin also does not affect the inhibition in phosphorylation observed by both cell signalling pathway inhibitors.

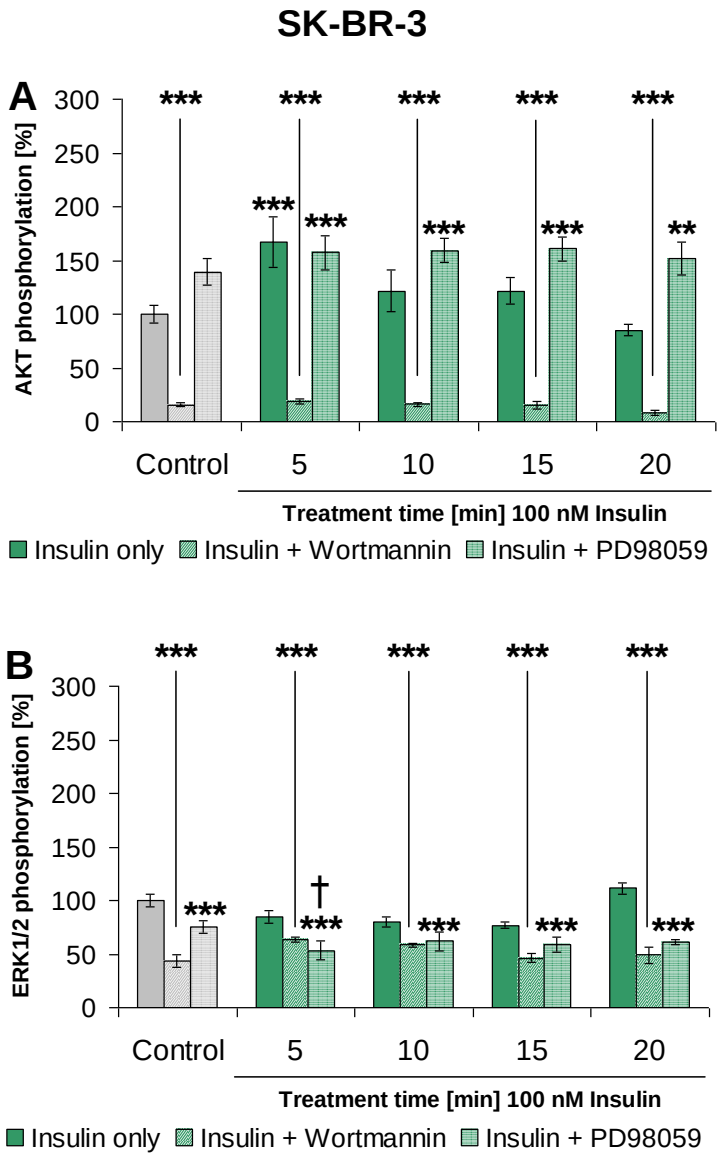

Figure 3-9: Changes in insulin induced cell signalling activity of A) PI3-kinase and B) MAP-kinase cell signalling pathways in SK-BR-3 breast cancer cells. Bars represent AKT-phosphorylation or ERK1/2-phosphorylation in relation to the respective control within each graph and are expressed as a percentage thereof. Error bars represent ± SEM of three experiments, each consisting of two replicates, *i.e.* six data points for each bar.
* Significance value compared to control, obtained using Dunnett's post-hoc t-test following univariate analysis of variance[5]. (** 0.01>p>0.001; *** p<0.001),
†significance value compared to its own control obtained using Tukey's HSD post-hoc test following univariate analysis of variance. († 0.05>p>0.01)

[5] Please note that "insulin only" treatment significantly decreased ERK1/2-phosphorylation after 5 min, 10 min and 15 min insulin treatment in SK-BR-3 cells as displayed in Figure 3-6, B. For comprehensiveness the asterisks indicting this significant difference have been omitted in Figure 3-9, B.

3.3.4 Effect of cell signalling pathway inhibitors on cell proliferation of insulin treated cells

The effect of wortmannin and PD98059 on cell proliferation in combination with insulin treatment for 24 h was assessed in MDA-MB-231 breast cancer cells and MCF-10A breast epithelial cells, but not in SK-BR-3 breast cancer cells (Figure 3-10). Untreated control cells were incubated in serum-free medium (Control) at all times. All results are presented as percentage change from this control.

In MDA-MB-231 cells, cell proliferation did not change with 100 nM wortmannin treatment for 24 h compared to control for "no inhibitor". Cell proliferation decreased by 29% after 24 h treatment with 50 μM PD98059 ($p<0.001$) compared to control for "no inhibitor". Cell proliferation did not change after 24 h combined treatment with 100 nM insulin and 100 nM wortmannin or with 100 nM insulin and 50 μM PD98059 compared to control for "no inhibitor". Cell proliferation increased significantly by 20% after combined treatment with 100 nM insulin and 50 μM PD98059 for 24 h ($p=0.002$) compared to "PD98059" control. Combined treatment with 100 nM insulin, 100 nM wortmannin and 50 μM PD98059 reduced cell proliferation by 15% ($p=0.007$) compared to control for "no inhibitor" (Figure 3-10, A).

Cell proliferation in MCF-10A cells was reduced with 100 nM wortmannin and 50 μM PD98059 treatment for 24 h by 66% and by 65% respectively compared to control for "no inhibitor" (both $p<0.001$). Combined treatment with 100 nM insulin and 100 nM wortmannin or 100 nM insulin and 50 μM PD98059 for 24 h increased cell proliferation by 31% ($p=0.001$) and 8% ($p=0.031$), respectively compared to control for "no inhibitor". Compared to their own control the increase in cell proliferation was 101%

($p<0.001$) for combined insulin/wortmannin treatment and 59% ($p<0.001$) for combined insulin/PD98059 treatment. Combined treatment with 100 nM insulin, 100 nM wortmannin and 50 μM PD98059 for 24 h decreased cell proliferation by 68% ($p<0.001$) compared to control for "no inhibitor" (Figure 3-10, B).

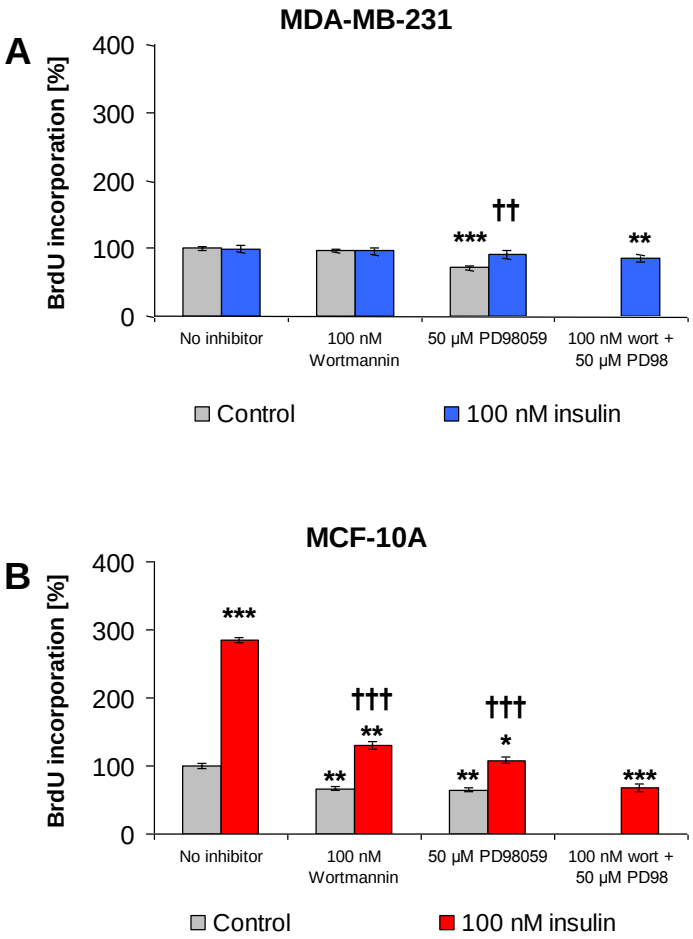

Figure 3-10: Changes in cell proliferation after treatment with insulin and cell signalling pathway inhibitors in A) MDA-MB-231 cells and B) MCF-10A cells. Bars represent BrdU-incorporation after treatment as indicates in relation to the control for "no inhibitor" and expressed as a percentage thereof. Error bars represent ± SEM of three experiments, each consisting of six replicates per treatment, *i.e.* 18 data points for each bar.
*significance value for treatment compared to "no inhibitor/control", obtained using Dunnett's post-hoc t-test following univariate analysis of variance.
(*0.05>p>0.01; **0.01>p>0.001; ***p<0.001),
†significance value compared for treatment to their own control, obtained using Tukey's HSD post-hoc test following univariate analysis of variance (†††p<0.001).

3.3.5 Effect of insulin on cell cycle progression

Cell cycle profiles for all cell lines were established after treatment with 100 nM insulin for 24 h. One initial experiment each was also performed after 3 h and 6 h of treatment with 100 nM insulin (data not shown), which did not indicate a significant change and these incubation times were not further pursued. Two controls were performed for instrument calibrations, one with cells incubated with fully supplemented growth medium at all times and one with cells incubated in serum-free medium as untreated control (see Appendix section 9.3.3.1). For the analysis presented in Figure 3-11, "control" refers to cells being incubated in serum-free medium, corresponding to the controls of all the other analyses presented is subsequent chapters.

The performed analysis allowed for estimating the impact insulin treatment had on the amount of cells within each cell cycle stage. Treatment with 100 nM insulin increased S-phase population by 1.5 percentage points (an increase of 13%[6]) after 24 h treatment ($p=0.002$) compared to S-phase population of control cells. Insulin treatment did not change any other cell cycle stage in MDA-MB-231 cells compared to the respective cell cycle stage population in control cells (Figure 3-11, A).

In MCF-10A (Figure 3-11, B) and SK-BR-3 (Figure 3-11, C) treatment with 100 nM insulin for 24 h did not change the amount of cells in any cell cycle stage compared to the respective cell cycle stage population in control cells.

[6] This number represents the percentage increase corresponding to a 1.5 percentage point increase between insulin treated and control cells. This is also valid for all additional chapters.

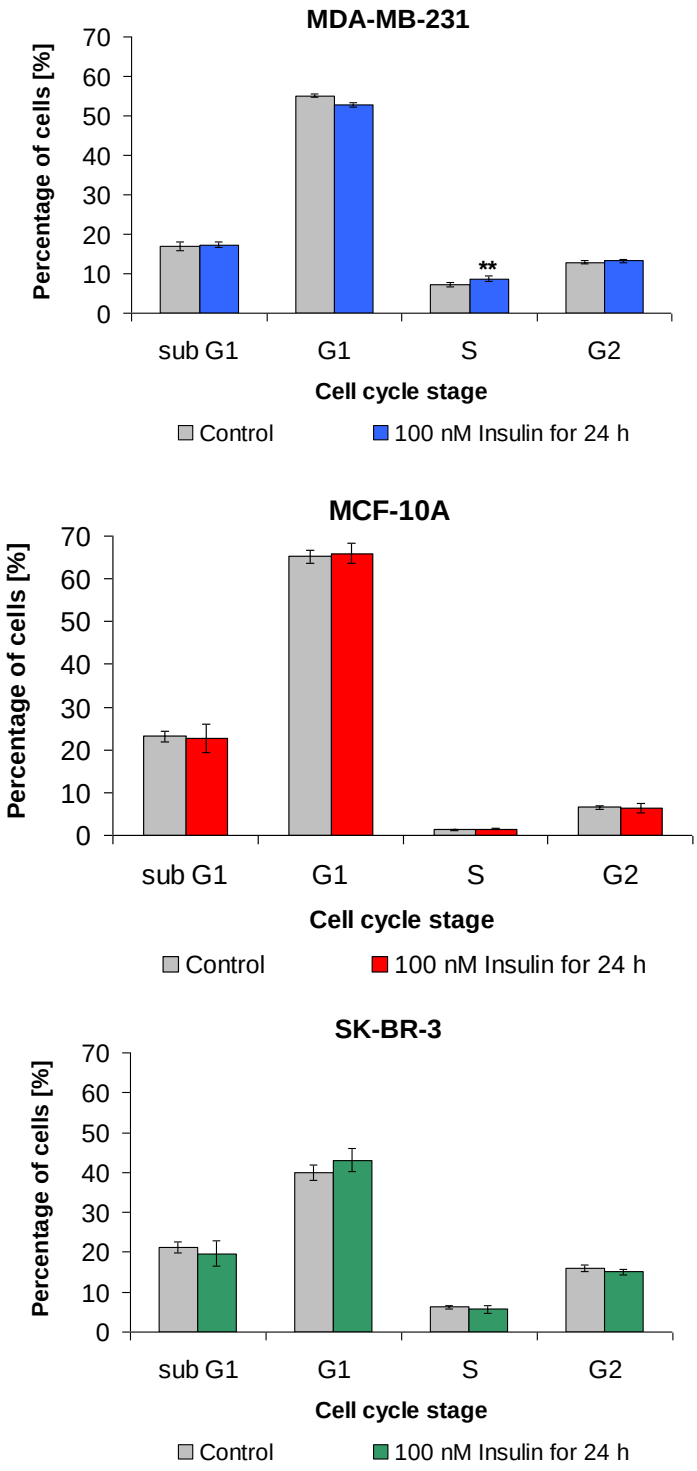

Figure 3-11: Changes of cell population distribution across cell cycle stages after 24 h treatment with 100 nM insulin treatment in A) MDA-MB-231 cells, B) MCF-10A cells and C) SK-BR-3 cells. Bars represent percentage of cells as obtained from a 10000 cell sample obtained, treated and analysed as described in section 2.8.1. Error bars represent ± SEM of three experiments with two replicates for each treatment, *i.e.* 6 data points for each bar. *significance value compared to control (grey) at that stage, obtained using Dunnett's t-test following univariate analysis of variance (** 0.05>p>0.01).

3.4 Discussion

3.4.1 The effect of insulin treatment on human MDA-MB-231 breast cancer cells

In the study presented here 100 nM insulin treatment for 24 h did not increase cell proliferation in MDA-MB-231 but decreased cell proliferation after 48 h of treatment (see section 3.3.1). Several studies had previously reported that insulin stimulation does not increase cell proliferation of MDA-MB-231 cells, using a variety of techniques to estimate cell proliferation (Bardon and Razanamahefa, 1998; Costantino *et al.*, 1993; Godden *et al.*, 1992; Osborne *et al.*, 1978). The study by Osborne and colleagues (1978) aimed at developing further their idea of insulin stimulated growth in breast cancer cells, which they had observed in a previous study with MCF-7 breast cancer cells (Osborne *et al.*, 1976). Examination included the ability of insulin to bind to its receptor and how long the insulin-IR interaction would be in place, before half of the administered insulin was degraded. In the study from 1978 this analysis included three additional breast cancer cell lines, including MDA-MB-231 breast cancer cells. Their first validating experiment was measuring incorporation of [^{14}C] labelled leucine and thymidine, which did not change in MDA-MB-231 breast cancer cells with 24 h insulin treatment at insulin concentrations between 0.06 ng/ml (0.103 nM) and 60 ng/ml (10.3 nM), but was significantly elevated in MCF-7 and ZR-75-1 breast cancer cells (Osborne *et al.*, 1978). The authors concluded that increased leucine and thymidine incorporation were indicative of increased proliferation, thus conclusively a lack of such incorporation corresponded to no effect of insulin on proliferation in MDA-MB-231 breast cancer cells. Thus the cell proliferation results in the study presented here correspond to the results observed by Osborne and colleagues (1978). The study by Costantino and colleagues (1993) is one of a number of studies by the same group on

the effect of insulin on breast cancer cells (see also section 8.4.2). Their focus shifted however to examining IR and IGF-1R in breast cancer cells in later studies. In the study from 1993, MDA-MB-231 breast cancer cells were specifically examined for [^{3}H] labelled thymidine incorporation after insulin treatment (range: 0.1 nM and 100 nM). Thus their experimental approaches of DNA synthesis and insulin concentration were similar to the experimental approach in the study presented here. Their incubation time in serum-free medium was however 48 h and insulin treatment time was 48 h, while in the study presented here, incubation time in serum-free medium was 24 h and insulin treatment time was either 24 h or 48 h. Their results were identical to the results presented in the study presented here, in that insulin treatment of 100 nM did not increase DNA synthesis in MDA-MB-231 breast cancer cells (Costantino *et al.*, 1993). Two additional studies (Bardon and Razanamahefa, 1998; Godden *et al.*, 1992) examined the effect of insulin on MDA-MB-231 as part of their studies. Unfortunately only abstracts were available for these last two studies, thus their measurement of cell proliferation and insulin treatment time and concentration are not known. Godden and colleagues' (1992) main finding was that insulin induced proliferation only in ER-positive breast cancer cells and not in ER-negative ones. Bardon and Razanamahefa's (1998) main finding was that retinoic acid inhibited insulin-induced cell proliferation in MCF-7 and T47D breast cancer cells but MDA-MB-231 breast cancer cells were not affected. It is not specified however, whether that meant that MDA-MB-231 cell proliferation did not increase with insulin treatment or whether retinoic acid treatment failed to decrease cell proliferation. In conclusion there is sufficient evidence suggesting that insulin does not affect cell proliferation in MDA-MB-231 breast cancer cells.

Interestingly, while studies, including the one presented here, continuously demonstrate that insulin does not affect cell proliferation in MDA-MB-231 breast cancer cells, insulin treatment does affect a number of other aspects in MDA-MB-231 breast cancer cells. Insulin is necessary for "normal" cell motility (Pan and Djamgoz, 2008) and mediates an increase HIF-1[7], which was MAP-kinase dependent, suggesting that insulin can affect the MAP-kinase pathway in these cells (Bartella *et al.*, 2008). These authors also observed that insulin treatment can increase leptin expression in these cells. This effect seems surprising as two reports suggested that insulin does not affect cell proliferation in MDA-MB-231 breast cancer cells, because of the expression of an inhibitor of IR-phosphorylation, membrane glycoprotein PC-1 (Belfiore *et al.*, 1996b; Costantino *et al.*, 1993). The study presented here, in addition to findings mentioned above, indicates however that insulin does have an effect on MDA-MB-231 breast cancer cells. Thus the effect of insulin on MDA-MB-231 breast cancer cells, suggesting a more complex effect than merely increased cell proliferation.

There was an interesting observation that blocking MEK1/2 phosphorylation without insulin treatment decreased cell proliferation in MDA-MB-231 cells, while in the presence of insulin the same effect was not observed (Figure 3-7, B). Thus insulin seemingly increased cell proliferation, if the MAP-kinase pathway was inhibited. The first aspect of this observation, *i.e.* a decrease in cell proliferation after MEK1/2 inhibition is readily explained by the hypothesis that the MAP-kinase pathway is responsible for mediating cell growth in MDA-MB-231, where the likelihood of

[7] HIF-1 is the hypoxia induced transcription factor, having been shown to promote transcription of the leptin gene (Cascio *et al.*, 2008; Grosfeld *et al.*, 2002; Wang *et al.*, 2008; Wu *et al.*, 2007 and section 1.1.4.3).

consecutively activated MAP-kinase pathway exists due to the mutations of RAS and RAF. The second part of the observation however, *i.e.* that insulin increases cell proliferation, if MEK1/2 is inhibited, is more complex to answer. Insulin treatment alone did not increase cell proliferation and indeed decreased proliferation after 48 h of treatment (Figure 3-4). The MAP-kinase pathway is a major growth promoting pathway the MAP-kinase pathway is probably the main pathway by which insulin can act as a mitogen. The only other possibility for growth promotion would be activation of the PI3-kinase pathway, but inhibiting this pathway did not result in growth inhibition (Figure 3-10). One possible explanation for this finding could be that MDA-MB-231 breast cancer cells may operate at their maximal proliferative limit after incubation in serum-free medium and that insulin treatment alone is not able to increase this proliferation any further. In the presence of a MEK1/2 inhibitor, insulin, through stimulation of a MAP-kinase independent pathway, is however able to return cell proliferation to its maximal level. This interpretation would explain several additional findings discussed later on. There is however a serious flaw in this interpretation in that, if there is such a maximal level of proliferation, one would not observe an increase beyond this level. MDA-MB-231 cells grown in growth medium, supplemented with FCS, easily surpassed this level of proliferation (see Appendix section 9.1.1 and section 9.1.2). Thus one may speculate that this proliferation maximum is "self-imposed" by these cells under circumstances imposed by incubation in serum-free medium. The study presented here however observed increases in cell proliferation with leptin treatment in cells grown in serum depleted medium (section 4.3.1). Thus there is no fully supported explanation for the observed insulin induced increase in cell proliferation after MEP-kinase cell signalling pathway inhibition. Alternatively

MDA-MB-231 cancer cells may produce an autocrine signal that utilises MAP-kinase signal, which is disrupted with PD98059 treatment. The increase in proliferation between the PD98059 control and the combined treatment of insulin and PD95059 however is not significant.

While the cell proliferation results of the study presented here were in agreement with the current findings in the literature, the results from the IR phosphorylation experiments were more controversial. The presented results show a 380% increase in IR tyrosine phosphorylation in MDA-MB-231 cells after 2 min treatment with 100 nM insulin. Other studies examined total IR-protein as opposed to changes in IR-phosphorylation. In 159 breast cancer samples, IR protein content was 6-fold elevated compared to samples from breast reduction surgery (Papa *et al.*, 1990). Additionally in breast cancer cell lines MCF-7 and ZR-75-1 IR content was elevated 5-fold and 3-fold, respectively (Milazzo *et al.*, 1992) and 5-fold in MDA-MB-231 breast cancer cells (Costantino *et al.*, 1993) compared to IR content in the non-malignant human breast epithelial cell line 184B5[8]. A subsequent study in MDA-MB-231 cells reported that the observed increased IR content is explained by an increase in IR-gene copy number, which is not usually observed in breast cancer cells or tumour samples (Papa *et al.*, 1997)[9]. Controversially, Costantino and colleagues (1993) reported that MDA-MB-231 cells overexpress IR, but they did not find an increase in IR phosphorylation with 100 nM insulin treatment for 1 min.

[8] IR-content was 29.1 ng/10^6 cells (Costantino *et al.*, 1993) and 5.3 ng/10^6 cells in 184B5 breast epithelial cell line (Milazzo *et al.*, 1992).

[9] MDA-MB-231 cells were the only cell line (of five examined in total) that showed an increase in IR-gene copy number, while the increase in IR expression in the other cell lines was suggested to be caused by changes in transcriptional activity at the IR promoter (Papa *et al.*, 1997).

Instead they reported a co-precipitate of the IR, which acted as a tyrosine kinase inhibitor, which was later identified as glycoprotein PC-1 (Belfiore *et al.*, 1996b). Thus there seems sufficient indication that IR phosphorylation in MDA-MB-231 breast cancer cells is significantly inhibited by an intrinsic factor. The results in the study presented here however show a significant increase in IR phosphorylation following insulin stimulation for 2 min in MDA-MB-231 breast cancer cells. A possible explanation is the difference in methodology. Costantino and colleagues (1993) used immunoprecipitation to isolate total IR protein, subjected the precipitate to western blotting and detected phosphorylated IR with an antiphosphotyrosine antibody, while the study presented here used a Phospho-Insulin ELISA assay. Still the observations presented here of IR phosphorylation are not supported by the findings of Costantino and colleagues (1993), with no conclusive explanation further evaluation may be needed to explore this interesting contradiction.

MDA-MB-231 breast cancer cells showed increased phosphorylation of AKT after 5 min and 10 min treatment with 100 nM insulin (Figure 3-6, A). In combination with the previous finding that IR phosphorylation was significantly increased, this result shows that MDA-MB-231 breast cancer cells do show a response to insulin treatment. Normal insulin signalling involves activation of the PI3-kinase pathway, thus, as the IR is strongly activated, it comes as no surprise that AKT shows a significant increase in phosphorylation. This suggests that normal insulin signalling is intact in MDA-MB-231 breast cancer cells and that there is no intrinsic inhibition. The signal however is short lived as the increase in AKT-phosphorylation after 10 min is only half as strong as after 5 min of 100 nM insulin treatment. One study examining PI3-kinase pathway activation

after insulin treatment in MDA-MB-231 breast cancer cells showed that inhibition of AKT decreased insulin-induced gene expression of leptin (Bartella *et al.*, 2008).

Activation of the MAP-kinase pathway in MDA-MB-231 cells was also observed. Compared to AKT-phosphorylation, ERK1/2-phosphorylation only significantly increased after 15 min treatment, when AKT-phosphorylation was decreasing after a 5 min maximum phosphorylation increase. This "lagging" behind may be an indication that the activation of the MAP-kinase pathway may be indirect, potentially even through the PI3-kinase pathway itself, via cross-activation from AKT to RAS. The phosphorylation results observed after combined treatment with insulin and wortmannin support this suggestion (Figure 3-7, B). Here, in the presence of 100 nM wortmannin, phosphorylation of ERK1/2 was not increased after treatment between 5 min and 20 min with 100 nM insulin. Thus intact PI3-kinase signalling was necessary for the observed increase in ERK1/2-phosphorylation after 15 min and 20 min treatment with 100 nM insulin. Alternatively ERK1/2 is further downstream in the MAP-kinase pathway than AKT is in the PI3-kinase pathway, thus it may take more time for the insulin signal to reach ERK1/2 (see section 1.6). Moreover RAF, MEK1/2 and ERK1/2[10] are cytosolic kinases, while AKT is phosphorylated at the cell membrane in close proximity to the original receptor site. In a study examining the potential for insulin to induce leptin expression, inhibition of ERK1/2 activation, reduced the observed leptin expression in MDA-MB-231 cells (Bartella *et al.*, 2008). This study however did not examine, if insulin increases ERK1/2-phosphorylation. Thus the study presented here is the first to demonstrate the ability of insulin to increase

[10] Only RAS is phosphorylated at the cell membrane, while subsequent members of the MAP-kinase pathway are not required to be at the cell membrane for phosphorylation.

ERK1/2-phosphorylation in MDA-MB-231 breast cancer cells. The pattern of MAP-kinase activation however indicates a complex inter-relationship with other pathways that may mediate this activation.

The effect of insulin treatment on cell population distribution by cell cycle stage is limited. Insulin treatment however does increase significantly the S-phase population in MDA-MB-231 cells and in turn seems to decrease the G0-phase population. This decrease is however not significant. During S-phase cells replicate their DNA content in preparation to undergo mitosis after passing through the G2/M checkpoint in the cell cycle. An increase in the S-phase population therefore may indicate insulin transferring more cells into a position to undergo mitosis, thus accelerating cell cycle progression. This finding may be interpreted that insulin increases cell cycle progression in MDA-MB-231 breast cancer cells. One would expect increased cell cycle progression in highly proliferating cells. The cell proliferation results from the study presented here in addition to others are not however in concordance with this observation.

3.4.2 The effect of insulin treatment on MCF-10A normal human breast epithelial cells

Cell proliferation increased in MCF-10A cells after treatment with 100 nM insulin for 24 h. Cell proliferation is still significantly increased after 48 h treatment with insulin compared to 48 h control[11]. Thus there is sufficient indication that insulin increased cell proliferation in MCF-10A cells. For the study presented here that raises the possibility that obesity may act through hyperinsulinaemia to promote cell proliferation in breast epithelial cells and thus increase the potential for carcinogenic development. There are

[11] See also Appendix section 9.2.

no comparable studies available that examined the effect of insulin on MCF-10A breast epithelial cells. Normal growth medium for MCF-10A cells however contains 10 µg/ml insulin (1.7 µM), which suggests that cell proliferation in these cells is affected by insulin (see also section 2.1.1 and section 8.8.1). Thus the study presented here may spur further investigation into the potential of insulin in promoting breast epithelial cell growth and thus act in a way to promote breast cancer development.

Assessment of insulin receptor content and activation for MCF-10A cells has not been examined elsewhere at the time of writing. The results presented here indicate a significant increase in IR phosphorylation after insulin treatment for 2 min. This confirms that insulin activates the IR in MCF-10A cells. Assessment of IR content showed no changes in IR expression after 2 min insulin treatment, thus the increase in phosphorylation is entirely caused by activation of the IR by insulin stimulus. Consequently, insulin exerts its effect, at least partly, through its own receptor in MCF-10A cells. In both MDA-MB-231 cells and MCF-10A cells, IR-protein expression decreased non-significantly after 2 min treatment with 100 nM insulin compared to untreated control. This is an expected finding, as phosphorylated IR cannot be de-phosphorylated, but instead is degraded in the proteasome, once it has undergone activation and replaced by newly synthesised IR. Thus this non-significant decrease in IR expression further supports an increase in IR-phosphorylation.

MCF-10A cells, AKT-phosphorylation increased significantly after 5 min treatment with 100 nM insulin. All further treatments with insulin did not increase AKT-phosphorylation. Thus insulin does activate the PI3-kinase cell signalling pathway. The

activation is short lived and has its maximum within the first ten minutes of the insulin stimulus[12]. Compared to MDA-MB-231 cells, AKT-phosphorylation in MCF-10A cells is lower and shorter lasting. The level of activation however may be expected given the finding of a lower increase in IR phosphorylation compared to MDA-MB-231 cells. Thus there may be a direct correlation between level of IR phosphorylation and level of AKT-phosphorylation. The only comparative study examined the effect of insulin on MCF-10A cells used MCF-10A cells as a control for measurements in ZR-75-1 breast cancer cells (Gliozzo *et al.*, 1998). Here insulin treatment stimulated AKT-activation, which was decreased after treatment with PI3-kinase inhibitor LY294002 in MCF-10A breast epithelial cells. This study however used this finding as comparison to ZR-75-1 breast cancer cells, in which insulin stimulated cell proliferation was PI3-kinase independent (Gliozzo *et al.*, 1998). Thus there is evidence from the results in the study presented here and the literature that insulin increases activation of the PI3-kinase cell signalling pathway in MCF-10A cells. Additionally, the effect of PI3-kinase inhibitor wortmannin in MCF-10A cells suggests that AKT is partly responsible for mediating insulin induced cell proliferation.

Phosphorylation of ERK1/2 was significantly increased after 10 min treatment with 100 nM insulin in MCF-10A cells. At all further time points, no significant change in ERK1/2-phosphorylation compared to control was observed. Thus insulin treatment does activate the MAP-kinase pathway in MCF-10A cells. Interestingly the phosphorylation of ERK1/2 is increased by about 30% compared to control, thus below

[12] The insulin receptor can become desensitised by high insulin concentrations, as demonstrated in liver cells, with significantly decreased ability to autophosphorylate after 16 h of 100 nM insulin treatment (Blake *et al.*, 1987). A similar mechanism may explain the quick drop in AKT-phosphorylation with insulin treatment.

the level of proliferation increase (~180% increase). Additionally ERK1/2-phosphorylation in MDA-MB-231 breast cancer cells is higher than in MCF-10A cells, but without the same increase in cell proliferation. Thus the level of insulin-induced ERK1/2-phosphorylation does not translate to the increase in cell proliferation, indicating either the involvement of additional signals in insulin induced cell proliferation in MCF-10A cells, or correspondingly inhibitory signals in MDA-MB-231 cells that do not allow ERK1/2 activation to translate into increased cell proliferation. Similarly to MDA-MB-231 cells, ERK1/2-phosphorylation does not occur at the same time as AKT-phosphorylation. While AKT-phosphorylation reaches its maximal increase within the first 10 min of insulin treatment, ERK1/2-phosphorylation reaches its maximum between 5 min and 15 min of insulin treatment. This may be an additional indication of insulin activation of the MAP-kinase cell signalling pathway being dependent on PI3-kinase pathway cross-talk. Observing the same finding in both breast cancer and breast epithelial cell lines indicates that this potential cross-talk may be a universal feature of insulin signalling.

Further evidence for a potential cross-talk between PI3-kinase pathway and MAP-kinase pathway is suggested by findings using cell signalling inhibitors on cell signalling pathway activation. Inhibition of PI3-kinase with wortmannin decreased insulin-induced increases in AKT-phosphorylation, while inhibition of the MEK1/2 with PD98059 had no effect on insulin induced phosphorylation of AKT. This observation is an indication that insulin induced AKT-phosphorylation is PI3-kinase dependent and not mediated by cross-talk from the MAP-kinase cell signalling pathway. On the other hand wortmannin eliminated the significant increase in ERK1/2-

phosphorylation after 10 min insulin treatment in MCF-10A cells. This indicates that insulin induced increases in ERK1/2-phosphorylation require intact PI3-kinase signalling, indicating a cross-talk between PI3-kinase and MAP-kinase cell signalling pathway. PD98059 also decreased ERK1/2-phosphorylation alone or after 5 min and 10 min in combination with 100 nM insulin treatment. The observation that PD98059 also decreases insulin induced ERK1/2-phosphorylation indicates that ERK1/2-phosphorylation is also MEK1/2 dependent. This also suggests that any potential cross-talk between AKT and the MAP-kinase pathway most likely occurs upstream of MEK1/2. Further investigation of this interesting suggestion may provide additional insight into the universality of this finding, the potential impact it has on cell proliferation and may be exploited therapeutically, without disrupting essential insulin signalling.

Insulin induced proliferation seems mediated through both the PI3-kinase and MAP-kinase pathway. Interestingly the pathways are not redundant but work synergistically, to increase cell proliferation in MCF-10A cells. By inhibiting either pathway, insulin-induced proliferation is reduced compared to insulin only treatment, but proliferation still increased with insulin treatment, compared to their control. Combined inhibition of both pathways however completely inhibits insulin induced cell proliferation. In fact a significant decrease in cell proliferation compared to untreated control was observed. This is a strong indication that insulin induced cell proliferation is mediated exclusively by the PI3-kinase and MAP-kinase cell signalling pathways without involvement of an additional pathway.

There was no effect of insulin on the distribution of the cell population across the cell cycle stages and thus there is no indication that insulin induces cell cycle progression in MCF-10A cells. This is surprising as insulin induced a significant increase in proliferation and cell signalling pathways. Thus one would also expect an increase in cell cycle progression. There is not a conclusive explanation to this finding. One suggestion might be that the distribution of cells across cell cycle stages is unaffected by incubation in serum-free medium and insulin, *i.e.* a slowing or increase of the cell cycle occurs unanimously across all cell cycle stages, leaving the distribution of the cell population unaffected, thus no change can be picked up with the method used in the study presented here. This suggestion is however unlikely as cell cycle arrest after incubation in serum-free medium should occur at specific points in the cell cycle, *i.e.* the cell cycle check points, which should result in an increase in G1 and possibly G2-phase population. This arrest should then be overcome by insulin and one would expect an increase in S-phase population similar to the one observed for MDA-MB-231 breast cancer cells. Thus this finding eludes a comprehensive explanation and is not supported by the additional findings for this cell line after insulin treatment, which suggested a strong proliferative effect of insulin on MCF-10A breast epithelial cells.

3.4.3 The effect of insulin treatment on SK-BR-3 human breast cancer cells

The effect of insulin on SK-BR-3 cells has not been described previously and the results observed in the study presented here do not indicate an involvement of insulin in cell proliferation in SK-BR-3 cells. Neither 24 h nor 48 h treatment revealed a difference in proliferation compared to control or between the two treatment times. In this respect SK-BR-3 cells display a similar response to insulin as MDA-MB-231 cells do, without

the decrease in proliferation after 48 h of treatment. This finding is therefore in line with previous observations that suggested that insulin does not increase in ER-negative breast cancer cell lines.

SK-BR-3 cells do possess a functioning IR, which significantly increased in phosphorylation in response to 100 nM insulin treatment for 2 min. The level of increase was similar to that of MCF-10A normal breast epithelial cells, which indicates insulin signalling at the level of IR phosphorylation in SK-BR-3 cells is similar to non-cancerous MCF-10A cells than to cancerous MDA-MB-231 breast cancer cells. This probably reflects IR overexpression in MDA-MB-231 cells and may also indicate that SK-BR-3 cells do not overexpress the IR. This is speculative as IR protein expression was not assessed due to time constraints.

AKT-phosphorylation is significantly increased in SK-BR-3 cells after treatment with 100 nM insulin for 5 min. Thus insulin acted similarly as in the other cell lines with a quick, high increase in phosphorylation followed by a just as quick a decrease. The level of AKT-activation was again similar to MCF-10A cells and not to MDA-MB-231 cells, the trend is however similar for all three cell lines.

ERK1/2-phosphorylation is significantly reduced in response to insulin treatment up to 20 min, at which ERK1/2-phosphorylation returned to baseline. This feature is not observed in any other examined cell line. An explanation for this observation may be that, if cross-talk from the PI3-kinase pathway to the MAP-kinase pathway is assumed then selective activation of the PI3-kinase pathway in response to insulin leads to

activation of insulin specific targets (*i.e.* GLUT-4) and not an unspecific cross-activation of MAP-kinase, which was observed in the two other cell lines.

This may be further supported by the findings of the cell signalling pathway inhibitors. AKT-phosphorylation is substantially decreased with wortmannin treatment alone. This suggests a high background activation of the PI3-kinase pathway, even in the absence of outside signals, *i.e.* during incubation in serum-free medium. It should be noted again that SK-BR-3 cells overexpress the erbb2 gene (HER receptor), whose intra-cellular target is PI3-kinase. Other studies found that PI3-kinase inhibition was highly effective in inhibiting cell proliferation in HER2 overexpressing SK-BR-3 breast cancer cells, even in herceptin-resistant cells (Junttila *et al.*, 2009). Thus the substantial decrease in AKT activation may point to a constitutively activated PI3-kinase pathway, either mediated by the mere existence of increased numbers of HER-receptors, or by HER-receptor mutations, leading to increased receptor activity, even in the absence of extra-cellular signals, or the presence of an autocrine signal, produced by SK-BR-3 cells themselves. Interestingly treatment with PD98059 increased AKT-phosphorylation significantly after treatment with 100 nM insulin for between 5 min and 20 min compared to the control without inhibitors. Insulin alone did not increase AKT-phosphorylation significantly after 10 min or 15 min of treatment. The possible explanation could be that RAS' normal target RAF cannot any longer be activated, as PD98059 is inhibiting MEK1/2 phosphorylation. Thus RAS may phosphorylate other targets, such as AKT. Consequently it may be suggested that in SK-BR-3 cells, different from the other two cell lines, there may be a cross-talk from RAS to AKT and that the PI3-kinase pathway is majorly responsible for mediating cell proliferation.

ERK1/2-phosphorylation is decreased with wortmannin alone and in combination with insulin treatment. The insulin-induced decrease in ERK1/2-phosphorylation however was no longer observed. Treatment with PD98059 alone also decreased ERK1/2-phosphorylation significantly. The insulin-induced decrease in ERK1/2-phosphorylation after 5 min treatment with 100 nM insulin was retained in the presence of PD98059. The same effect however was no longer observed after 10 min and 15 min of insulin treatment in the presence of PD98059.

The effect of cell signalling pathway inhibitors on cell proliferation was not examined for SK-BR-3 cells. This assessment however would be interesting after having observed the substantial effect of wortmannin on AKT-phosphorylation.

Insulin treatment had no significant effect on the distribution of the cell population across the cell cycle stages. There may be a trend that insulin could shift the cell population by reducing the G2-population and increasing the G1-population. This may indicate an increased progression from the G2-phase into mitosis, thus increasing cell cycle progression. This observation however did not reach statistical significance.

3.4.4 Implications on the understanding of function of cell signalling pathways in breast cancer in response to insulin stimulation

In addition to its normal function of glucose transport, several studies have indicated an involvement of the PI3-kinase pathway in cancer aetiology and progression (Brader and Eccles, 2004; Fresno Vara *et al.*, 2004; McCubrey *et al.*, 2006; Scheid and Woodgett, 2001b). The activation of PI3-kinase pathway involves nuclear translocation of AKT (Meier and Hemmings, 1999). AKT is an extraordinary versatile kinase affecting

multiple downstream targets. Besides its effects on glucose metabolism, such as GSK3 activation (Cross *et al.*, 1995) and GLUT4 activation (Kohn *et al.*, 1996), AKT activation induces different cell survival mechanisms necessary for tumour survival (Testa and Bellacosa, 2001; see section 1.6.1). The here presented results indicate that in all three examined cell lines normal insulin signalling, *i.e.* increased phosphorylation of AKT, is intact (Figure 3-6). Activation of AKT in MDA-MB-231 cells was higher and more sustained than in the other two cell lines. There may be several explanations for this observation. While AKT gene mutations have not been reported, AKT gene amplifications are common in breast and ovarian cancer (Bellacosa *et al.*, 1995; Cheng *et al.*, 1996). Similarly, phosphatase and tensin homologue deleted on chromosome 10 (PTEN) is the complementary phosphatase to PI3-kinase. It de-phosphorylates PIP3 to become PIP2 (see Figure 1-4). PTEN is known as a tumour suppressor gene (Lu *et al.*, 1999; Weng *et al.*, 1999) and is frequently mutated in breast cancer (Hollestelle *et al.*, 2007). Moreover carriers of PTEN germ-line mutations have a notably higher risk of developing breast cancer (Simpson and Parsons, 2001). While PTEN is not mutated in MDA-MB-231 cells (Hollestelle *et al.*, 2007), either of the two mechanisms, amplification of AKT and/or suppression of PTEN, may be mechanisms explaining the increase in AKT activation after insulin treatment observed in MDA-MB-231 cells. Additionally the increased activation of AKT may be a trickle-down effect of the increased phosphorylation of IR in MDA-MB-231 breast cancer cells.

The activation of AKT after insulin treatment supports the finding of increased IR phosphorylation, which is a feature that in MDA-MB-231 breast cancer cells had not been observed previously (Costantino *et al.*, 1993). Examination of insulin-induced

activation of AKT in the presence of wortmannin indicated PI3-kinase-specific activation of AKT. Interestingly MEK1/2 inhibition did not decrease AKT activation in all cell lines. In SK-BR-3 cells, in particular, MEK1/2 inhibition increased insulin-induced phosphorylation of AKT (Figure 3-9, A). The cross-talk between RAS and PI3-kinase is well known. So it may be that inhibiting a RAS downstream target, MEK1/2, diverts RAS kinase activity to secondary substrates such as PI3-kinase, which in turn increases AKT activation.

Similarly to the PI3-kinase pathway, the MAP-kinase pathway has received increased attention in mediating many cancer-specific signals of growth and mitogenic factors. Its involvement in cancer aetiology and progression has been extensively reviewed (McCubrey *et al.*, 2007; Reddy *et al.*, 2003; Santen *et al.*, 2002). None of the insulin induced increases in ERK1/2 activation have been observed if PD98059 was present in any cell line, indicating that insulin-induced ERK1/2 activation in MDA-MB-231 cells and MCF-10A cells is exclusively MEK1/2 dependent. Additionally inhibition of PI3-kinase with wortmannin also decreased ERK1/2 activation in these cells, indicating that additionally insulin-induced ERK1/2 activation is dependent at least in part on intact PI3-kinase signalling. It is routinely observed that RAS can activate PI3-kinase (Rodriguez-Viciana *et al.*, 1994; Yan *et al.*, 1998). A reverse mechanism is however not well documented. Nonetheless one study reported on AKT-induced RAF/MEK/ERK mediated Bad and caspase-9 phosphorylation (Cardone *et al.*, 1998). This however only allows an explanation for the observed decrease, if insulin-induced activation of ERK1/2 is exclusively dependent on PI3-kinase activity. An inhibition of PI3-kinase with wortmannin would only affect ERK1/2 activation, if IR and/or IRS fail to activate

RAS in response to insulin stimulation. Unfortunately with the present results this cannot be evaluated, however as the activation of RAS by IR and IRS is well-supported by the literature (White and Kahn, 1994) it seems unlikely to have failed in MDA-MB-231 cells and MCF-10A cells. Another explanation may be that the wortmannin concentration used was cytotoxic and instead of merely inhibiting PI3-kinase signalling, it destroyed the cells itself. In fact cell proliferation was decreased with wortmannin treatment alone, but only in MCF-10A cells (Figure 3-10). Likewise PD98059 alone decreased cell proliferation in MCF-10A cells, but did not inhibit cells to activate AKT (Figure 3-10). Hence the cytotoxic potential of the used wortmannin concentration may be negligible. Thus a cross-talk from AKT to RAS or RAF seems the most likely explanation of the observed findings, which would be a novel mechanism of insulin signalling in breast cancer cells and breast epithelial cells. This suggestion needs however to be validated by additional investigations into the exact mechanism by which PI3-kinase may mediate insulin induced activation of ERK1/2.

3.4.5 Rationale for the approach

Measuring cell proliferation is accessible by a number of ways: cell count, [^{3}H]-thymidine incorporation, MTT-assay amongst others. In the here presented study BrdU-incorporation was chosen. It is the non-radioactive equivalent of [^{3}H]-thymidine incorporation. It allowed for measurement of DNA-replication, which was thought to be the best measure of cell proliferation for the study presented here. It also had the advantage of not requiring the handling of radioactive material and did not require a scintillator.

Immunoprecipitation was initially used to assess IR-phosphorylation before it was decided to use the *in vitro* kit instead. The reason for change in this approach was an inability to successfully isolate the IR-protein using immunoprecipitation. The *in vitro* kit worked essentially on the same theoretical basis as immunoprecipitation and delivered reproducible results.

Similarly it was initially attempted to detect ERK1/2-phosphorylation using western blotting, which yielded quantifiable results (see section 7.3.2). However using this approach for the detection of AKT-phosphorylation, it was not possible to identify AKT-protein. Instead it was decided to use commercial *in vitro* kits for detection of phosphorylation of both proteins, which yielded good reproducibility.

The used insulin concentration was higher than physiological concentrations (100 nM vs. <0.8 nM). This concentration was chosen by a number of previous investigators, who intended similar investigations (see sections 3.1 and 3.4). It was also not intended to re-create a physiological environment, but rather an examination of the specific effects insulin had on the cell culture system. In order to maximise this effect, a high insulin concentration seemed appropriate.

3.5 Summary

The effects of insulin on MDA-MB-231 cells generally indicate a potential for cancer progression in these cells. These cells overexpress the IR and, as a result, show highly increased IR-phosphorylation upon insulin stimulation. Insulin increases activation of major cell signalling pathways and cell cycle progression, it lacks however the potential

to increase cell proliferation. Similarly cell signalling pathways are increased in MCF-10A cells, but with no observed change in cell cycle progression. Additionally cell proliferation was significantly increased in these cells with insulin, suggesting that insulin acts as a mitogen on these cells. SK-BR-3 cells showed a contradictory reaction to insulin treatment. IR phosphorylation increased, resulting in an increase in PI3-kinase cell signalling activation. Cell proliferation was however not affected by insulin treatment. A significant finding was the drastic decrease in AKT-phosphorylation after wortmannin treatment, indicating a constitutively activated PI3-kinase pathway in these cells, probably caused by HER2 overexpression, or by a yet unidentified autocrine signalling mechanism. Another surprising finding was the significant and sustained increase in AKT-phosphorylation after MAP-kinase inhibition. Furthermore MAP-kinase activation was decreased after insulin treatment, which is unexpected. More surprise comes from the finding that ERK1/2-phosphorylation is decreased with insulin treatment even when MEK1/2 is inhibited, indicating that insulin treatment may reduce ERK1/2-phosphorylation in a MEK1/2 independent manner. Thus insulin acts differently on the three examined cell lines with no clear distinction between cancer cells and non-cancer cells. Insulin may be a major affector of the signalling pathways within the cells. Both PI3-kinase and MAP-kinase cell signalling pathways are affected by insulin stimulation.

Chapter 4
Leptin

4 LEPTIN

In this chapter the effect of leptin on several molecular markers in MDA-MB-231 breast cancer cells, MCF-10A breast epithelial cells and SK-BR-3 breast cancer cells is presented. It was aimed to determine the effect of leptin on cell proliferation, activation of PI3-kinase and MAP-kinase cell signalling pathways and cell cycle progression in this cell culture system.

4.1 Introduction

Leptin is a hormone linked to body weight homeostasis in mammals. It is a 16 kDa peptide hormone identified as the product of the obese gene, first described in spontaneous obese mice strains (Ingalls *et al.*, 1950), and subsequently cloned in mice and humans (Zhang *et al.*, 1994). Its production was initially thought to be exclusive to adipocytes, but was subsequently found to be secreted by a number of other cell types, including breast epithelial cells (Margetic *et al.*, 2002). Adipocyte secretion of leptin however is the major source for circulatory leptin and provides a quantitative measure of body fatness. After the discovery of leptin it was found that serum leptin concentrations were ~7.5 ng/ml (0.47 nM) in normal weight individuals (BMI<27.3 kg/m^2 for men and <27.8 kg/m^2 for women) and ~31.3 ng/ml (1.96 nM) in obese[1] individuals, thus leptin concentrations are increased in obese individuals and leptin concentrations correlate positively with BMI (Considine *et al.*, 1996). Additionally to the primary function of body weight homeostasis, it was observed that leptin exerts a number of additional functions, including a role in immune response

[1] This study used a BMI cut-off point of 27.3 kg/m^2 for men and 27.8 kg/m^2 for women to distinguish between normal weight and obese, thus slightly below the now accepted cut off point of 30 kg/m^2 for both genders. Leptin concentrations between these two groups were significantly different.

(Lord *et al.*, 1998), angiogenesis (Sierra-Honigmann *et al.*, 1998) and reproduction (Masuzaki *et al.*, 1997). Leptin was also found to play a role in puberty and sexual maturation in women, including breast development (Kiess *et al.*, 1999; Neville *et al.*, 2002). The combination of increased leptin levels in the obese and its involvement in breast development and reproduction has led to the suggestion that leptin may be responsible for the increased risk of breast cancer in obese postmenopausal women (Surmacz, 2007).

4.1.1 Leptin and leptin receptor (Ob-R) expression in breast cancer cells

Expression of the leptin receptor (Ob-R) was found in breast tumour samples. Additionally only breast tumours expressing Ob-R had metastasised and displayed a more malignant phenotype (Ishikawa *et al.*, 2004). Additionally, over 90% of examined breast tumours have shown increased expression of leptin, compared to corresponding normal epithelial tissue, suggesting the possibility of autocrine leptin signalling in breast cancer (Ishikawa *et al.*, 2004). Ob-R was also detected in several breast cancer cell lines, including T47-D cells (Hu *et al*., 2002), MCF-7 cells (Dieudonne *et al.*, 2002) and MDA-MB-231 cells (Garofalo *et al.*, 2006). Additionally leptin mRNA was detected in all three cell lines. Leptin mRNA was also detected in MCF-10A normal breast epithelial cells (O'brien *et al.*, 1999). The presence of Ob-R in this cell line however was not established. Leptin expression is under the control of the HIF-1 transcription factor (Ambrosini *et al.*, 2002; Grosfeld *et al.*, 2002), which is activated by hypoxia. In chapter 1 hypoxia was introduced as being a possible cause for increased leptin secretion from obese adipocytes (section 1.1.4.3). Similarly hypoxia is of major concern to solid tumours, which grow extensively without adequate neovascularisation.

Thus increased leptin expression in tumours may be a product of this phenomenon. Alternatively leptin expression in cancer cells may be increased in breast cancer cells by hyperinsulinaemia and insulin resistance as insulin induced gene and protein expression of leptin in MDA-MB-231 breast cancer cells (Bartella *et al.*, 2008).

An interesting set of experiments further emphasised the role of leptin and its receptor in breast cancer aetiology. In *db/db* (homozygous deletion of the Ob-R gene) and *ob/ob*[2] (homozygous deletion of the leptin gene) mice, virus-induced mammary cancer failed to develop in either mouse strain, but developed readily in non-mutagenic comparison strains (Cleary *et al.*, 2003; Cleary *et al.*, 2004). This finding suggests that leptin signalling may be necessary for the initiation of breast cancer development in mice. Thus a possible theory of leptin's involvement could include an initiation phase in which high leptin concentrations, as found in obese individuals, may promote or facilitate breast cancer aetiology (see also section 8.5). This may be a potential explanation for the growth of triple negative breast cancer, which manages to survive without expressing any of the three common growth receptors (ER, HER2-receptor and the progesterone receptor), and thus evading common anti-cancer treatments (Irvin and Carey, 2008). A recent study also added to this assertion by demonstrating that overweight and obesity are risk factors for the development of triple-negative breast cancer (Trivers *et al.*, 2009).

[2] The ob-gene codes for the leptin protein, while the db-gene codes for the leptin-receptor protein (Ob-R) in mouse genetics. Mutations in these genes result in mice either being deficient of leptin (*ob/ob*-mice) or without functioning leptin-receptor (*db/db*-mice). Both mutations lead to disruption in intra-cellular leptin signalling and to phenotypically obese and diabetic mice. These mouse models are generally used to examine the effects of obesity and/or diabetes.

4.1.2 Leptin and breast cancer cell proliferation

Several studies have examined the effect of different concentrations of leptin on cell proliferation in different breast cancer cells. Leptin treatment (20 nM (320 ng/ml) or 50 nM (800 ng/ml), 24 h) significantly increased cell proliferation ([^{3}H]-thymidine incorporation) in ER-positive MCF-7 breast cancer cells (Dieudonne *et al.*, 2002). Treatment with 50 ng/ml (3.125 nM), 100 ng/ml (6.25 nM) or 1000 ng/ml (62.5 nM) leptin for 24 h significantly increased ^{3}H-thymidine incorporation in T47D breast cancer cells and 100 ng/ml (6.25 nM) and 1000 ng/ml (62.5 nM) leptin also significantly increased Formazan production of MTT, indicating that high leptin concentrations are able to stimulate cell proliferation in T47D breast cancer cells (Laud *et al.*, 2002). Leptin treatment also affected cell numbers in MDA-MB-231 and SK-BR-3 breast cancer cells (Ray *et al.*, 2007). Cell numbers in MDA-MB-231 breast cancer cells significantly increased after 24 h or 48 h treatment with 50 ng/ml (3.125 nM) or 100 ng/ml (6.25 nM) leptin, following incubation in serum-free medium for 18 h (Ray *et al.*, 2007). Ray and colleagues (2007) also observed a positive relationship between PCNA protein expression, a marker of cell proliferation, and increasing leptin concentrations (5 ng/ml (0.31 nM), 10 ng/ml (0.63 nM), 25 ng/ml (1.6 nM), 50 ng/ml (3.13 nM), and 100 ng/ml (6.25 nM)) in MDA-MB-231 breast cancer cells. These authors did not comment however, if their treatment significantly increased PCNA expression, but rather reported on observed trends. Cell numbers in SK-BR-3 breast cancer cells were significantly increased after both 24 h and 48 h leptin treatments with 5 ng/ml (0.31 nM), 10 ng/ml (0.63 nM), 25 ng/ml (1.6 nM) and 50 ng/ml (3.13 nM) leptin (Ray *et al.*, 2007). Leptin treatment (25 ng/ml (1.56 nM) or 125 ng/ml (7.81 nM), 48 h) also increased Formazan production from MTT in ZR-75-1 breast cancer cells

(Chen *et al.*, 2006a). Additionally increased cell proliferation was also observed in MDA-MB-231[3] breast cancer cells after 40 ng/ml (2.5 nM) leptin treatment for 48 h (Frankenberry *et al.*, 2006). Thus there is evidence suggesting a proliferative effect of leptin on breast cancer cells. No results on the effect of leptin on breast epithelial cells however have been reported, which will be added to in the study presented here.

4.1.3 Leptin and cell signalling pathways

In human breast cancer cells, leptin can activate a possible three different cell signalling pathways: the Janus–activated kinase 2/signal transducers and activators of transcription 3 (JAK2/STAT3) pathway, the MAP-kinase pathway and the PI3-kinase pathway (Cirillo *et al.*, 2008).

JAK2 inhibitor AG940 inhibited leptin-induced cell proliferation of ZR-75-1 breast cancer cells, suggesting the JAK2/STAT3 cell signalling pathway may contribute in leptin mediated cell proliferation (Chen *et al.*, 2006a). Similarly in MCF-7 breast cancer cells, AG940 inhibited leptin-induced cell cycle progression (Saxena *et al.*, 2007b). Additionally a leptin-induced increase in cell proliferation of MCF-7 cells was inhibited by AG940 treatment (Yin *et al.*, 2004). Others also found an activation of STAT3 with 20 nM (320 ng/ml) leptin treatment in MCF-7 cells (Dieudonne *et al.*, 2002). It was also noted that STAT3 was responsible for leptin-induced expression of survivin, an anti-apoptotic protein, after treatment with 10 nM leptin for 24 h in MCF-7 cells (Jiang *et al.*, 2008). In MDA-MB-231 breast cancer cells, 100 ng/ml (6.25 nM) leptin

[3] Frankenberry and colleague (2006) use the term "HTB-26 cells" in reference to the ATCC catalogue number for MDA-MB-231 breast cancer cells. Other researchers have cited Frankenberry and colleague's (2006) use of HTB-26 as referring to MDA-MB-231 breast cancer cells, *e.g.* Ray and colleagues (2007).

treatment increased significantly the phosphorylation of STAT3 and increased expression of JAK2 after 24 h leptin treatment in SK-BR-3 breast cancer cells and increased expression and phosphorylation of STAT3 in MCF-7 cells (Ray *et al.*, 2007). Activation of the MAP-kinase cell signalling pathway with leptin treatment was observed in MCF-7 breast cancer cells, where 1000 ng/ml (62.5 nM) leptin treatment for 48 h significantly increased ERK1/2-phosphorylation (Catalano *et al.*, 2004). Inhibition of the MAP-kinase pathway using 10 μM PD98059 blocked the leptin induced (100 ng/ml (6.25 nM, 6 days) increase in cell proliferation (MTT-assay) in ZR-75-1 breast cancer cells (Chen *et al.*, 2006a). Others also observed activation of ERK1/2 with 20 nM (320 ng/ml) leptin treatment in MCF-7 cells (Dieudonne *et al.*, 2002). Furthermore the MAP-kinase pathway was also activated in the T47-D breast cancer cell line and was also found to mediate leptin-induced cell proliferation of 1000 ng/ml (62.5 nM) leptin treatment for 24 h (Laud *et al.*, 2002). Additionally treatment with 80 ng/ml (5 nM) leptin significantly increased MAP-kinase activation in ZR-75-1 breast cancer cells (Frankenberry *et al.*, 2006).

In ZR-75-1 breast cancer cells 80 ng/ml (5 nM) leptin treatment increased activation of the PI3-kinase pathway. Inhibition of PI3-kinase with wortmannin additionally inhibited leptin-induced cell proliferation (Frankenberry *et al.*, 2006). Chen and colleagues (2006a) also found that PI3-kinase inhibition, using 200 nM wortmannin, blocked leptin-induced (100 ng/ml (6.25 nM), 6 days) cell proliferation (MTT-assay) in ZR-75-1 breast cancer cells. In MCF-7 and SK-BR-3 breast cancer cells, 100 ng/ml (6.25 nM) leptin treatment for 24 h increased PI3-kinase activity (Ray *et al.*, 2007).

4.1.4 Leptin and cell cycle progression

Leptin increased cell cycle progression in several breast cancer cell lines. In ZR-75-1 breast cancer cells, 100 ng/ml (6.25 nM) leptin treatment increased expression of cyclin D1, a protein involved in mediating cell cycle progression across the G1/S cell cycle checkpoint (Chen *et al.,* 2006a). Similarly cell population in S-phase and cyclin D1 expression were increased after 100 ng/ml (6.25 nM) leptin treatments for 24 h in MCF-7 breast cancer cells compared to untreated cells (Saxena *et al.,* 2007b). Additionally, gene expression of cell cycle proteins cyclin D1, cyclin G and CDK-2 was increased in response to 500 ng/ml (31.25 nM) leptin treatment in MCF-7 cells (Perera *et al.,* 2008). In MDA-MB-231 cells 100 ng/ml (6.25 nM) leptin treatment for 24 h also increased cyclin D1 expression (Ray *et al.,* 2007).

4.2 Materials and Methods

4.2.1 Cell lines and leptin treatment

MDA-MB-231, MCF-10A and SK-BR-3 cells were cultured and treated as described in Materials and Methods sections 2.1.1 and 2.1.2. Formulation of leptin treatment medium and final leptin treatment concentration is described in Materials and Methods section 2.1.3.2.

4.2.2 Cell proliferation assay

The cell proliferation assay was performed as described in section 3.2.2, with the exception that for a 48 h experiment, 10 µM/well BrdU was added at the beginning of the 48 h incubation instead of only for the last 24 h. There were no alterations for a 24 h experiment. Four experiments were performed for MDA-MB-231 cells, MCF-10A cells and SK-BR-3 cells each. Each experiment contained six replicate wells for each treatment. Subsequently a range of leptin concentrations were tested and made by adding 200 µl of 1 µM (16000 ng/ml) to 4 ml serum-free medium, resulting in 50 nM (800 ng/ml) leptin. The remaining concentrations were obtained through 1-in-2 serial dilution from 800 ng/ml to 400 ng/ml to 200 ng/ml to 100 ng/ml to 50 ng/ml to 25 ng/ml to 12.5 ng/ml to 6.25 ng/ml leptin (see Table 4-1 for corresponding molar concentrations). Two experiments examining the effect of the leptin concentrations range on cell proliferation in all three cell lines were performed. During each experiment, each leptin concentration and the control was performed in six replicates.

Table 4-1: Leptin concentrations used to assess cell proliferation and corresponding unit conversion, assuming leptin as 16 kDa protein.

Leptin concentration [ng/ml]	6.25	12.5	25	50	100	200	400	800	1600
Leptin concentration [nM]	0.390625	0.78125	1.5625	3.125	6.25	12.5	25	50	100

4.2.3 Cell signalling pathway assays

The cell signalling pathways were analysed as described in section 3.2.5 but with 100 nM leptin as treatment medium. Two experiments examining AKT-phosphorylation after leptin treatment each with two replicates for control and each treatment time-point were performed for MDA-MB-231 cells and MCF-10A cells. Three experiments were performed for SK-BR-3 cells. ERK1/2-phosphorylation was assessed in two experiments for MDA-MB-231 cells and SK-BR-3 cells, and in three experiments for MCF-10A cells.

4.2.4 Cell cycle analysis by flow cytometry

Changes in the cell distribution across cell cycle stages was assessed as described in section 3.2.6 but with 100 nM leptin as treatment medium. Three experiments were performed for each cell line.

4.3 Results

4.3.1 Effect of leptin treatment on cell proliferation

Cell proliferation of all cell lines was assessed after 100 nM leptin treatment for 24 h or 48 h. Untreated control cells were incubated in serum-free medium (Control) at all times. All results are presented as percentage change from this control.

In MDA-MB-231 breast cancer cells, cell proliferation increased by 21% after 24 h ($p<0.001$) treatment with 100 nM leptin. Cell proliferation did not change significantly after 48 h of leptin treatment (Figure 4-1, A).

In MCF-10A normal breast epithelial cells, cell proliferation did not change significantly after 24 h treatment with 100 nM leptin. Cell proliferation increased significantly by 14% after 48 h ($p=0.013$) treatment with 100 nM leptin (Figure 4-1, B).

In SK-BR-3 breast cancer cells, cell proliferation increased non-significantly by 17% after 24 h ($p=0.157$) treatment with 100 nM leptin. Cell proliferation did not change significantly after 48 h treatment with 100 nM leptin (Figure 4-1, C).

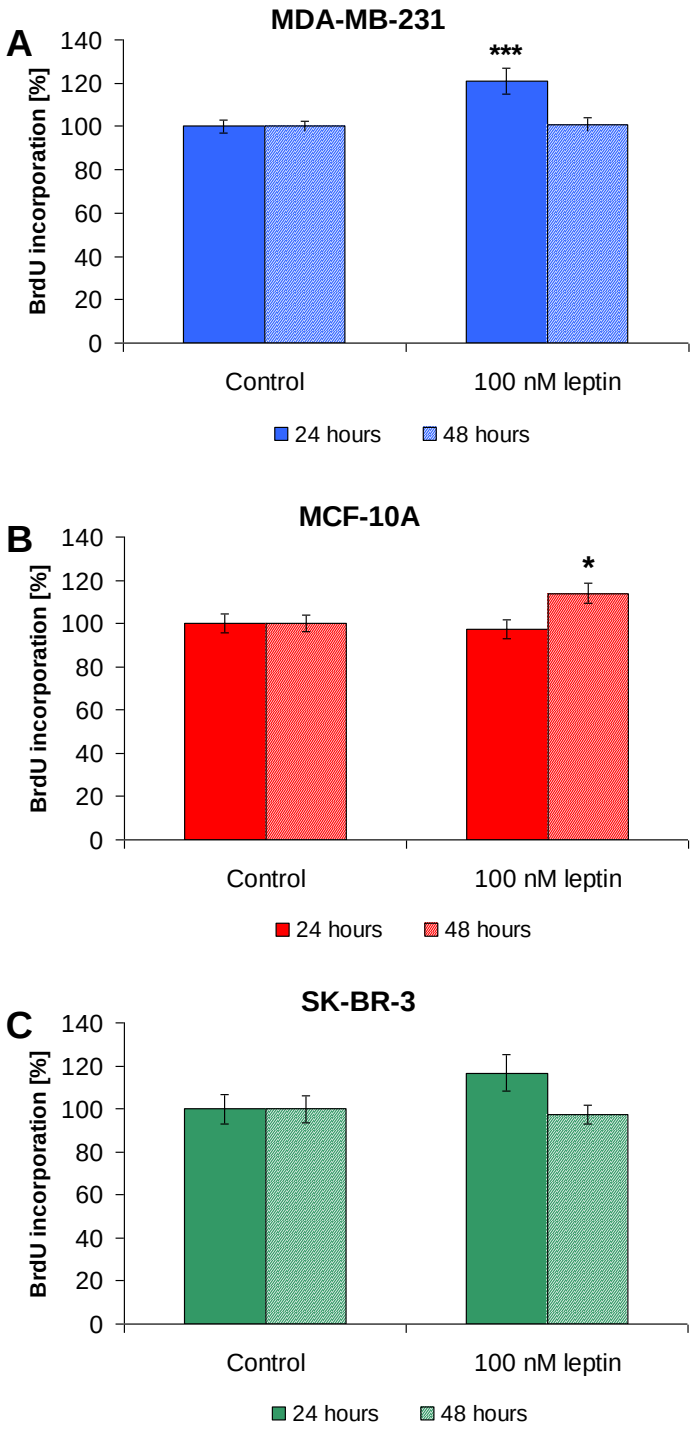

Figure 4-1: Changes in cell proliferation after treatment with 100 nM leptin for 24 h or 48 h in A) MDA-MB-231breast cancer cells (blue), B) MCF-10A breast epithelial cells (red) and C) SK BR-3 breast cancer cells (green). Bars represent BrdU-incorporation in relation to the respective control within each graph and are expressed as a percentage thereof. Error bars represent ± SEM of three experiments, each consisting of six replicates, *i.e.* 18 data points for each bar.
* Significance value compared to control, obtained using One-way ANOVA analysis (* 0.05>p>0.01; *** p<0.001).

4.3.2 Effect of a range of leptin concentrations on cell proliferation

Cell proliferation of all cell lines was assessed after leptin treatment with several concentrations (see Table 4-1) for 24 h or 48 h. Untreated control cells were incubated in serum-free medium (Control) at all times. All results are presented as percentage change from this control.

In MDA-MB-231 breast cancer cells cell proliferation did not change significantly with any of the examined leptin concentration after 24 h treatment compared to untreated control (Figure 4-2, A). Cell proliferation significantly decreased by 11% after treatment with 400 ng/ml ($p=0.023$) leptin and by 26% after treatment with 800 ng/ml ($p<0.001$) leptin treatment for 48 h compared to untreated control (Figure 4-2, B). Cell proliferation did not change at any other leptin concentration after treatment for 48 h.

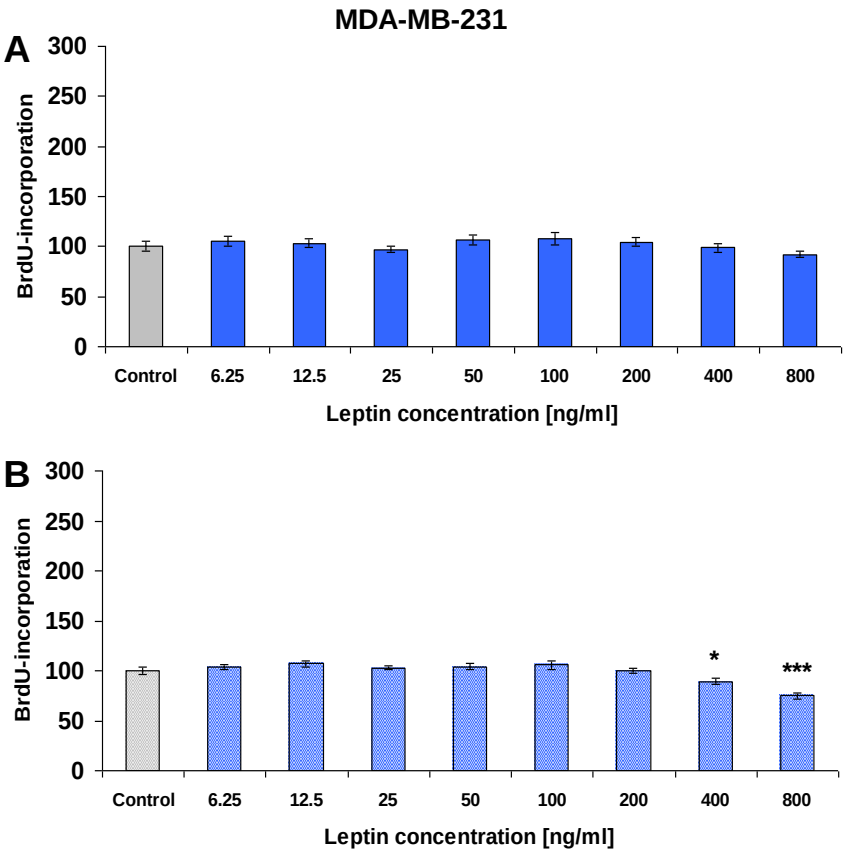

Figure 4-2: Changes in cell proliferation after treatment with a range of leptin concentrations as indicated for A 24 h and B 48 h in MDA-MB-231 breast cancer cells. Bars represent BrdU-incorporation in relation to the respective control within each graph and are expressed as a percentage thereof. Error bars represent ± SEM of two experiments, each consisting of six replicates, *i.e.* 12 data points for each bar.
* Significance value compared to control, obtained using Dunnett's post-hoc t-test following ANOVA analysis (* $0.05>p>0.01$; *** $p<0.001$).

In MCF-10A breast epithelial cells, cell proliferation increased significantly by 19% after treatment with 6.25 ng/ml leptin (p=0.048) and decreased significantly by 25% after treatment with 800 ng/ml leptin (p=0.006) for 24 h compared to control (Figure 4-3, A). Cell proliferation increased by 14%, 14% and 17% after treatment with 12.5 ng/ml (p=0.047), 50 ng/ml (p=0.036) and 100 ng/ml (p=0.006) leptin for 48 h, respectively compared to control (Figure 4-3, B).

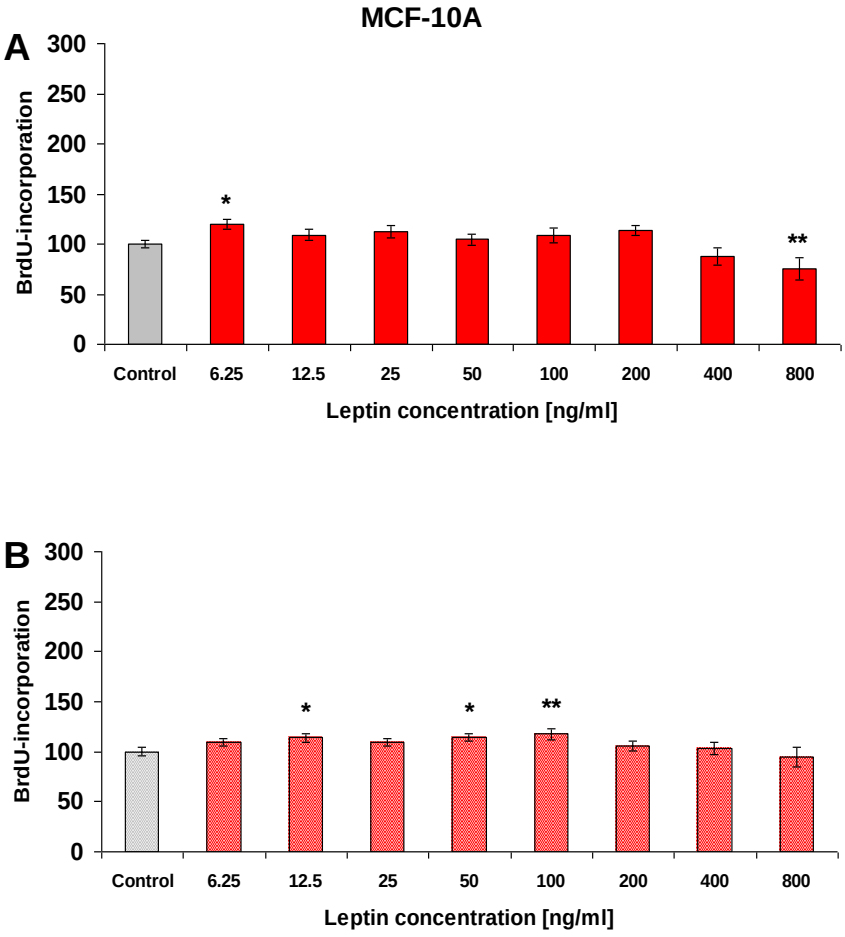

Figure 4-3: Changes in cell proliferation after treatment with a range of leptin concentrations as indicated for A 24 h and B 48 h in MCF-10A breast epithelial cells. Bars represent BrdU-incorporation in relation to the respective control within each graph and are expressed as a percentage thereof. Error bars represent ± SEM of two experiments, each consisting of six replicates, *i.e.* 12 data points for each bar.
* Significance value compared to control, obtained using Dunnett's post-hoc t-test following ANOVA analysis (* 0.05>p>0.01; ** 0.01<p<0.001).

In SK-BR-3 breast cancer cells cell proliferation increased significantly by 61%, 96%, 104%, 115%, 115%, 110% and 51% after treatment with 6.25 ng/ml, 12.5 ng/ml, 25 ng/ml, 50 ng/ml, 100 ng/ml, 200 ng/ml and 400 ng/ml leptin for 24 h (all p-values <0.001), respectively compared to control (Figure 4-4, A). After 48 h treatment cell proliferation increased significantly by 44%, 53%, 53%, 69%, 75%, 69%, 52% and 33% after treatment with 6.25 ng/ml, 12.5 ng/ml, 25 ng/ml, 50 ng/ml, 100 ng/ml, 200 ng/ml, 400 ng/ml (all p-values <0.001) and 800 ng/ml (p=0.009) leptin treatment for 48 h, respectively compared to control (Figure 4-4, B).

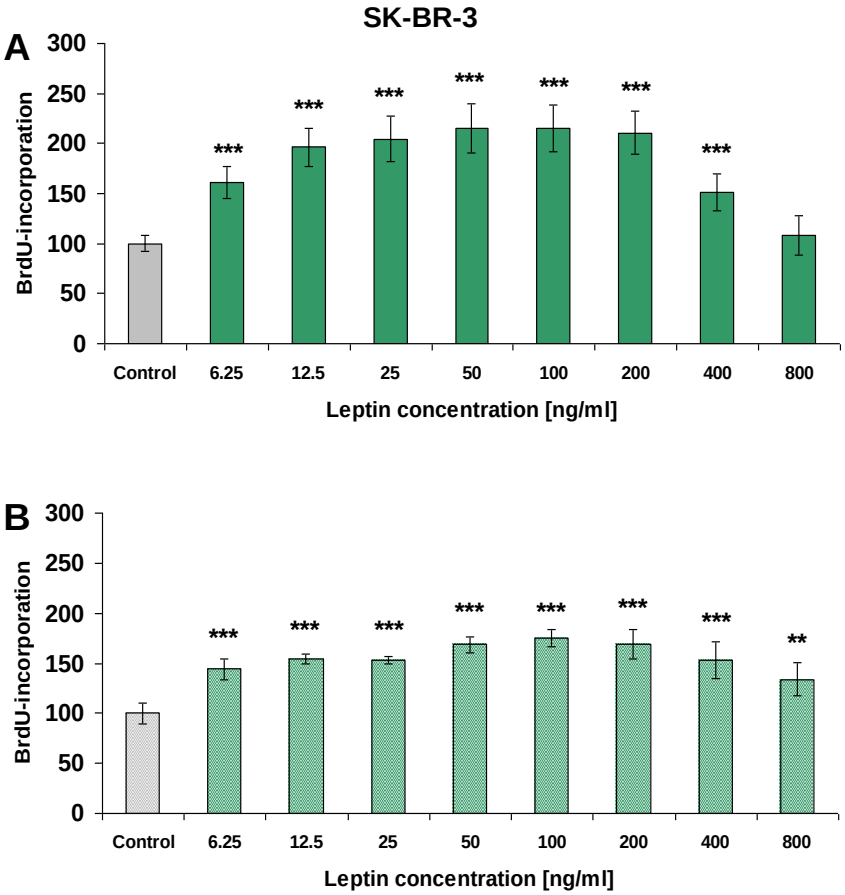

Figure 4-4: Changes in cell proliferation after treatment with a range of leptin concentrations as indicated for A 24 h and B 48 h in SK-BR-3 breast cancer cells. Bars represent BrdU-incorporation in relation to the respective control within each graph and are expressed as a percentage thereof. Error bars represent ± SEM of two experiments, each consisting of six replicates, *i.e.* 12 data points for each bar.
* Significance value compared to control, obtained using Dunnett's post-hoc t-test following ANOVA analysis (** 0.01<p<0.001; *** p<0.001).

4.3.3 Effect of leptin treatment on activation of downstream cell signalling pathways

Phosphorylation of AKT or ERK1/2, representative of activation of the PI3-kinase or MAP-kinase cell signalling pathway, respectively, was measured after treatment with 100 nM leptin for between 5-20 min (Figure 4-5). Untreated control cells were incubated in serum-free medium (Control) at all times. All results are presented as percentage change from this control.

In MDA-MB-231 cells, AKT-phosphorylation did not change significantly after treatment with 100 nM leptin for 5 min, 10 min, 15 min or 20 min compared to control (Figure 4-5, A). Phosphorylation of ERK1/2 did not change significantly after 5 min and 10 min treatment with 100 nM leptin. ERK1/2-phosphorylation decreased significantly by 17% and 20% after 15 min ($p=0.026$) and 20 min ($p=0.011$) treatment with 100 nM leptin, respectively compared to control (Figure 4-5, B).

In MCF-10A cells AKT-phosphorylation did not change significantly after treatment with 100 nM leptin for 5 min, 10 min, 15 min or 20 min compared to control (Figure 4-5, C). Phosphorylation of ERK1/2 increased non-significantly by 42% after 5 min ($p=0.151$) treatment with 100 nM leptin compared to control. ERK1/2-phosphorylation increased significantly by 78%, 75% and 58% after 10 min ($p=0.005$), 15 min ($p=0.007$) or 20 min ($p=0.035$) treatment with 100 nM leptin, respectively, compared to control (Figure 4-5, D).

In SK-BR-3 cells AKT-phosphorylation did not change significantly after treatment with 100 nM leptin for 5 min, 10 min, 15 min or 20 min compared to control (Figure

4-5, E). Phosphorylation of ERK1/2 did not change significantly after 5 min treatment with 100 nM leptin compared to control. ERK1/2-phosphorylation decreased however significantly by 32% and 34% after 10 min ($p<0.001$) and 15 min ($p<0.001$) treatment with 100 nM leptin, respectively, compared to control. After treatment with 100 nM leptin for 20 min, ERK1/2-phosphorylation was not significantly different from control (Figure 4-5, F)

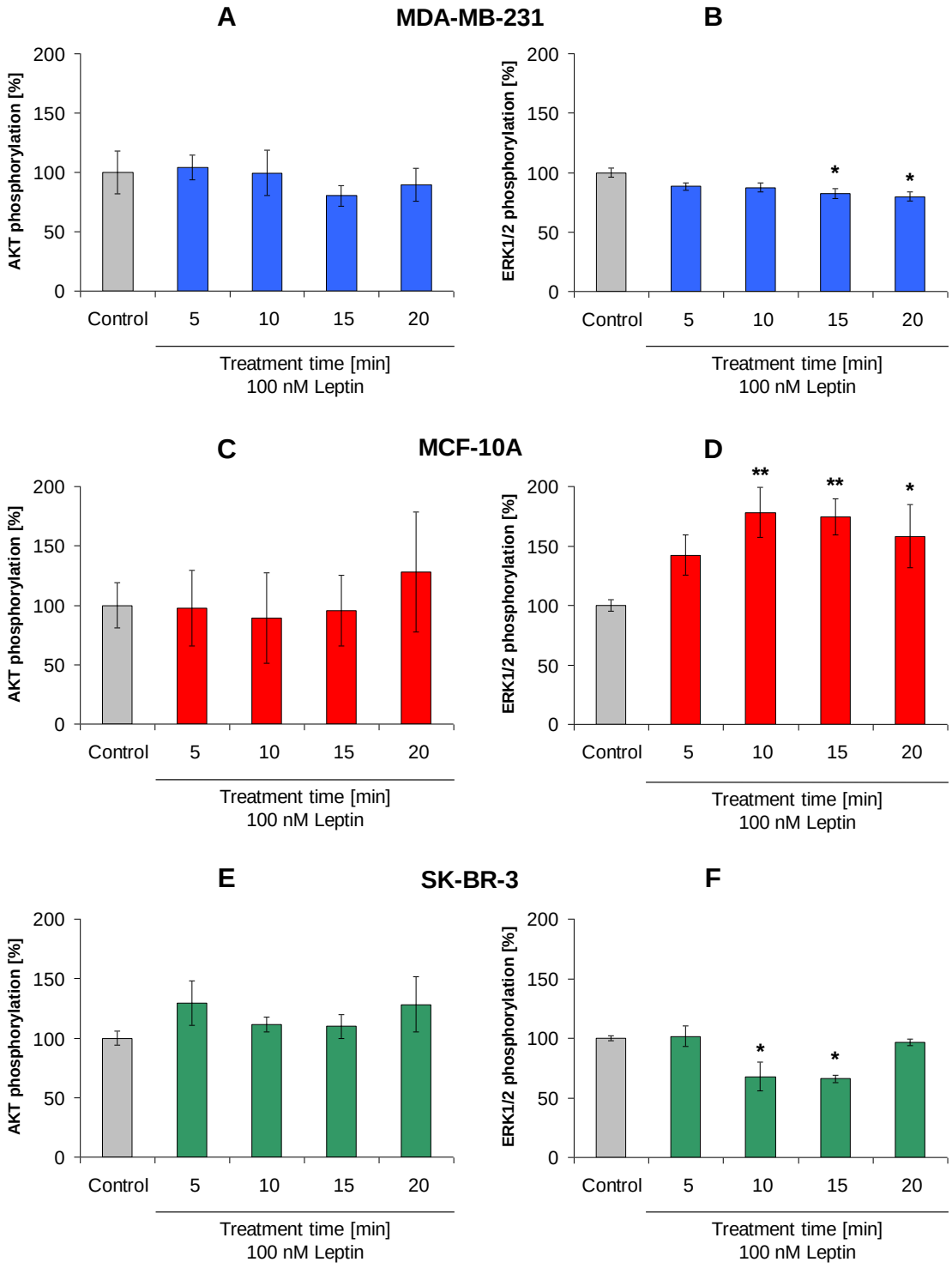

Figure 4-5: Changes in cell signalling pathway activation (PI3-kinase (A, C, E), MAP-kinase (B, D, F)) with 100 nM leptin treatment in A, B) MDA-MB-231 breast cancer cells, C, D) MCF-10A breast epithelial cells and E, F) SK-BR-3 breast cancer cells. Bars represent AKT-phosphorylation or ERK1/2-phosphorylation in relation to the respective control within each graph and are expressed as a percentage thereof. Error bars represent ± SEM of three experiments, each consisting of two replicates, *i.e.* six data points for each bar.
* Significance value compared to control, obtained using Dunnett's post-hoc t-test following univariate analysis of variance (* 0.05>p>0.01; ** 0.01>p>0.001).

4.3.4 Effect of leptin treatment on breast cancer cell cycle progression

Cell cycle profiles for all cell lines were established after treatment with 100 nM leptin for 24 h. One initial experiment each was also performed after 3 h and 6 h of treatment with 100 nM leptin (data not shown), which did not indicate a significant change and these incubation times were not further pursued. The controls are the same as described in section 3.3.5. Leptin treatment for 24 h did not change the distribution of cell cycle stages in any of the examined cells compared to control. In all stages (including sub G1) no significant difference between leptin treatment and untreated control cells was observed. Several non-significant trends were however observed.

In MDA-MB-231 cells 100 nM leptin treatment increased subG1-phase population at the expense of G1, which were non-significantly different to control (Figure 4-6, A). SubG1-phase was increased by 1.2 percentage points (a 7% increase), G1 was decreased by 1.0 percentage point (a 2% decrease).

In MCF-10A cells 100 nM leptin treatment decreased subG1-phase population and non-significantly increased the S-phase population by 0.3 percentage points (a 20% increase) (Figure 4-6, B).

In SK-BR-3 cells 100 nM leptin treatment may increase the G1-phase population and decrease the G2-phase population (Figure 4-6, C). Cells in G1-phase increased non-significantly by 4.5 percentage points (an 11% increase) and cells in G2-phase decreased by 2.0 percentage points (a 12% decrease).

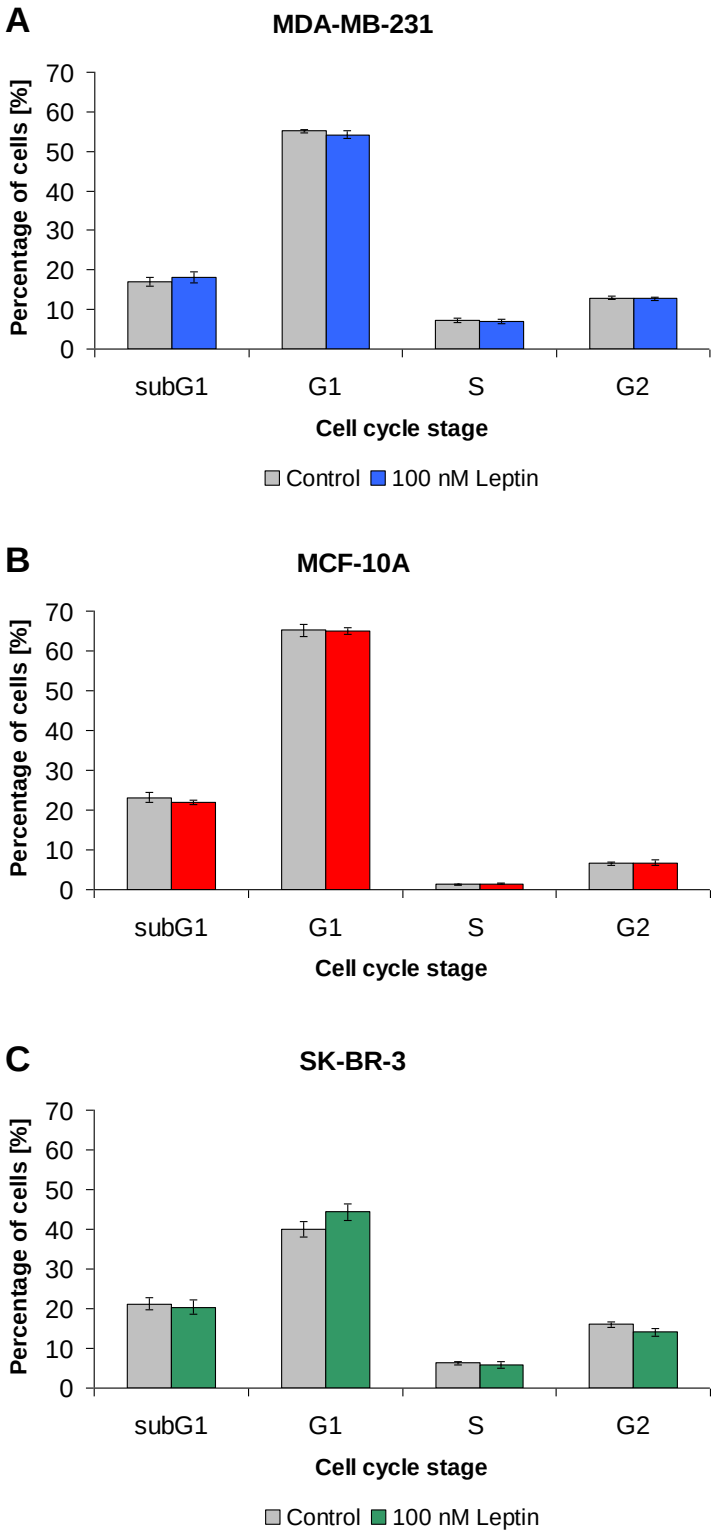

Figure 4-6: Changes of cell population distribution across cell cycle stages after 24 h treatment with 100 nM leptin in A) MDA-MB-231 cells, B) MCF-10A cells and C) SK-BR-3 cells. Bars represent percentage of cells as obtained from a 10000 cell sample obtained, treated and analysed as described in section 2.8.1. Error bars represent ± SEM of three experiments with two replicates for each treatment, *i.e.* 6 data points for each bar.

4.4 Discussion

4.4.1 Effect of leptin treatment on human MDA-MB-231 breast cancer cells

In MDA-MB-231 breast cancer cells leptin treatment increased cell proliferation significantly after 24 h treatment with 100 nM leptin, but not after 48 h (Figure 4-1, A). At lower concentrations however cell proliferation did not increase after 24 h treatment and decreased after 48 h treatment (Figure 4-2). Conversely, Ray and colleagues (2007) observed an increase in proliferation after treatment with 50 ng/ml (3.125 nM) and 100 ng/ml (6.25 nM) leptin for 24 h. These authors also observed increased cell proliferation after 48 h treatment. Likewise Frankenberry and colleagues (2006) observed an increase in proliferation after 48 h treatment with 40 ng/ml (2.5 nM) compared to cells grown in full growth medium, corresponding to "unsynchronised" cells. Both these studies observed increased cell proliferation in MDA-MB-231 breast cancer cells at low leptin concentrations, which was not observed in the study presented here, where an increase at high leptin concentration after 24 h treatment was observed. There were some methodological differences between these two published studies and the study presented here. The study presented here used BrdU-incorporation, *i.e.* rate of DNA synthesis, while Ray and colleagues (2007) used cell counts, *i.e.* cell cycle progression and Frankenberry and colleagues (2006) used MTT, *i.e.* metabolic rate. Frankenberry and colleagues' (2006) treatment medium also contained serum[4], which further hinders direct comparison of their results to the study presented here. This difference may explain the difference in them observing cell proliferation increases at low leptin concentrations, while the study presented here observed cell proliferation increases at high leptin concentrations. Thus results from the study presented here and

[4] Treatment was however preceded by 20 h incubation in serum-free medium, before the experiment was performed in serum-supplemented medium (Frankenberry *et al*., 2006).

previously published studies indicate that leptin is a significant growth factor able to modify and accelerate cell growth in MDA-MB-231 breast cancer cells. Different from insulin, high leptin concentrations increased cell proliferation in these cells. Taken the results presented in the following chapters into consideration, high leptin concentrations were the only treatment that increased cell proliferation in MD-MB-231 breast cancer cells, suggesting that leptin may be an important mitogenic factor for these cells. At 400 ng/ml and 800 ng/ml leptin treatment however cell proliferation decreased significantly after 48 h treatment, thus the proliferative effects of leptin manifest within the first 24 h of treatment. Interestingly the findings by Ray and colleagues (2007), where low leptin concentrations increased proliferation, are not supported by the findings in the study presented here. Thus the results observed in the here presented study are not unanimously supporting a proliferative effect of leptin on MDA-MB-231 breast cancer cells. The observed changes in cell proliferation are small, thus the physiological effect of leptin on these cells may be negligible.

In MDA-MB-231 breast cancer cells, AKT-phosphorylation did not change in response to 100 nM leptin treatment for between 5-20 min (Figure 4-5, A). It has been suggested that leptin can activate the PI3-kinase cell signalling pathway in prostate cancer cells (Hoda and Popken, 2008) and colon cancer cells (Hoda *et al.*, 2007; Ogunwobi and Beales, 2007). In the study presented here, leptin did not increase AKT-phosphorylation in MDA-MB-231 breast cancer cells. Frankenberry and colleagues (2006) however observed increases in AKT-phosphorylation in MDA-MB-231 breast cancer cells after 4 h treatment with 4 ng/ml (0.25 nM) leptin using western blotting and antibodies specific for phosphorylated AKT. The main difference compared to the study presented

here are the timepoint (20 min vs. 4 h) and the treatment concentration (100 nM vs. 0.25 nM), which could explain the difference in observation. In the study presented here the chosen times were used in concordance with the findings of chapter 3, where AKT-phosphorylation reached its maximum within the first ten minutes of treatment (see section 3.3.3.1). Frankenberry and colleagues (2006) also observed an increase in phosphorylated AKT after 30 min of treatment with 40 ng/ml leptin (2.5 nM) with a maximum at 2 h. Different from insulin, AKT may not be a primary target for leptin signalling in MDA-MB-231 breast cancer cells. Regarding the treatment concentration, Frankenberry and colleagues (2006) observed an increase in phosphorylated AKT at leptin concentrations of 4 ng/ml (0.25 nM), 40 ng/ml (2.5 nM) and 80 ng/ml (5 nM) leptin. Moreover, these authors obtained their results without incubation in serum-free medium utilising phospho-AKT and total-AKT specific antibodies in a Western Blotting analysis. They also present no statistical analysis of their Western Blotting data and did not comment on the reproducibly of these findings. Hence their observed increase may not be statistically significant. There is therefore considerable difference in the experimental set-up and analysis between the results presented here the results presented by Frankenberry and colleagues (2006), which are different in the use of incubation in serum-free medium, treatment time, treatment concentration, analysis of phosphorylation and data analysis. Hence the finding of Frankenberry and colleagues' (2006) study may not be directly comparable to the here presented results and thus do not necessarily contradict the findings presented here. Ray and colleagues (2007) measured protein expression of PI3-kinase after leptin treatment (at increasing concentrations of 5 ng/ml (0.31 nM), 10 ng/ml (0.63 nM), 25 ng/ml (1.6 nM), 50 ng/ml (3.13 nM), 100 ng/ml (6.25 nM) leptin), but did not observe a linear increase with

increasing leptin concentrations and did not suggest that AKT protein expression was a target for leptin signalling in MDA-MB-231 breast cancer cells and did not examine changes in AKT-phosphorylation.

In MDA-MB-231 breast cancer cells, ERK1/2-phosphorylation decreased significantly after 15 min and 20 min 100 nM leptin treatment (Figure 4-5, B), suggesting that leptin treatment does not activate the MAP-kinase cell signalling pathway and even reduces its basal activity. This is a similar finding as observed for the activation of ERK1/2 in SK-BR-3 breast cancer cells after insulin treatment, providing the possibility of a similar explanation (see section 3.4.3). Alternatively the high leptin concentrations used in the study presented here may exert a different effect than initially thought. The high leptin concentrations were chosen to maximise any effect leptin may have on the cell culture model. It may however be that instead of maximising leptin signalling in these cells, high leptin concentrations reduced the activity of their own receptor. A similar effect has been observed by leptin decreasing the expression of the adiponectin receptor in MDA-MB-231 breast cancer cells (Dos Santos *et al.*, 2008). On the other hand, in other studies the MAP-kinase pathway has been described as a target for leptin-induced intra-cellular signalling (Fruhbeck, 2006). Frankenberry and colleagues (2006) observed an increase in ERK1/2-phosphorylation in MDA-MB-231 breast cancer cells after treatment with 4 ng/ml (0.25 nM) leptin for 4 h. Thus the results presented here are not supported by results published by others. This may possibly be caused by the high leptin concentrations used in the study presented here.

Another possible explanation could be that leptin induced cell proliferation in MDA-MB-231 breast cancer cells, as observed at 100 nM leptin after 24 h treatment, is mediated independently of MAP-kinase pathway activation, possibly through JAK2/STAT3. In support of this suggestion, activation of JAK2/STAT3 was observed with 20 nM (320 ng/ml) leptin treatment in MCF-7 cells (Dieudonne *et al.*, 2002). Leptin-induced activation of JAK2/STAT3 was also observed in endometrial cancer cells (Sharma *et al.*, 2006), hepatocellular carcinoma cells (Saxena *et al.*, 2007a) and gastric cancer cell (Pai *et al.*, 2005). Activation of JAK2/STAT3 in MDA-MB-231 breast cancer cells after leptin treatment had not been observed previously and given the findings presented here may be a valid target for further investigation. Furthermore cell proliferation experiments using PD98059 to inhibit MAP-kinase signalling and AG940 to inhibit JAK2/STAT3 signalling should help identify the pathway responsible for the observed leptin-induced cell proliferation in MDA-MB-231 cells.

The finding that leptin treatment decreases ERK1/2-phosphorylation is interesting as prior to leptin treatment, these cells were incubated in serum-free medium for 24 h, which is thought to synchronise cell cycle and decrease all cell signalling activities as it removes all exogenous factors that could potentially activate cell signalling pathways, including the MAP-kinase pathway. Thus, theoretically, after incubation in serum-free medium all kinases of the MAP-kinase pathway, including ERK1/2 should not be phosphorylated.

Consequently any treatment should not be able to decrease phosphorylation even further[5]. This was observed however after leptin treatment for ERK1/2-phosphorylation, which could only have been the case, if ERK1/2 was still partly phosphorylated after incubation in serum-free medium. This in turn requires an exogenous signal to continue phosphorylating ERK1/2, which points to autocrine signalling in MDA-MB-231 breast cancer cells. Alternatively, ERK1/2 may be aberrantly phosphorylated through mutated kinases upstream of ERK1/2. In fact, in MDA-MB-231 breast cancer cells both RAS and RAF are mutated and possess constitutively increased kinase activities (Hollestelle *et al.*, 2007). This may point to constitutively active MAP-kinase cell signalling in these cells, either through aberrant RAS and RAF signalling, or through the presence of autocrine signalling (see also section 8.5). Both hypotheses are also supported by the finding in section 3.3.4, where inhibition of the MAP-kinase pathway, using PD98059 alone, decreased cell proliferation significantly.

In MDA-MB-231 breast cancer cells, leptin treatment did not significantly change the cell cycle population distribution (Figure 4-6, A). Conversely, Ray and colleagues (2007) found an increase in cyclin D1 gene expression after 24 h and 48 treatments with increasing concentrations of 5 ng/ml (0.31 nM), 10 ng/ml (0.63 nM), 25 ng/ml (1.6 nM), 50 ng/ml (3.13 nM), 100 ng/ml (6.25 nM) leptin, the authors however did not quantify this increase. Thus there is no convincing evidence suggesting that leptin

[5] For comparison, optical density of ERK1/2-phosphorylation values (and total ERK1/2 protein values) for the control cells, i.e. cells after incubation in serum-free medium, of the three different cell lines were 59.0±5.2 (923.5±60.0) for MDA-MB-231 breast cancer cells, 13.3±1.5 (980.5±89.6) for MCF-10A breast epithelial cells and 24.8±2.3 (880.4±148.4) for SK-BR-3 breast cancer cells. This indicates that all three cell lines express roughly identical amounts of ERK1/2, but background phosphorylation is highest in MDA-MB-231 cells and lowest in MCF-10A cells.

induces cell cycle progression in MDA-MB-231 breast cancer cells. In fact the results presented here indicate no effect of leptin on cell cycle progression in these cells. Given the generally observed leptin induced increase in cell proliferation however, one is inclined to also suspect an increase in cell cycle progression. It is to be remembered that cell cycle analysis using PI is a measurement of the amount of cells in each cell cycle stage, not a measurement for the speed of transition from one stage to the next. Thus it essentially measures the impact leptin treatment has on cell cycle checkpoints. If the impact on both checkpoints is exactly equal, the distribution of cells across the stages may be unaffected, thus even though the speed may have increased, it cannot be observed using DNA staining. Thus the increased attention on leptin as a mediator of breast cancer in obese postmenopausal women may be justified and has received further validation from the study presented here. The exact molecular effect of leptin in breast cancer cells however may be more complex than a straight forward mitogenic effect.

4.4.2 Effect of leptin treatment on human MCF-10A normal breast epithelial cells

Treatment of MCF-10A normal breast epithelial cells with 100 nM leptin did not change cell proliferation after 24 h treatment and increased significantly after 48 h of treatment (Figure 4-1, B). Cell proliferation however increased with lower concentrations of leptin (6.25 ng/ml) after 24 h treatment and with a number of additional lower concentrations after 48 h (Figure 4-3). This may indicate that leptin is a mitogenic factor for these epithelial cells, but may act slower than insulin, which increased cell proliferation rapidly after 24 h. The literature has not provided any similar studies yet for this cell line, making the study presented here the first to report on leptin-induced cell proliferation. Supportively, the presence of Ob-R was observed in this cell

line (O'brien *et al.*, 1999), suggesting that these cells have intact leptin signalling, which could increase cell proliferation. The increase in proliferation after 48 h treatment with 100 nM leptin is comparable to the increase in cell proliferation after 24 h in the MDA-MB-231 breast cancer cell line (14% increase in MCF-10A cells compared to 21% in MDA-MB-231 cells), thus suggesting a universal effect of leptin on breast epithelial cells and breast cancer cells. This is interesting as it would suggest that leptin could be involved in mediating both: increased risk of developing breast cancer and increased breast cancer progression in postmenopausal obese women.

In MCF-10A breast epithelial cells, AKT-phosphorylation did not change with 100 nM leptin after treatment between 5 min and 20 min. There was however higher variation between the repeated experiments resulting in higher standard error values, which may have masked a significant change. After 20 min of treatment, for example, AKT-phosphorylation was almost 30% higher than at baseline (untreated control, Figure 4-5, C). Activation of PI3-kinase cell signalling pathway in MCF-10A breast cancer cells cannot therefore confidently be excluded. Hence a further experiment with combined treatment of wortmannin and leptin may indicate if PI3-kinase signalling is involved in leptin-induced cell proliferation.

In MCF-10A breast epithelial cells, ERK1/2-phosphorylation increased significantly after 10 min and remained significantly phosphorylated until 20 min after treatment start (Figure 4-5, D). This is the first study to observe an increase in ERK1/2-phosphorylation after leptin treatment in MCF-10A breast epithelial cells. Whether this leptin induced increase in ERK1/2-phosphorylation is responsible for the

increase in cell proliferation after 48 h of leptin treatment may be questionable, as there was no indication of a leptin induced increase in cell proliferation after 24 h, which may be expected, if the MAP-kinase pathway is activated after a relatively short 10 min treatment time. Thus either the leptin induced increase in cell proliferation in MCF-10A cells after 48 h is caused by a multitude of factors, including activation of the MAP-kinase and JAK2/STAT3 pathways that only manifest themselves after 48 h treatment or the increase in ERK1/2-phosphorylation is unrelated to the increase in cell proliferation. Activation of the JAK2/STAT3 pathway after leptin treatment however was not investigated. It is more likely that leptin exerts rather weak mitogenic properties on MCF-10A cells, given the absence of all the growth factors in MCF-10A cells' normal growth medium (insulin, hydrocortisone, EGF, horse serum). Thus continuous activation of ERK1/2 may only achieve significantly increased cell proliferation after 48 h of treatment, even at high concentrations of 100 nM leptin.

The cell population distribution across the cell cycle stages was not significantly affected by leptin treatment in MCF-10A cells (Figure 4-6, B). No comparison to published data is available. A non-significant trend for leptin to decrease the sub-G1 population and increase the S-phase population was observed. This complements earlier findings of increased cell proliferation and activation of MAP-kinase cell signalling pathways in the study presented here. Further evaluation of cell cycle progression with leptin treatment in MCF-10A normal breast epithelial cells may be necessary however to determine the significance of the influence of leptin on cell cycle progression.

4.4.3 Effect of leptin treatment on human SK-BR-3 breast cancer cells

In SK-BR-3 breast cancer cells, 24 h and 48 h treatment with 100 nM leptin did not change cell proliferation significantly (Figure 4-1, C). Treatment at lower leptin concentrations however showed increased proliferation after both 24 h and 48 h leptin treatment (Figure 4-4). Interestingly, cell proliferation in SK-BR-3 breast cancer cells did not indicate a dose-response of cell proliferation, but rather indicated an optimum leptin concentration of about 100 ng/ml. Both higher and lower leptin concentrations did not increase cell proliferation to the level observed at 100 ng/ml. This suggests a more complex mode of action of leptin on SK-BR-3 breast cancer cells. The high increase in cell proliferation after 24 h and 48 h leptin treatment suggests that leptin acts mitogenic in these cells, especially when compared to the results for the other two cell lines. Ray and colleagues (2007) also observed a significant increase in cell numbers compared to untreated control cells after 24 h and 48 h treatment with 5 ng/ml (0.31 nM), 10 ng/ml (0.63 nM), 25 ng/ml (1.6 nM) and 50 ng/ml (3.13 nM) leptin. Thus there is evidence that cell proliferation increases in SK-BR-3 breast cancer cells with leptin treatment and that the increase of cell proliferation is linear at low leptin concentrations (6.25 ng/ml-100 ng/ml) but the increase in cell proliferation decreases thereafter.

One may speculate on the observation that high leptin concentrations did not increase cell proliferation, but lower leptin concentrations increased cell proliferation significantly. If leptin is an important mitogen, it would be expected that cell proliferation should increase linearly with increasing concentrations, reaching a plateau at high concentrations, when the mitogenic effect is at its maximum. A decrease back to

the baseline of cell proliferation indicates that leptin is not a straight forward mitogen in these cells, but that it is affecting at least two opposing mechanisms: one mechanism to increase cell proliferation and one to reduce cell proliferation. At low leptin concentrations the effect of leptin on the proliferative mechanism prevails, increasing cell proliferation with increasing leptin concentrations. At higher leptin concentrations (for SK-BR-3 breast cancer cells, the results presented here suggest 100 ng/ml leptin to be the turning point), when cell proliferation would be expected to plateau, the inhibiting mechanism becomes increasingly prevalent, reducing the increase in cell proliferation until it has reached baseline again. From the results presented here, two additional observations indicate that at 1600 ng/ml (100 nM) leptin, an inhibitory effect on cell proliferation is dominating in SK-BR-3 breast cancer cells. First, ERK1/2-phosphorylation decreased significantly with 100 nM leptin after 10 min and 15 min treatment (Figure 4-5, F). Second, G1-phase population increased non-significantly after 24 h treatment with 100 nM leptin (Figure 4-6, C), suggesting increased cell cycle arrest after treatment with high leptin concentrations. Additionally, another study observed decreased proliferation in T47D breast cancer cells at high leptin concentrations (62.5 nM (1000 ng/ml)) (Laud *et al*., 2002). If further support of this explanation is desired, it may be prudent to increase leptin concentrations further in order to identify, if the inhibitory mechanism keeps decreasing cell proliferation and may even increase apoptotic rate.

It may be that leptin has two opposing effects on its own receptor as suggested for MDA-MB-231 breast cancer cells. This could be due to an overload of leptin on the leptin-receptor so that at high leptin concentrations newly synthesised leptin receptor

molecules are immediately saturated by leptin and are immediately labelled for degradation at the proteasome. If normal leptin signalling requires the input of multiple leptin receptors, leptin signalling could be seriously impeded. At lower leptin concentrations, the receptor is not immediately saturated with leptin, thus the necessary number of leptin receptors can accumulate to exert normal leptin signalling. Alternatively, the observed increase in cell proliferation may be dependent on an Ob-R-HER2 cross-talk, which is inhibited at high leptin concentrations[6]. A leptin-HER2-receptor transactivation has been observed in SK-BR-3 cells, to the effect that leptin increased HER2-receptor activation (Soma *et al.*, 2008). Additionally in MCF-7 cells, which co-expressed Ob-R and HER2, the two receptors would co-localise and co-precipitate, indicating that the Ob-R could interact with HER2 signalling in response to leptin stimulation (Fiorio *et al.*, 2008). These authors observed that leptin treatment (200 ng/ml (12.5 nM)) for 15 min increased HER2 activity (Fiorio *et al.*, 2008). Furthermore HER2 signalling is mediated by the MAP-kinase cell signalling pathway (Kurebayashi, 2001). Thus the observed decrease in MAP-kinase signalling may indicate that a possible HER2-Ob-R cross-talk is inhibited at high leptin concentrations. From the results presented here and the available literature however neither conclusion can be drawn with a high degree of confidence.

In SK-BR-3 cells no significant changes in AKT-phosphorylation after 5 min, 10 min, 15 min or 20 min of 100 nM leptin treatment was observed (Figure 4-5, E). By comparison, Ray and colleagues (2007) observed an increase in the total protein

[6] The SK-BR-3 cell line overexpresses the erbb2 gene product, HER2, which increases cell proliferation (Hudziak *et al.*, 1997). The mechanism is similar to that of ER, except that HER2 expression is graded, while ER expression is definite.

expression of PI3-kinase with increasing concentrations of leptin (concentration range was between 5 ng/ml (0.31 nM), 10 ng/ml (0.63 nM), 25 ng/ml (1.6 nM), 50 ng/ml (3.13 nM) and 100 ng/ml (6.25 nM) leptin). This result was however not quantified, as the authors were interested, if protein expression showed a trend with increased leptin concentration, which was observed. For determining activation of the PI3-kinase pathway by leptin, measuring the phosphorylation of PI3-kinase or AKT would be a more valid target. Thus there seems to be no indication in the literature or the study presented here that leptin increases phosphorylation of AKT in SK-BR-3 breast cancer cells. In the study presented here, AKT-phosphorylation was non-significantly increased at all time points, this increase was not linear however (Figure 4-5, E). AKT-phosphorylation was equally increased after 5 min and 20 min of treatment, while after 10 min and 15 min, the increase was lower. An explanation of the observed findings would be that AKT is not a target for leptin signalling, similar to the findings observed in MDA-MB-231 breast cancer cells, where the same conclusion was suggested. Alternatively, this finding could be caused by the same effect that was suggested for cell proliferation, *i.e.* that high leptin concentrations inhibit cell growth in SK-BR-3 breast cancer cells

In SK-BR-3 cells ERK1/2-phosphorylation did not change after 5 min, decreased significantly after 10 min and 15 min and did not change after 20 min treatment with 100 nM leptin (Figure 4-5, F). None of the published studies examining the effect of leptin on SK-BR-3 cells investigated a leptin-induced increase in ERK1/2-phosphorylation. Thus the here presented observations indicate that leptin decreased activity of the MAP-kinase, indicating a similar situation as in MDA-MB-231

breast cancer cells. In SK-BR-3 cells however the possibility that this observation is caused by mutated upstream kinases can be excluded, as it was observed that SK-BR-3 cells express wild-type RAS and RAF (Hollestelle *et al.*, 2007). Thus the possibility that an exogenous signal is secreted by the SK-BR-3 breast cancer cells themselves is a possibility. This would point to the possibility of autocrine signalling in these cells, as was similarly suggested for MDA-MB-231 cells. Alternatively, this finding could be caused by the inhibitory mechanism that is stimulated in SK-BR-3 breast cancer cells by high leptin concentrations as suggested earlier.

In SK-BR-3 cells 24 h treatment with 100 nM leptin did not significantly change the distribution of cells across cell cycle stages compared to the untreated control values (Figure 4-6, C). A trend for 100 nM leptin to increase the cell population of the G1-phase and decrease the G2-phase population was observed. An increase in G1-phase may indicate that leptin treatment increases arrest of the cell cycle at the G1/S cell cycle checkpoint, which could point to the inhibitory mechanism of high leptin concentrations, as suggested earlier. A decrease in G2 could point to an influence of high leptin concentrations on the G2/M cell cycle checkpoint. High leptin concentrations may need to affect cell cycle only at either checkpoint in order to explain the observed findings. If leptin treatment slows cell cycle progression at the G1/S checkpoint, more cells are retained in G1-phase and fewer cells enter S-phase and thus G2-phase, accounting for both phenomena. Likewise increased passage past the G2/M checkpoint would decrease cells retained in G2 and more cells entering mitosis and thus G1-phase, where they encounter an intact G1/S checkpoint and accumulate. One can easily see that the first explanation would warrant cell cycle slowing properties of

leptin, while the second would suggest increased cell cycle progression with leptin treatment in SK-BR-3 breast cancer cells. In support of the suggestion that high leptin concentrations may act inhibitory on SK-BR-3 cell growth, it could be concluded that leptin treatment may indeed act inhibitory on the G1/S cell cycle checkpoint and decrease cell cycle progression in SK-BR-3 breast cancer cells. This idea however not supported by a statistical significance of this finding.

4.4.4 Rationale for the approach

The rationale for the chosen experimental methodology is the same as explained in section 3.4.5.

The chosen leptin concentration used for the majority of the experiments in the study presented here (100 nM (1600 ng/ml)) was higher than physiological concentrations; mean leptin concentrations in moderately obese (see footnote 1) individuals were 31.3 ng/ml (1.96 nM) (Considine *et al.*, 1996). Thus the chosen leptin concentration was similarly above the physiological concentration as insulin. Assuming a physiological insulin concentration of 0.3 nM (see footnote 1 in chapter 3), the insulin treatment concentration was 333-times higher. By comparison the used leptin concentration was 51-times higher than the mean obese leptin concentration of 31.3 ng/ml. It was again not aimed to re-create a physiological environment, but rather to maximise the effect of leptin on the cell culture system to identify even small effects of leptin. As the breast tissue is largely composed of adipose tissue, the microenvironment of the breast tumour may show increased leptin concentrations compared to circulating concentrations. A similar situation has been demonstrated for oestrogen, which was ten-times higher than

circulating concentrations in the breast tumour microenvironment (van Landeghem *et al.*, 1985). In the literature comparable leptin studies in breast cancer cell models use less leptin than in the study presented here[7]. In order to investigate, if lower leptin concentrations may affect cell proliferation in the used cell culture system a range of lower leptin concentrations was also used (6.25 ng/ml-800 ng/ml). For comparison significant changes in cell proliferation in MDA-MB-231 cells with 50 ng/ml (3.125 nM) leptin after 24 h and 48 h and with 5 ng/ml (0.3125 nM) leptin after 24 h and 48 h in SK-BR-3 cells were reported (Ray *et al.*, 2007). Frankenberry and colleagues (2006) reported significant increases in cell proliferation in MDA-MB-231 cells with 40 ng/ml (2.5 nM) leptin treatment for 48 h and increases in ERK1/2 and AKT-phosphorylation with 4 ng/ml (0.25 nM) leptin after 4 h treatment. Thus the study presented here is unique in the used leptin concentration, addressing the effects of leptin at the very high end of the previously used leptin concentration.

4.5 Summary

The three cell lines reacted differently to leptin treatment and no significant change in one cell line was observed in either of the other two, except for the decrease in ERK1/2-phosphorylation after 10 min in MDA-MB-231 cells and SK-BR-3 cells. MDA-MB-231 cells increased in proliferation after 24 h treatment, but not after 48 h treatment; MCF-10A cells did not increase in proliferation after 24 h but did increase after 48 h treatment; SK-BR-3 cell proliferation did not change in proliferation at either timepoint. Using a range of leptin concentrations indicated in all three cell lines that high leptin

[7] The highest concentration used to treat breast cancer cells in the literature was 62.5 nM (1000 ng/ml) by Laud and colleagues (2002) and Catalano and colleagues (2004). This concentration is about 2/3 of that used in the study presented here (100 nM (1600 ng/ml)).

concentrations (>50 nM) may have no effect on cell proliferation or even a growth inhibiting effect, but that lower leptin concentration may have a growth promoting effect.

AKT-phosphorylation was not affected in any of three cell lines after 100 nM leptin treatment. There was some cohesion in the observation of ERK1/2-phosphorylation changed with leptin treatment. Both cancer cell lines, MDA-MB-231 and SK-BR-3 cells, showed a decrease in ERK1/2-phosphorylation in response to leptin treatment, while the normal breast epithelial cell line MCF-10A increased in ERK1/2-phosphorylation with leptin treatment. Leptin did not have a significant effect on cell cycle in all three cell lines.

The finding for MDA-MB-231 cells, that leptin increased proliferation but decreased the activation of the MAP-kinase pathway, indicated that high leptin concentrations increase cell proliferation by a different cell signalling pathway. Since there was also no activation of the PI3-kinase pathway, the JAK2/STAT3 pathway is a major candidate. A similar mechanism may be found in SK-BR-3 breast cancer cells. The non-linear change in ERK1/2-phosphorylation and a similar non-significant trend for the AKT-phosphorylation over time with leptin treatment however indicate a more complex system of leptin signalling in SK-BR-3 cells, suggesting both growth promoting (at low leptin concentrations) and growth inhibitory (at high leptin concentrations) effects of leptin. In MCF-10A breast epithelial cells, cell proliferation did not increase until 48 h after leptin treatment had started, but showed a marked increase in ERK1/2-phosphorylation, which was higher than the observed increase with insulin

treatment. Thus leptin exerted growth promoting effects on MCF-10A breast cancer cells, which may indicate a progression towards malignant growth. The available literature seems to neglect this aspect of leptin signalling as no studies are available examining the effect of leptin on normal breast epithelial cells as a possible link between obesity and breast cancer aetiology. The here presented results indicate a high influence of leptin on growth promotion in breast epithelial cells, suggesting that further research should include leptin as a factor of breast cancer aetiology rather than exclusively focus on its influence on increased progression of breast cancer.

Chapter 5
Tumour Necrosis Factor-α

5 TUMOUR NECROSIS FACTOR-ALPHA

In this chapter the effect of TNF-α on several molecular markers in MDA-MB-231 breast cancer cells, MCF-10A breast epithelial cells and SK-BR-3 breast cancer cells is presented. It was aimed to determine the effect of TNF-α on cell proliferation, activation of PI3-kinase and MAP-kinase cell signalling pathways and cell cycle progression in this cell culture system. Thus in this chapter the same molecular mechanisms are investigated after TNF-α treatment as for leptin in chapter 4.

5.1 Introduction

Tumour necrosis factor-α (TNF-α) is a cytokine involved in the induction of inflammation and initiation of an acute phase immune response. When cells are stimulated to secrete TNF-α, membrane-bound precursor molecules on the surface of the secreting cell are cleaved to form 51 kDa circulating TNF-α (Black *et al.*, 1997). Its name derived from the initial observation that this cytokine was able to induce cell death in the murine fibrosarcoma L-929 cell line (Carswell *et al.*, 1975). Endocrine signalling is accomplished by circulating TNF-α binding to and activating its specific receptor TNF-receptor 1 (TNF-R1) on target cells. The receptor is expressed ubiquitously in all human tissues, including breast epithelial cells (Krajewska *et al.*, 1998). Additionally epithelial cells also express the TNF-R2, which can be activated by TNF-α, but whose intra-cellular signalling is not as well understood as TNF-R1 signalling (Gaur and Aggarwal, 2003). The TNF-R1 contains a "death domain", which, if activated is able to induce apoptosis by activation of the autoproteolytic process of caspase 8 and downstream caspases (Gaur and Aggarwal, 2003). Indeed in MCF-7 breast cancer cells, TNF-α induces apoptosis and inhibits cell proliferation (Burow *et*

al., 1998). The TNF-R1 death domain however is not routinely activated following TNF-α binding (Gaur and Aggarwal, 2003). TNF-α's main intra-cellular target is the NF-κB transcription factor, which is involved in mediating the expression of proteins involved in cell survival and anti-apoptosis, through activation of the JAK2/STAT3 pathway, the PI3-kinase pathway and the MAP-kinase pathway in the breast cancer cell line T47D (Rivas *et al.*, 2008). NF-κB has been suggested as an important link in inflammation-induced cancer development (Karin, 2009). Constitutive activation of NF-κB has also been linked to increased cancer progression in breast cancer cell lines (Nakshatri *et al.*, 1997).

As such TNF-α is a pleiotropic cytokine, which exerts contradictory impulses on target cells. Thus it has been observed that TNF-α can activate cell proliferation and apoptosis in the same cell line (Gaur and Aggarwal, 2003). Increased cell survival is possibly mediated through MAP-kinase cell signalling pathways, the complex action of TNF-α is likely to involve however a number of interacting pathways (Gaur and Aggarwal, 2003). The connection of increased TNF-α secretion by white adipose tissue with increased BMI could provide a link to explain the connection of obesity and postmenopausal breast cancer (see also section 1.5.3.2).

5.1.1 TNF-α and cell proliferation in breast cancer

In line with the opposing effects of TNF-α, contrasting results on TNF-α's impact on cell proliferation in breast cancer cells were observed. Rivas and colleagues (2008) demonstrated that 10 ng/ml TNF-α treatment for 48 h increased cell proliferation significantly in T47D human breast cancer cells. Conversely in MCF-7 breast cancer

cells, TNF-α treatment reduced cell proliferation (Lee and Nam, 2008). Interestingly different MCF-7 subtypes reacted differently to treatment with 10 ng/ml TNF-α for 24 h to 72 h, indicating that TNF-α exerts different and indeed opposing effects on the same cell line (Burow *et al.*, 2000). Cell proliferation in MDA-MB-231 breast cancer cells was not affected by 0.25 nM (12.75 ng/ml) TNF-α, decreased proliferation however was observed in MCF-7, ZR-75-1 and T47-D breast cancer cells (Mueller *et al.*, 1996). This study also observed a decrease in cell proliferation in MDA-MB-231 breast cancer cells after combined treatment of TNF-α and interferon-γ. A similar co-treatment decreased cell proliferating in SK-BR-3 breast cancer cells, which was suggested to be caused by forced reduction in HER2 protein expression (Kumar and Mendelsohn, 1994).

5.1.2 TNF-α and cell signalling pathways in breast cancer

In T47D human breast cancer cells, 20 ng/ml TNF-α induced significant activation of PI3-kinase pathway, MAP-kinase pathway and JAK2/STAT3 pathway (Rivas *et al.*, 2008). Additionally in MDA-MB-231 cells, TNF-α induced activation of MAP-kinase pathway and the JAK2/STAT3 pathway after induction of an autocrine stimulation of TNF-α by exposure of cells to ionising radiation (Dent *et al.*, 1999). In SK-BR-3 breast cancer cells, induction of apoptosis, using TRAIL, a protein mimicking the apoptosis inducing effects of TNF-α, was dependent on normal PI3-kinase functioning (Dubska *et al.*, 2005). In MCF-7 breast cancer cells, TNF-α treatment increased activation of NF-κB by activation of PI3-kinase and AKT (Burow *et al.*, 2000).

5.1.3 TNF-α and cell cycle progression in breast cancer

Treatment with 10 ng/ml TNF-α for 24 h increased G1-phase population in T47D cells, while reducing the population in S-phase and G2-phase, thus creating an inhibition of cell cycle progression at the G1/S cell cycle checkpoint (Pusztai *et al.*, 1993). A similar increase in G1-phase cell population was observed after treatment with 10 ng/ml TNF for 36 h in MCF-7 cells, suggesting cell cycle arrest at the G1/S cell cycle checkpoint (Bogin *et al.*, 1998). Furthermore cell cycle related and anti-mitogenic proteins p21, p53 and Retinoblastoma (Rb) were stimulated and suggested to mediate G0/G1 cell cycle arrest after 10 ng/ml TNF-α treatment for 25 h (Jeoung *et al.*, 1995). These studies suggest that TNF-α inhibits cell cycle progression in ER-positive breast cancer cells.

5.2 Materials and Methods

5.2.1 Cell lines and TNF-α treatment

MDA-MB-231, MCF-10A and SK-BR-3 cells were cultured and treated as described in Materials and Methods sections 2.1.1 and 2.1.2 . Formulation of TNF-α treatment medium and final TNF-α concentration is described in Materials and Methods section 2.1.3.3.

5.2.2 Cell proliferation assay

The cell proliferation assay was performed as described in section 3.2.2, with the exception that for a 48 h experiment, 10 μM/well BrdU was added at the beginning of the 48 h incubation instead of only for the last 24 h. There were no alterations for a 24 h experiment. Three experiments were performed for each cell line and each timepoint. Each experiment consisted of six replicates for each treatment, *i.e.* six wells for control and six wells for treatment.

5.2.3 Cell signalling pathway assays

The cell signalling pathways were analysed as described in section 3.2.5, but with 10 ng/ml TNF-α as treatment medium. For each cell line, MDA-MB-231, MCF-10A and SK-BR-3, two experiments were performed to assess AKT-phosphorylation and two to assess ERK1/2-phosphorylation. Each experiment included two replicates for each treatment and the control, *i.e.* two values for control, two for 5 min, two for 10 min, two for 15 min and two for 20 min treatment in each experiment.

5.2.4 Cell cycle analysis by flow cytometry

Changes in the cell distribution across cell cycle stages was assessed as described in section 3.2.6 , but with 10 ng/ml TNF-α as treatment medium.. Three experiments were performed for each cell line.

5.3 Results

5.3.1 Effect of TNF-α treatment on cell proliferation

Cell proliferation of all cell lines was assessed after 10 ng/ml TNF-α treatment for 24 h or 48 h. Untreated control cells were incubated in serum-free medium (Control) at all times. All results are presented as percentage change from this control.

In MDA-MB-231 breast cancer cells, cell proliferation did not significantly change after 24 h or 48 h treatment with 10 ng/ml TNF-α (Figure 5-1, A).

In MCF-10A normal breast epithelial cells, cell proliferation increased significantly by 26% after 24 h ($p<0.001$) treatment and by 38% after 48 h ($p=0.002$) treatment with 10 ng/ml TNF-α (Figure 5-1, B).

In SK-BR-3 breast cancer cells, cell proliferation increased significantly by 31% after 24 h ($p=0.016$) and by 59% after 48 h ($p<0.001$) treatment with 10 ng/ml TNF-α (Figure 5-1, C).

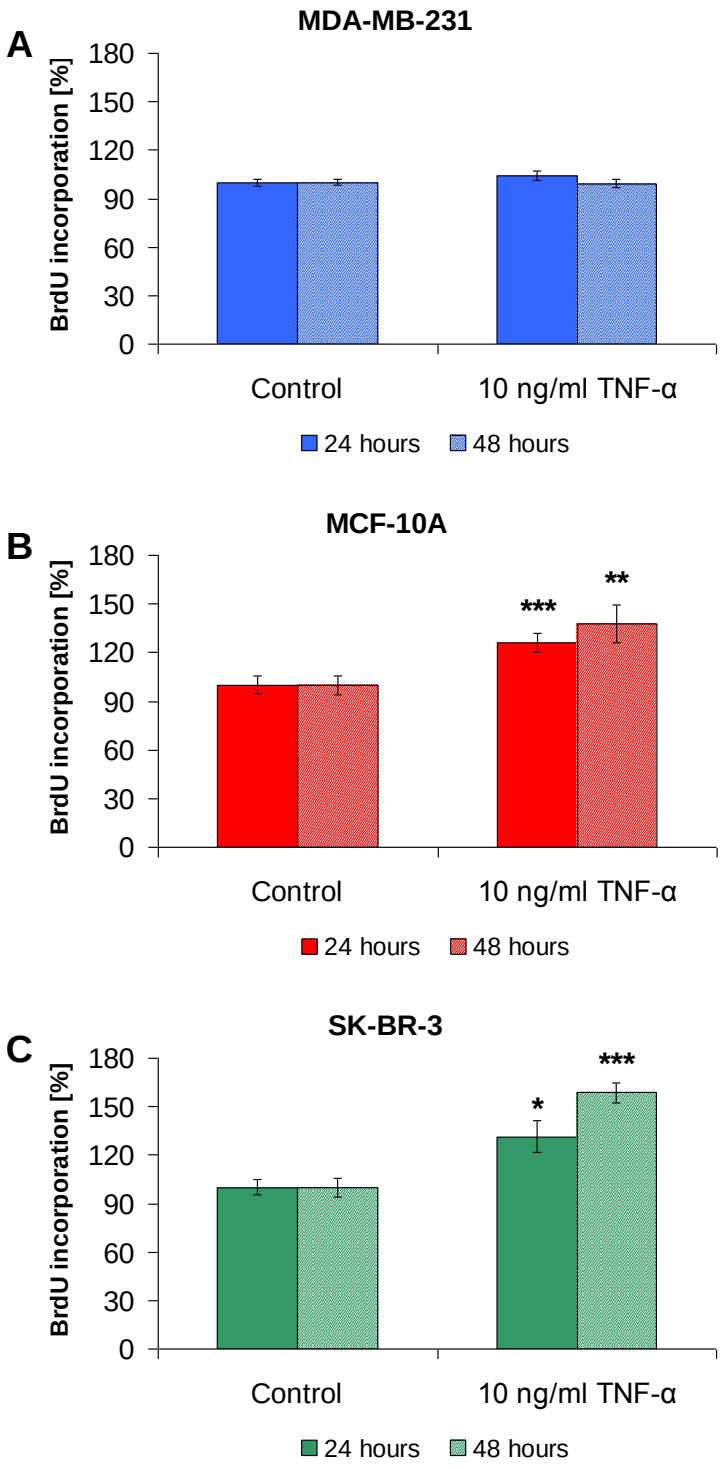

Figure 5-1: Changes in cell proliferation after treatment with 10 ng/ml TNF-α for 24 h or 48 h in A) MDA-MB-231 breast cancer cells (blue), B) MCF-10A breast epithelial cells (red) and C) SK BR-3 breast cancer cells (green). Bars represent BrdU-incorporation in relation to the respective control within each graph and are expressed as a percentage thereof. Error bars represent ± SEM of three experiments, each consisting of six replicates, *i.e.* 18 data points for each bar.
* Significance value compared to control, obtained using Dunnett's post-hoc t-test following ANOVA analysis. (*0.05>p>0.01; **0.01>p>0.001; ***p<0.001)

5.3.2 Effect of TNF-α treatment on activation of downstream cell signalling pathways

Phosphorylation of AKT or ERK1/2, representative of activation of the PI3-kinase or MAP-kinase cell signalling pathway, respectively, was measured after treatment with 10 ng/ml TNF-α for between 5-20 min. Untreated control cells were incubated in serum-free medium (Control) at all times. All results are presented as percentage change of this control.

In MDA-MB-231 breast cancer cells, AKT-phosphorylation did not change significantly after treatment between 5 min and 20 min with 10 ng/ml TNF-α (Figure 5-2, A). ERK1/2-phosphorylation was significantly decreased by 22% after 5 min ($p=0.044$) treatment with 10 ng/ml TNF-α (Figure 5-2, B).

In MCF-10A normal breast epithelial cells, AKT-phosphorylation did not change significantly after treatment between 5 min and 20 min with 10 ng/ml TNF-α (Figure 5-2, C). A non-significant increase of 77% ($p=0.169$) after 20 min of treatment with 10 ng/ml TNF-α compared to untreated control was however observed. ERK1/2-phosphorylation increased significantly by 144%, 103% and 108% after 10 min ($p=0.003$), 15 min ($p=0.025$) and 20 min ($p=0.019$) treatment with 10 ng/ml TNF-α compared to untreated control (Figure 5-2, D).

In SK-BR-3 breast cancer cells, AKT-phosphorylation increased significantly by 66% and 72% after 5 min ($p=0.04$) and 10 min ($p=0.025$) treatment with 10 ng/ml TNF-α, respectively compared to untreated control. AKT-phosphorylation increased non-significantly by 60% after 15 min ($p=0.06$) treatment with 10 ng/ml TNF-α

compared to untreated control. AKT-phosphorylation increased significantly by 91% after 20 min ($p=0.006$) treatment with 10 ng/ml TNF-α (Figure 5-2, E). ERK1/2-phosphorylation increased significantly by 47% after 5 min ($p=0.027$) treatment with 10 ng/ml TNF-α (Figure 5-2, F).

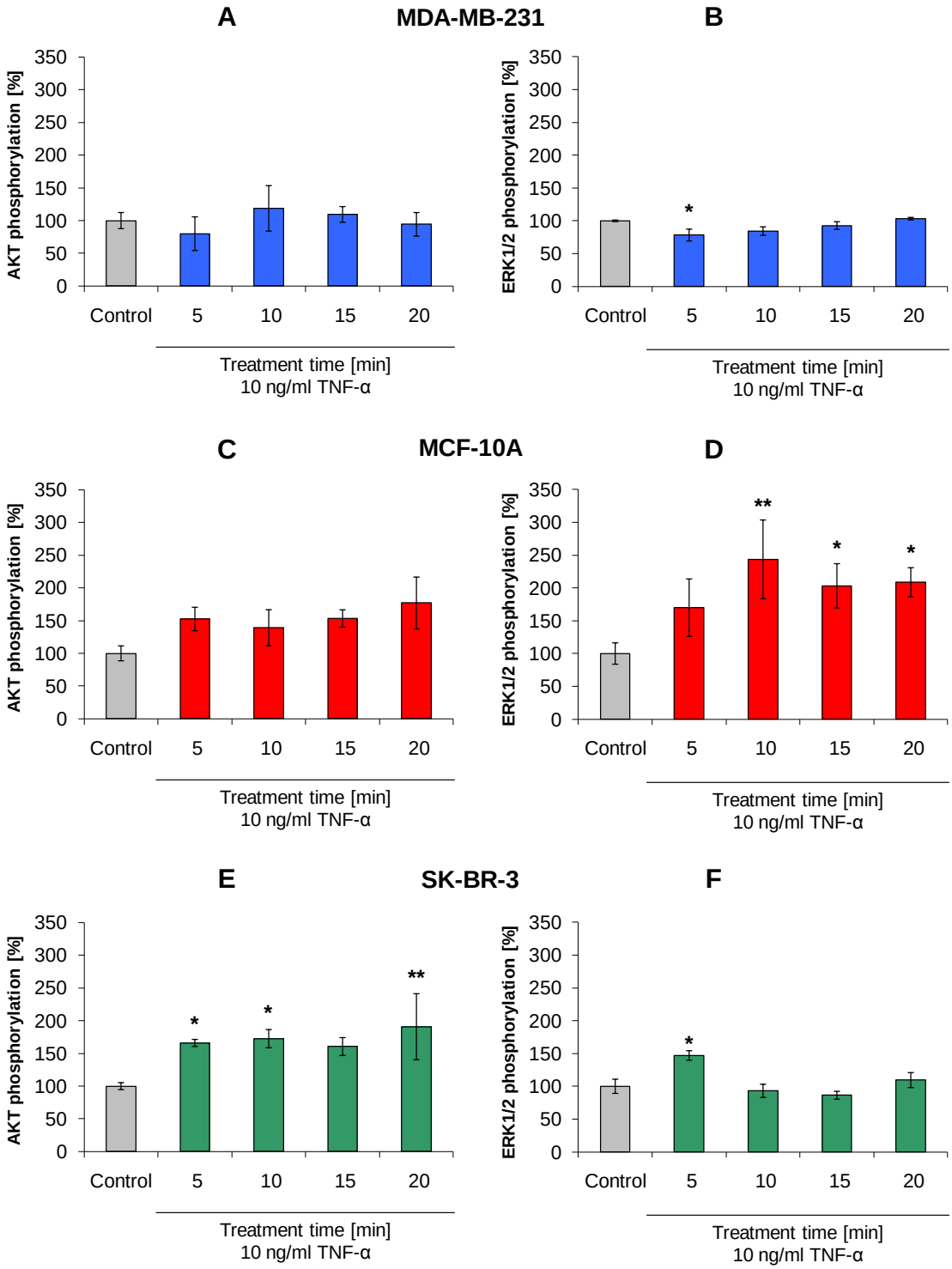

Figure 5-2: Changes in cell signalling pathway activation (PI3-kinase (A, C, E), MAP-kinase (B, D, F)) with 10 ng/ml TNF-α treatment in A, B) MDA-MB-231 breast cancer cells, C, D) MCF-10A breast epithelial cells and E, F) SK-BR-3 breast cancer cells. Bars represent AKT-phosphorylation or ERK1/2-phosphorylation in relation to the respective control within each graph and are expressed as a percentage thereof. Error bars represent ± SEM of three experiments, each consisting of two replicates, *i.e.* six data points for each bar.
* Significance value compared to control, obtained using Dunnett's post-hoc t-test following univariate analysis of variance. (* 0.05>p>0.01; ** 0.01>p>0.001)

5.3.3 Effect of TNF-α treatment on breast cancer cell cycle progression

Cell cycle profiles for all cell lines were established after treatment with 10 ng/ml TNF-α for 24 h. One initial experiment each was also performed after 3 h and 6 h of treatment with 10 ng/ml TNF-α (data not shown), which did not indicate a significant change and these incubation times were not further pursued. The controls are the same as described in section 3.3.5.

In MDA-MB-231 breast cancer cells, the subG1 population increased significantly by 4.6 percentage points (a 27% increase) after 24 h ($p=0.001$) treatment with 10 ng/ml TNF-α compared to the subG1 population of control cells. Population of the G1-phase decreased significantly by 3.5 percentage points (a 6% decrease) after 24 h ($p=0.006$) treatment with 10 ng/ml TNF-α compared to G1-phase population of control cells. S-phase population decreased significantly by 1.8 percentage points (a 15% decrease) after 24 h treatment ($p<0.001$) with 10 ng/ml TNF-α compared to S-phase population of control cells. No significant difference in G2-phase population after 24 h treatment ($p=0.233$) with 10 ng/ml TNF-α compared to G2-phase population of control cells was observed (Figure 5-3, A).

In MCF-10A normal breast epithelial cells no significant difference in any cell cycle phase and the subG1 population was observed after 24 h treatment with 10 ng/ml TNF-α compared to control. In the G1-phase population a non-significant decrease of 6.6 percentage points (a 12% decrease) after 24 h treatment ($p=0.051$) with 10 ng/ml TNF-α compared to G1-phase population of control cells was observed, the significance value being minimally above the significance level for the study presented here (Figure

5-3, B). This is a strong indication that TNF-α may promote cell cycle progression past the G1/S checkpoint.

In SK-BR-3 breast cancer cells subG1 population (p=0.372) and G1-phase population (p=0.811) were not significantly different between 24 h treatment with 10 ng/ml TNF-α and the respective control. Population of the S-phase decreased significantly by 1.5 percentage points (a 25% decrease) after 24 h (p=0.011) treatment with 10 ng/ml TNF-α compared to S-phase population of control cells. Population of the G2-phase decreased significantly by 3.8 percentage points (a 24% decrease) after 24 h (p=0.011) treatment with 10 ng/ml TNF-α compared to G2-phase population of control cells (Figure 5-3, C).

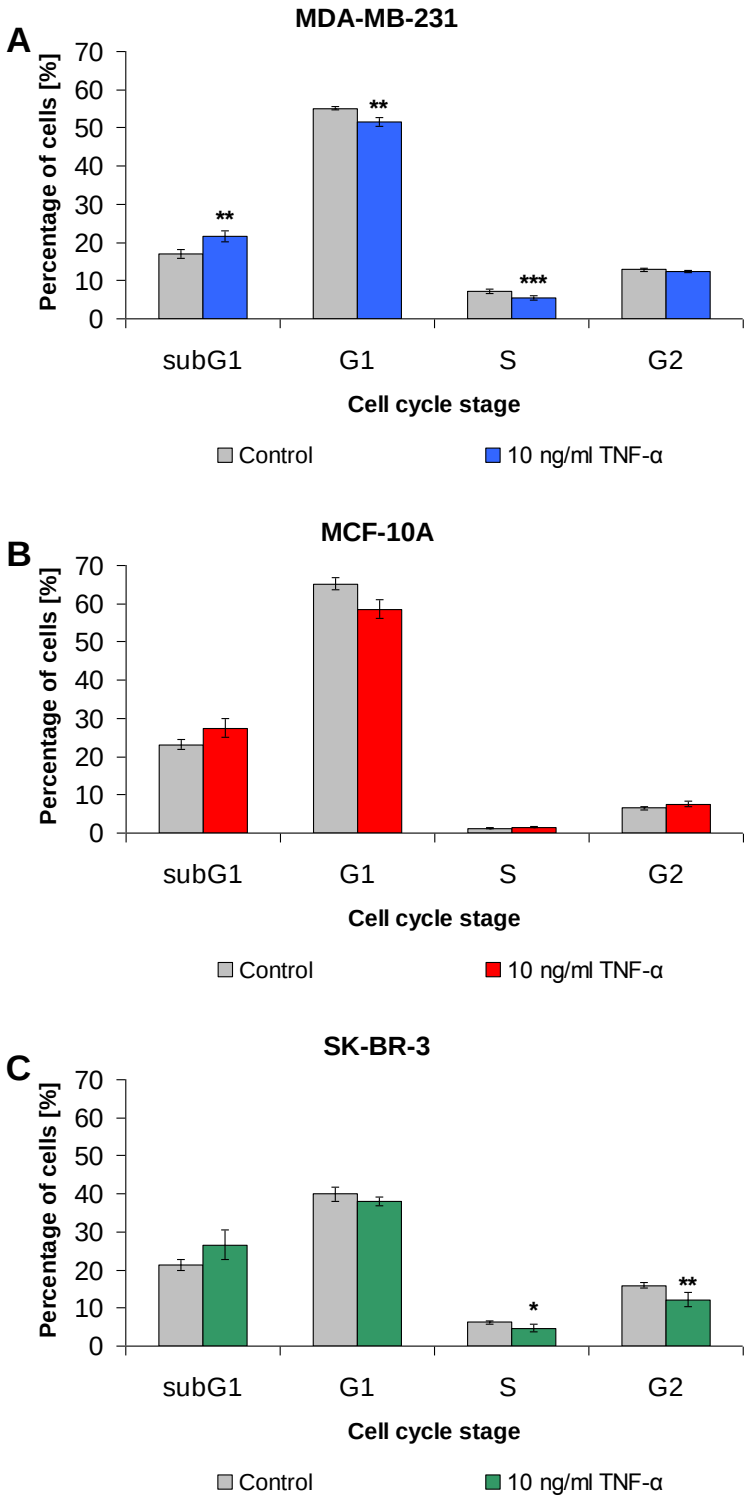

Figure 5-3: Changes of cell population distribution across cell cycle stages after 24 h treatment with 10ng/ml TNF-α in A) MDA-MB-231 cells, B) MCF-10A cells and C) SK-BR-3 cells. Bars represent percentage of cells as obtained from a 10000 cell sample obtained, treated and analysed as described in section 2.8.1. Error bars represent ± SEM of three experiments with two replicates for each treatment, *i.e.* 6 data points for each bar.
* Significance value compared to control (grey) at that stage, obtained using Dunnett's post-hoc t-test following univariate analysis of variance (* 0.05>p>0.01; ** 0.01>p>0.001; *** p<0.001),

5.4 Discussion

5.4.1 Effect of TNF-α treatment on human MDA-MB-231 breast cancer cells

In MDA-MB-231 breast cancer cells, cell proliferation did not change after 24 h or 48 h treatment with 10 ng/ml TNF-α (Figure 5-1, A). Similarly Mueller and colleagues (1996) observed that 0.25 nM (12.75 ng/ml) TNF-α treatment alone did not change cell proliferation in these cells. Thus at the time of writing there is no indication that TNF-α treatment has an effect on cell proliferation in MDA-MB-231 breast cancer cells. TNF-α has a wide variety of effects on cell growth, including inducing cell proliferation and apoptosis (Gaur and Aggarwal, 2003). Thus the effects of TNF-α may cancel each other out so that outwardly no change in cell proliferation in MDA-MB-231 breast cancer cells is observable. That however would imply that the impact of TNF-α on opposing mechanisms is essentially equal, for them to cancel each other.

In MDA-MB-231 breast cancer cells, 10 ng/ml TNF-α treatment did not change phosphorylation of AKT (Figure 5-2, A). By comparison, the only other study examining PI3-kinase activation after TNF-α treatment in breast cancer cells observed that in T47D breast cancer cells, 20 ng/ml TNF-α increased AKT-phosphorylation after 5 min of treatment, and was highest after 30 min (Rivas *et al.*, 2008). No other studies have reported on the impact of TNF-α on PI3-kinase pathway in breast cancer cells, thus there is no indication that the PI3-kinase cell signalling pathway is activated in response to TNF-α treatment in MDA-MB-231.

In MDA-MB-231 breast cancer cells, 10 ng/ml TNF-α treatment significantly decreased phosphorylation of ERK1/2 after 5 min of treatment (Figure 5-2, B). In the only

comparable study, treatment of MDA-MB-231 breast cancer cells with 10 ng/ml TNF-α for 24 h induced expression of MMP-9[1], which was reduced after inhibition of MEK1/2 by 10 μM U0126[2], suggesting that TNF-α activates ERK1/2 to exert this effect (Kim *et al.*, 2008). This study however did not examine ERK1/2-phosphorylation directly. Moreover, while ERK1/2-phosphorylation was significantly reduced after 5 min treatment, phosphorylation increased steadily back to baseline after 20 min[3]. Additional investigation at later time points might determine whether this rise continues further, which might indicate increased activation of the MAP-kinase cell signalling pathway after longer TNF-α treatments. The rationale for doing so would be that Kim and colleagues' (2008) increased MMP-9 expression was observed after 24 h TNF-α treatment.

In MDA-MB-231 breast cancer cells, 10 ng/ml TNF-α treatment for 24 h significantly increased the subG1 population and decreased the G1 and S-phase population of the cell cycle profile (Figure 5-3, A). Cell cycle has not been examined in MDA-MB-231 cells before. By comparison, in T47D breast cancer cells, 10 ng/ml TNF-α treatment 24 h increased G1-phase population at the expense of S-phase and G2-phase populations, leading the authors to conclude that TNF-α treatment arrests cell cycle at the G1/S cell cycle checkpoint (Pusztai *et al.*, 1993). They also suggest that TNF-α is not cytotoxic, *i.e.* it does not induce apoptosis, but rather cytostatic, *i.e.* it induces growth arrest, in

[1] MMP-9 is a protein involved in facilitating invasion of cancer cells in tumour surrounding tissue and according to the authors of the here discussed study, MMP-9 expression is pivotal for breast cancer cells to metastasise (Kim *et al.*, 2008).

[2] U0126 is a MEK1/2 kinase inhibitor, similar to PD98059 used in the study presented here. It inhibits MAP-kinase cell signalling.

[3] Using Tukey HSD post-hoc test as described in section 2.9, it was observed that ERK1/2-phosphorylation was significantly increased after 20 min of treatment compared to 5 min treatment ($p=0.042$).

T47D cells[4]. Compared to findings in the study presented here, the higher subG1 population indicates that TNF-α treatment increases apoptosis in MDA-MB-231 cells. The additional finding that G2-phase population is not changed with TNF-α treatment may indicate that apoptosis may occur predominantly during mitosis or before entering mitosis, with the remaining cell population advancing further in the cell cycle, but G1-phase and S-phase not being "replenished" from cells that have undergone mitosis and thus the overall cell number in these phases is decreased. By comparison the results from the cell proliferation experiments (see section 5.3.1) in the study presented here however do not indicate a decrease in cell proliferation, which would be conducive to an increase in apoptosis. Thus the cell proliferation results do not support the increased apoptosis observed with the cell cycle experiments. On the other hand the decrease in ERK1/2-phosphorylation may indicate a decrease in cell cycle progression and thus be more agreeable with the cell cycle results. Conclusively in line with the potential for TNF-α to exert different and sometimes opposing effects on cells, the results obtained for MDA-MB-231 breast cancer cells do not allow to conclude a simplistic mechanism by which TNF-α may alter cell growth in these cells.

5.4.2 Effect of TNF-α treatment on human MCF-10A normal breast epithelial cells

In MCF-10A breast epithelial cells, treatment with 10 ng/ml TNF-α increased cell proliferation after 24 h and 48 h. This is a novel finding as the impact of TNF-α on cell proliferation in these, or other breast epithelial cell lines, has not been described previously. In rat mammary epithelial cells however TNF-α treatment increased proliferation (Ip *et al.*, 1992, Varela *et al.*, 2001). Freshly prepared rat mammary

[4] This conclusion is drawn from additional results presented by Pusztai and colleagues (1993).

epithelial cells were isolated, incubated in primary culture and treated with TNF-α for 21 days. Cell growth and morphogenesis were significantly increased compared to control cells (Varela *et al.*, 2001). The here presented results indicate that TNF-α treatment has a substantial and sustained effect on promoting cell proliferation in MCF-10A cells. A further investigation should include extending the treatment time beyond the 48 h mark to determine, if cell proliferation is further increased by a single dose of TNF-α in order to investigate, if TNF-α can permanently increase proliferation. This would indicate that TNF-α is not only a mitogen for these cells, but might affect a permanent change to increase proliferation, which is a feature of cancer cells. These findings indicate that TNF-α is an important factor in contributing to the increased risk of breast cancer in obese postmenopausal women and may contribute to breast cancer aetiology.

In MCF-10A breast epithelial cells, 10 ng/ml TNF-α treatment did not change AKT-phosphorylation significantly between 5 min and 20 min treatment time. All treatment times however indicate a non-significant increase, including AKT-phosphorylation being 77% higher after 20 min treatment with 10 ng/ml TNF-α compared to untreated control (Figure 5-2, C). Further repetition of the experiment may reduce the large standard error, which contributes to the observed non-significance of the obtained results. Thus from the results presented here, it cannot be concluded that TNF-α signalling involves AKT-phosphorylation, however further investigation is warranted. There are no studies that have investigated the change in phosphorylation of AKT after treatment with TNF-α in MCF-10A or other breast epithelial cells.

In MCF-10A breast epithelial cells, 10 ng/ml TNF-α increased phosphorylation of ERK1/2 significantly after between 10 min and 20 min treatment. The highest increase was observed after 10 min of treatment (Figure 5-2, D). No significant differences between treatment time points were detected[5], indicating no significant further increase in activation beyond 10 min treatment. This indicates a sustained activation of at least 10 min and probably beyond. A further experiment could include examining TNF-α induced activation of ERK1/2 beyond the 20 min mark to assess the sustained increase in ERK1/2-phosphorylation with TNF-α treatment in MCF-10A cells, which may support the idea that high TNF-α concentrations permanently change MCF-10A metabolism. With no additional information available on the influence of TNF-α on MCF-10A cells, it could be concluded that MAP-kinase activation may be responsible in mediating TNF-α induced cell proliferation in these cells. Further investigation should include the use of MEK1/2 inhibitors to estimate the contribution the activation of ERK1/2 makes to TNF-α induced cell proliferation in these cells.

In MCF-10A breast epithelial cells, 10 ng/ml TNF-α did not change the distribution of the cell population across cell cycle phases. G1-phase population decreased after TNF-α treatment ($p=0.051$, Figure 5-3, B). A decrease in G1-phase population combined with an increase in S-phase population ($p=0.349$) could be interpreted as an increase in cell cycle progression

Although not a statistical significant observation, this finding complements the cell proliferation findings and MAP-kinase pathway activation, suggesting a significant

[5] Using Tukey's HSD post-hoc test

involvement of TNF-α in promoting cell growth in MCF-10A breast epithelial cells. These initial findings should promote further investigation to validate these results and further the insight of the role of TNF-α on carcinogenic transformation of breast epithelial cells and its potential contribution to the obesity postmenopausal breast cancer link.

5.4.3 Effect of TNF-α treatment on human SK-BR-3 breast cancer cells

In SK-BR-3 breast cancer cells, 10 ng/ml TNF-α treatment increased cell proliferation after 24 h and 48 h of treatment. The highest increase was observed after 48 h treatment (Figure 5-1, C). Similarly, Rivas and colleagues (2009) observed an increase in SK-BR-3 cell proliferation following 20 ng/ml TNF-α treatment for 48 h, using [^{3}H]-thymidine incorporation. The authors also concluded that herceptin treatment was not able to inhibit the cross-activation of HER2 by TNF-α and the resulting increase in cell proliferation[6]. This may suggest an important mechanism of herceptin-resistance and provide a mechanistic link between obesity and herceptin-resistance. The similar findings of the study presented here and previously published results suggest a substantial impact of TNF-α on cell proliferation of SK-BR-3 breast cancer cells.

In SK-BR-3 breast cancer cells, 10 ng/ml TNF-α treatment increased phosphorylation of AKT after 5 min, 10 min and 20 min of treatment. The highest increase in AKT-phosphorylation was observed after 20 min treatment (Figure 5-2, E). No studies are available that investigated the phosphorylation of AKT after treatment with TNF-α

[6] Herceptin is a monoclonal antibody, developed and approved for treatment of HER2 overexpressing breast cancer, of which SK-BR-3 cells are the prime example. Efficacy of herceptin is limited to about 30% of patients, whose tumours overexpresses HER2 (Rivas *et al.*, 2009).

in these cells. Activation of AKT has been linked to activation of proliferation stimulating transcription factor NF-κB in T47D breast cancer cells (Rivas *et al.*, 2008). Thus activation of AKT may be a pathway for TNF-α stimulated cell proliferation in SK-BR-3 breast cancer cells.

In SK-BR-3 breast cancer cells, 10 ng/ml TNF-α treatment significantly increased phosphorylation of ERK1/2 after 5 min of treatment (Figure 5-2, F). The increase is lower than that of AKT-phosphorylation (47% increase in ERK1/2-phosphorylation after 5 min treatment compared to 91% increase in AKT-phosphorylation after 20 min treatment), however, as mentioned in section 3.4.2, the level of ERK1/2-phosphorylation increase does not relate to the level of increase in cell proliferation. Moreover AKT-phosphorylation is stably increased from 5 min treatment onwards, while ERK1/2-phosphorylation returns to baseline after 10 min treatment and remains unaffected at later treatment points. Thus the MAP-kinase pathway may not play as significant a role in mediating TNF-α intra-cellular signalling in SK-BR-3 cells as the PI3-kinase cell signalling pathway does.

In SK-BR-3 breast cancer cells, 10 ng/ml TNF-α treatment significantly decreased S-phase and G2-phase population after 24 h treatment. As compensation the subG1 population increased, however this increase was not significant (Figure 5-3, C). A similar result was observed for T47D breast cancer cells, where S-phase and G2-phase but not G1-phase populations decreased after treatment with 10 ng/ml TNF-α for 24 h (Pusztai *et al.*, 1993). These authors concluded that TNF-α induced cell cycle arrest at the G1/S cell cycle checkpoint (Pusztai *et al.*, 1993). If this is applicable to SK-BR-3

cells, then earlier findings showing an increase in cell proliferation (Figure 5-1, C) and significant increases in cell signalling pathways (Figure 5-2, E, F) with 10 ng/ml TNF-α treatment are not supported by this finding. These interpretations suggest a pleiotropic effect of TNF-α on these cells, both increasing cell proliferation and cell cycle arrest. An alternative suggestion, in line with a growth promoting action of TNF-α on SK-BR-3 cells, would be that TNF-α specifically increased cell cycle progression across the G2/M cell cycle checkpoint, thus increasing the number of cells entering mitosis. With an intact G1/S cell cycle checkpoint, one would imagine a decrease in S-phase and G2-phase populations, just as observed after TNF-α treatment. As a result, one would also expect an increase in G1-phase population where cells would accumulate after undergoing mitosis, this was however not observed. Thus this alternative explanation is not fully supported by the here presented findings.

5.4.4 Rationale for the approach

The rationale for the chosen experimental methodology is the same as explained in section 3.4.5.

The TNF-α concentration used in our experiments (10 ng/ml) was within the range (0.1 ng/ml-200 ng/ml) used by Rivas and colleagues (2008). Physiological circulating concentrations of TNF-α in obese (mean BMI 30.4 kg/m^2 ± 1.3 kg/m^2) postmenopausal (mean age 49.0 years ± 5.3 years) breast cancer patients were 22.0 pg/ml ± 9.77 pg/ml (Gonullu *et al.*, 2005). Thus the used TNF-α concentration was about 45-times higher than physiological concentrations. This concentration was similarly above physiological concentrations as leptin in the previous chapter. It was decided to concentrate on a

single TNF-α concentration rather than a range in order to examine a number of possible changes and get an initial overview of the potential action of TNF-α on the cell culture system. The TNF-α concentration used for the study presented here significantly increased cell proliferation in T47D breast cancer cells (Rivas *et al.*, 2008).

5.5 Summary

Across the three cell lines (MDA-MB-231 breast cancer cells, MCF-10A breast epithelial cells, SK-BR-3 breast cancer cells) and the four tested variables (cell proliferation, AKT-phosphorylation, ERK1/2-phosphorylation, cell cycle profile) it may be suggested that MCF-10A breast epithelial cell and SK-BR-3 breast cancer cells seem to be affected similarly by TNF-α treatment. The effect on MDA-MB-231 breast cancer cells on the other hand was different. This suggests that TNF-α is a pleiotropic cytokine, with different effects on specific cell types. It remains an interesting fact that the two breast cancer cell lines show very different responses, *i.e.* results that can be interpreted both as growth promoting and growth inhibiting, to TNF-α treatment, while MCF-10A cells react more uniformly, *i.e.* growth promoting, to this treatment.

Cell proliferation was not influenced by TNF-α treatment in MDA-MB-231 cell, but increased similarly in MCF-10A cells and SK-BR-3 cells. MDA-MB-231 may naturally have a resistance to growth promoting abilities of TNF-α. No studies are available on the TNF-R in these cells, thus MDA-MB-231 cells may have renounced the growth promoting effects TNF-R expression in favour of protection from its apoptosis promoting effect. This however may not be a valid explanation as the results from the cell cycle experiments (see section 5.3.3) indicate an increase in apoptosis. Thus the

effect of TNF-α on cell proliferation is cell specific and is not limited to either breast epithelial or breast cancer cells.

AKT-phosphorylation was significantly increased in SK-BR-3 cells only, the high standard error of the mean between the experiments performed for the MCF-10A cells however makes the true insignificance of the observed findings doubtful. There was no indication that TNF-α treatment increased AKT-phosphorylation in MDA-MB-231 cells. Thus TNF-α induced AKT-phosphorylation may affect the cells similarly as TNF-α induced cell proliferation. AKT activation following TNF-α treatment has been linked to increased activity of NF-κB transcription factor, known to increase transcription of cell proliferative genes (Rivas *et al.*, 2008). This could thus be the main cell signalling pathway by which TNF-α increases cell proliferation in SK-BR-3 cells and maybe in MCF-10A breast epithelial cells. Further investigations using PI3-kinase inhibitor wortmannin could provide an insight into the exact mechanisms.

ERK1/2-phosphorylation was significantly increased in MCF-10A and SK-BR-3 cells. Only MCF-10A cells however exhibited a sustained increase in ERK1/2-phosphorylation, while SK-BR-3 cell's ERK1/2-phosphorylation returned to baseline after 10 min TNF-α treatment. In MDA-MB-231 breast cancer cells, TNF-α initiated a significant decrease in ERK1/2-phosphorylation after 5 min treatment, possibly indicating a growth inhibitory effect. If cell proliferation in MCF-10A cells is indeed not mediated by the PI3-kinase pathway, it is promoted most certainly by the MAP-kinase pathway. Hence further investigations using MEK1/2 inhibitors (U0126 or

PD98059) could provide further insight into the mechanistic of intra-cell mediation of proliferative signals from TNF-α in MCF-10A cells.

In line with the decrease in ERK1/2-phosphorylation MDA-MB-231 breast cancer cells show the highest change in cell population distribution across cell cycle stages with TNF-α treatment, indicating an apoptotic effect of TNF-α on these cells. Also SK-BR-3 cells exhibit a decrease in cell cycle progression after TNF-α treatment. Cell cycle distribution in MCF-10A cells on the other hand is not affected by TNF-α treatment. In fact the near-significant decrease ($p=0.051$) in the G1-phase population further supports the conclusion that TNF-α acts highly prolific on these cells. Interestingly the results presented here may suggest that TNF-α arrests cell cycle at the G2/M cell cycle checkpoint in the MDA-MB-231 breast cancer cell line and at the G1/S cell cycle checkpoint in SK-BR-3 breast cancer cells. The result on cell cycle arrest is the same, it emphasises however the different effect that TNF-α has on the two examined breast cancer cells. The finding that SK-BR-3 cells show a cell cycle arrest after TNF-α treatment remains in contradiction to the other observed findings of increased proliferation and increased activation of cell signalling pathways following TNF-α treatment.

In conclusion, TNF-α affects breast cancer cells and breast epithelial cells. Each examined cell line however exhibited an individual response to TNF-α treatment. In MDA-MB-231 cells TNF-α seems to exert no effect or a slightly growth inhibiting and apoptosis inducing effect. In MCF-10A breast epithelial cells, TNF-α increases cell proliferation, with no indication of cell cycle arrest or onset of apoptosis. SK-BR-3 cells

show no conclusive growth promoting or apoptotic reactions to TNF-α treatment. TNF-α treatment increased cell proliferation and cell signalling pathway activation, but also indicates increased cell cycle arrest. Thus TNF-α may contribute to the obesity breast cancer link, but its exact mechanistic effect on mediating breast cancer aetiology or breast cancer progression remains elusive.

Chapter 6
Adiponectin and Interleukin-6

6 ADIPONECTIN AND INTERLEUKIN-6

In this chapter the effect of two additional adipokines, adiponectin and IL-6 on cell proliferation is presented. It is the result of the intention to investigate several molecular markers that may potentially be involved in contributing to the link between obesity and postmenopausal breast cancer. It was aimed to determine the effect of adiponectin and high IL-6 concentrations on cell proliferation in MDA-MB-231 breast cancer cells, MCF-10A breast epithelial cells and SK-BR-3 breast cancer cells.

6.1 Introduction

6.1.1 Adiponectin

Adiponectin is a 26 kDa adipocyte-derived polypeptide. At the time of writing, adipocytes are the only source for circulating adiponectin. Physiologically adiponectin appears in two configurations: either as a trimer, known as low-molecular weight (LMW) adiponectin or in polymers of up to 18 adiponectin molecules, termed high-molecular weight adiponectin (HMW). It has been suggested that HMW adiponectin may be more important in mediating the effects of adiponectin than total adiponectin (Inoue *et al.*, 2007; Arita *et al.*, 1999). Different from the majority of other adipokines, adiponectin is negatively correlated with body weight and BMI, *i.e.* as BMI increases, adiponectin concentrations decrease. In normal weight individuals the mean circulating adiponectin level was 8.9 μg/ml, while it was reduced to 3.7 μg/ml adiponectin in obese individuals (Arita *et al.*, 1999[1]). These authors also demonstrated that adiponectin concentrations decrease exponentially with increased BMI. Individuals

[1] This study is inconsistent in labelling adiponectin concentration and the authors described the adiponectin concentrations in the text as mg/ml, μg/ml and ml/ml. However citing Arita and colleagues' (1999) study, Arditi and colleagues (2006) confirmed adiponectin concentrations were in μg/ml.

with decreased adiponectin concentrations were found to have an increased risk of developing breast cancer, independent of BMI (Mantzoros *et al.*, 2004). Moreover low adiponectin concentrations were also found in patients with more severe types of breast cancer as well as more advanced breast cancer (Miyoshi *et al.*, 2003). Thus decreased concentrations of adiponectin, associated with obesity, could explain the increased breast cancer risk in obese postmenopausal women. Conversely high concentrations of adiponectin may decrease proliferation, induce cell cycle arrest or increase apoptosis of breast cancer cells.

Adiponectin treatment of breast cancer cell lines has generally resulted in decreased proliferation, supporting the above assertion. A growth inhibitory effect of adiponectin however was only observed at low adiponectin concentrations, far below the physiological concentrations. Proliferation was decreased in MDA-MB-231 breast cancer cells after treatment with 25 ng/ml adiponectin for 24 h. Interestingly 250 ng/ml adiponectin or 500 ng/ml adiponectin treatment for 24 h did not significantly decrease cell proliferation (Dos Santos *et al.*, 2008). These authors suggested that the adiponectin receptor is degraded quicker at adiponectin concentrations >25 ng/ml and thus decreasing intra-cellular adiponectin signalling. Grossmann and colleagues (2008) examined the influence of 50-5000 ng/ml adiponectin on five breast cancer cell lines. Cell proliferation, measured using the MTT cell viability test after 48 h treatment, decreased in MCF-7, T47D and SK-BR-3 breast cancer cells at 500 ng/ml and higher, but not in MDA-MB-231 or MDA-MB-361 breast cancer cells at up to 5000 ng/ml (Grossmann *et al.*, 2008). Thus the growth inhibitory effects of adiponectin on MDA-MB-231 breast cancer cells were not universally observed. In addition induction

of apoptosis was observed after treatment of MDA-MB-231 breast cancer cells with adiponectin (Kang *et al.*, 2005). Others however did not observe an increase of apoptosis in MDA-MB-231 breast cancer cells after adiponectin treatment, instead reported growth inhibitory effects (Nakayama *et al.*, 2008). These authors measured cell cycle progression with 10 μg/ml adiponectin after 24 h, 48 h and 72 h treatment finding continuous increases in G1-phase populations (Nakayama *et al.*, 2008). Two other studies found growth inhibitory effects after adiponectin treatment in MCF-7 breast cancer cells (Dieudonne *et al.*, 2006; Arditi *et al.*, 2007). Dieudonne and colleagues (2006) also observed an induction of apoptosis in MCF-7 breast cancer cells after 96 h treatment with 250 ng/ml adiponectin, using DNA-fragmentation, while Arditi and colleagues (2007) did not observe increase apoptosis with 15 μg/ml adiponectin for 48 h.

6.1.2 Interleukin-6

Interleukin-6 (IL-6) is a 24 kDa inflammatory cytokine. It is primarily produced by macrophages and T-cells to stimulate inflammation and initiate an immune response, especially in the event of burns and other trauma. Conversely, its pleiotropic function is seen by the observation that IL-6 can also mediate an anti-inflammatory effect (Pedersen *et al.*, 2003). IL-6 however acted as anti-inflammatory only as a result of increased IL-6 myocyte expression following exercise and not adipocyte derived IL-6. Thus, while short-term IL-6 secretion may be beneficial, the chronic elevation of IL-6 circulating concentrations in the obese may promote some of the obesity-associated metabolic disorders, including increased susceptibility to breast cancer (Gonullu *et al.*, 2005).

Concordantly, adipocyte-derived IL-6 stimulated cell motility and invasion in MCF-7 and MDA-MB-231 breast cancer cells (Walter *et al.*, 2009). On the other hand, another study, where the authors treated T47D breast cancer cells with 10 ng/ml IL-6 for 10 d, observed a decrease in the number of colonies formed after treatment compared to equally long incubated control cells, indicating a decrease in cell proliferation (Badache and Hynes, 2001). Interestingly, in MCF-7 breast cancer cells treatment with 10 ng/ml IL-6 not only promoted growth, but also triggered IL-6 secretion by MCF-7 breast cancer cells themselves, indicating that IL-6 may increase growth and, as a subsequent or independent mechanism, initiate a self-sustained autocrine loop of IL-6 promoted cell growth (Conze *et al.*, 2001; Sansone *et al.*, 2007). This may point to a different mode of action of IL-6 on breast cancer growth, compared to other adipokines.

6.2 Materials and Methods

6.2.1 Cell lines and treatment with adiponectin or interleukin-6

MDA-MB-231, MCF-10A and SK-BR-3 cells were cultured and treated as described in Materials and Methods sections 2.1.1 and 2.1.2. Formulation of adiponectin and IL-6 treatment media and final treatment concentrations are described in Materials and Methods sections 2.1.3.4 and 2.1.3.5, respectively.

6.2.2 Cell proliferation assay

The cell proliferation assay was performed as described in section 3.2.2, but with 250 ng/ml or 500 ng/ml adiponectin or 10 ng/ml IL-6 as treatment medium, with the additional exception that for a 48 h experiment, 10 μM/well BrdU was added at the beginning of the 48 h incubation instead of only for the last 24 h. There were no alterations for a 24 h experiment. Three experiments were performed for each treatment, each cell line and each time point. Each experiment consisted of six replicates for each treatment, *i.e.* six wells for control and six wells for treatment.

6.3 Results

6.3.1 Effect of adiponectin treatment on cell proliferation

Cell proliferation of all cell lines was assessed after 250 ng/ml adiponectin and 500 ng/ml adiponectin treatment for 24 h or 48 h. Untreated control cells were incubated in serum-free medium (Control) at all times. All results are presented as percentage change from this control (Figure 6-1).

In MDA-MB-231 breast cancer cells, 250 ng/ml adiponectin treatment did not significantly change cell proliferation after 24 h or 48 h treatment. Treatment with 500 ng/ml adiponectin did not significantly change cell proliferation after 24 h or 48 h treatment. A non-significant 5% decrease in cell proliferation (p=0.077) after 250 ng/ml adiponectin treatment for 48 h was the highest change observed (Figure 6-1, A).

In MCF-10A breast epithelial cells, 250 ng/ml adiponectin treatment did not significantly change cell proliferation after 24 h or 48 h. Likewise treatment with 500 ng/ml adiponectin did not significantly change cell proliferation after 24 h or 48 h treatment. A non-significant 9% decrease in cell proliferation (p=0.210) after 500 ng/ml adiponectin treatment for 48 h was the highest change observed (Figure 6-1, B).

In SK-BR-3 breast cancer cells, 250 ng/ml adiponectin treatment decreased cell proliferation significantly by 17% after 24 h treatment (p=0.028), but no significant change in cell proliferation was observed after 48 h treatment. Treatment with 500 ng/ml adiponectin decreased cell proliferation significantly by 17% after 24 h

treatment ($p=0.024$), but no significant change in cell proliferation after 48 h of treatment was observed (Figure 6-1, C).

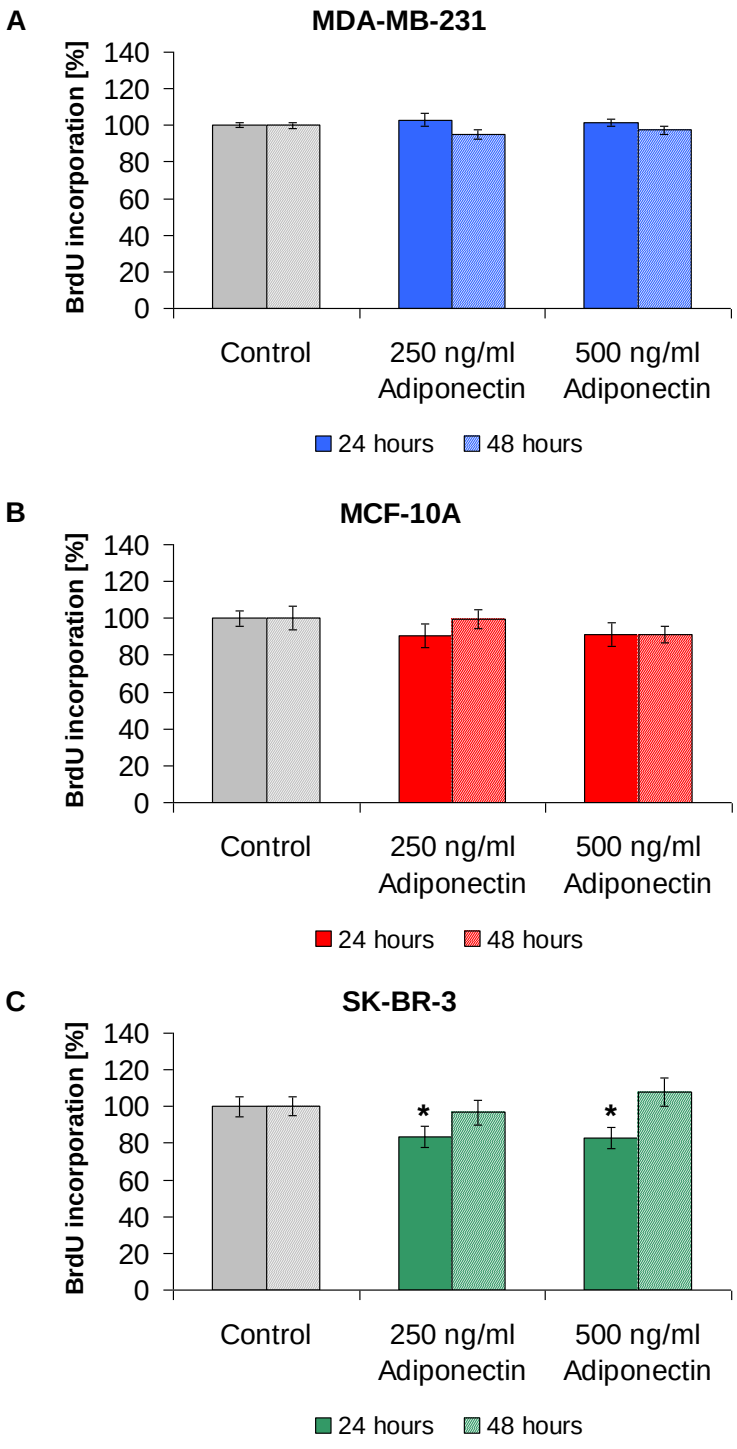

Figure 6-1: Changes in cell proliferation after treatment with 250 ng/ml or 500 ng/ml Adiponectin for 24 h or 48 h in A) MDA-MB-231 breast cancer cells (blue), B) MCF-10A breast epithelial cells (red) and C) SK BR-3 breast cancer cells (green). Bars represent BrdU-incorporation in relation to the respective control within each graph and are expressed as a percentage thereof. Error bars represent ± SEM of three experiments, each consisting of six replicates, *i.e.* 18 data points for each bar.
* Significance value compared to control, obtained using One-way ANOVA analysis. (* 0.05>p>0.01)

6.3.2 Effect of Interleukin-6 on cell proliferation

Cell proliferation of all cell lines was assessed after 10 ng/ml IL-6 treatment for 24 h or 48 h. Untreated control cells were incubated in serum-free medium (Control) at all times. All results are presented as percentage change from this control (Figure 6-2).

In MDA-MB-231 breast cancer cells, 10 ng/ml IL-6 treatment increased cell proliferation significantly by 4% after 24 h treatment ($p=0.045$), but not after 48 h of treatment compared to control (Figure 6-2, A).

In MCF-10A breast epithelial cells, 10 ng/ml IL-6 treatment did not significantly change cell proliferation after 24 h treatment, but increased cell proliferation significantly by 38% after 48 h of treatment ($p<0.001$) compared to control (Figure 6-2, B).

In SK-BR-3 breast cancer cells, 10 ng/ml IL-6 treatment did not change cell proliferation after 24 h or 48 h. The highest change observed was a non-significant 16% decrease in proliferation after 24 h of treatment ($p=0.105$) compared to control (Figure 6-2, C).

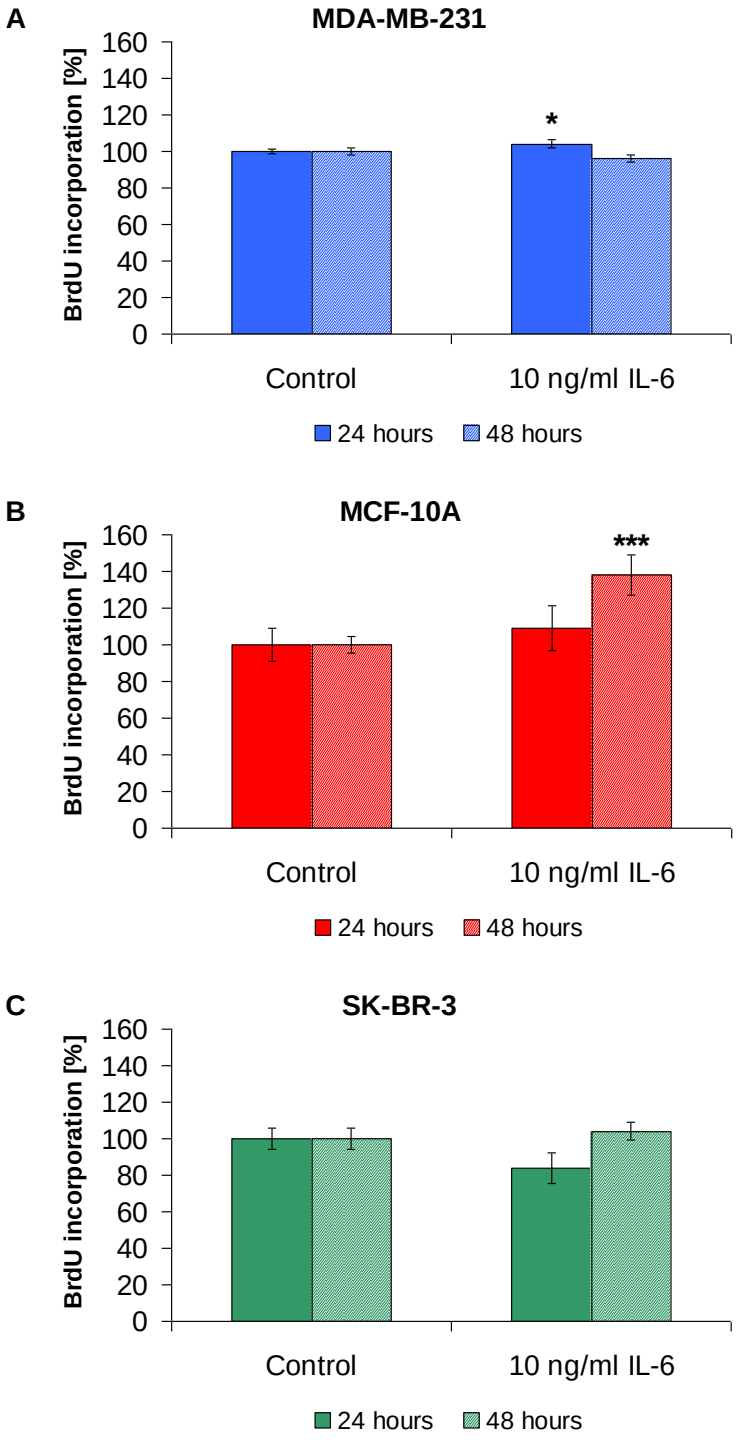

Figure 6-2: Changes in cell proliferation after treatment with 10 ng/ml IL-6 for 24 h or 48 h in A) MDA-MB-231 breast cancer cells (blue), B) MCF-10A breast epithelial cells (red) and C) SK BR-3 breast cancer cells (green). Bars represent BrdU-incorporation in relation to the respective control within each graph and are expressed as a percentage thereof. Error bars represent ± SEM of three experiments, each consisting of six replicates, *i.e.* 18 data points for each bar.
* Significance value compared to control, obtained using One-way ANOVA analysis.
(* $0.05>p>0.01$; *** $p<0.001$)

6.4 Discussion

6.4.1 Effect of adiponectin on cell proliferation

In MDA-MB-231 breast cancer cells, adiponectin treatment with either concentration (250 ng/ml or 500 ng/ml) did not change cell proliferation after 24 h or 48 h treatment (Figure 6-1, A). Thus the results do not indicate that adiponectin may induce a growth inhibiting effect on these cells. Similarly non-significant changes in cell proliferation, using [^{3}H]-thymidine incorporation, were observed when MDA-MB-231 breast cancer cells were treated by 250 ng/ml or 500 ng/ml adiponectin for 24 h (Dos Santos *et al*., 2008). Assessment of the adiponectin receptor led the authors to suggest that high (>250 ng/ml) adiponectin concentrations may decrease normal adiponectin signalling, since they observed a significant decrease adiponectin receptor expression at this concentration (Dos Santos *et al.*, 2008). Thus their study may provide an explanation for the results observed in the study presented here, as the same adiponectin concentrations were used. This study (Dos Santos *et al.*, 2008) however incubated cells for 48 h in medium containing 5% FCS, which could affect the cells differently prior to treatment than the protocol used in the study presented here, where no FCS supplementation was used for incubation in serum-free medium. Conversely higher concentrations of adiponectin, than the ones used in the study presented here, induced cell cycle arrest and apoptosis. Treatment of MDA-MB-231 breast cancer cells with 10 µg/ml and 30 µg/ml adiponectin decreased cell proliferation after 24 h, 48 h and 72 h treatment, using cell proliferation reagent WST-1, which works similar to MTT (Nakayama *et al.*, 2008). The same study also observed no difference in proliferation with 0.1 µg/ml or 1 µg/ml adiponectin, thus in fact supporting the here presented findings. These authors however did not incubate in serum-free medium prior to

adiponectin treatment (Nakayama *et al.*, 2008). Incubating MDA-MB-231 breast cancer cells in serum-free medium for 18-24 h prior to 48 h adiponectin treatment, Grossmann and colleagues (2008) observed no changes in cell proliferation of with adiponectin concentrations between 50 ng/ml and 5000 ng/ml, using the MTT-assay. Previous findings of cell proliferation in adiponectin treatment employed very different protocols and treatment concentrations. In the study presented here no changes in cell proliferation at 250 ng/ml and 500 ng/ml adiponectin was observed. Using different methods all other studies agreed with this finding at these adiponectin concentrations. At both lower and higher concentrations however adiponectin decreased proliferation in MDA-MB-231 breast cancer cells, thus suggesting a complex system of interaction between adiponectin and cell proliferation in MDA-MB-231 breast cancer cells.

In MCF-10A breast epithelial cells, adiponectin treatment with either concentration (250 ng/ml or 500 ng/ml) did not change cell proliferation after 24 h or 48 h treatment (Figure 6-1, B). Cell proliferation in MCF-10A cells was decreased compared to control after treatment with 10 µg/ml adiponectin for 4 and 6 days but not after 3 days (Treeck *et al.*, 2008). This indicates that adiponectin may affect MCF-10A breast epithelial cells only after a longer incubation period and at a higher adiponectin concentration than the ones used in the study presented here. The long incubation period used by Treeck and colleagues (2008) however may create some problems with cell growth, as these authors used 2500 cells/well in a 96-well plate for up to 6-days in serum-supplemented growth medium (by comparison the study presented here uses 5000 cells/well in serum-free medium). By comparison in the study presented here, 4×10^5 MCF-10A cells were able to grow to full confluence in 75 cm^2 flasks in 4 days in growth medium, as observed

during routine cell maintenance. Thus there might be some uncertainties in the findings of Treeck and colleagues (2008). The here presented results do not indicate that cell proliferation of MCF-10A cells is decreased with adiponectin treatment.

In SK-BR-3 breast cancer cells adiponectin treatment decreased cell proliferation after 24 h with either concentration of 250 ng/ml or 500 ng/ml adiponectin (Figure 6-1, C). Similarly, Grossmann and colleagues (2008) observed a decrease in cell proliferation after 48 h of treatment with 500 ng/ml adiponectin in these cells. Pfeiler and colleagues (2008) however did not observe a change in cell proliferation in these cells following treatment with up to 15 µg/ml adiponectin for up to five days (Pfeiler *et al.*, 2008). Thus it seems that lower adiponectin concentrations rather than high concentrations may be able to decrease cell proliferation in SK-BR-3 breast cancer cells, which is surprising as it is generally thought that high adiponectin concentrations should increase growth inhibition more than low concentrations, given the inverse relation of adiponectin concentrations and BMI.

The large variety of adiponectin concentrations used in the literature does not seem to cause concern by the authors of these studies. They also did not justify the adiponectin concentration they used. Thus it will presumably take a number of additional studies to find a consensus, at which concentrations adiponectin shows its highest inhibitory effect.

6.4.2 Effect of IL-6 on cell proliferation

In MDA-MB-231 breast cancer cells, IL-6 treatment increased cell proliferation significantly after 24 h (Figure 6-2, A). By comparison leptin increased cell proliferation by 21% after 24 h of treatment (see section 4.3.1). Further repetition of the experiment or employment of additional methods of measuring cell proliferation might be needed to evaluate the statistical significance of this finding. Even if the statistical significance was to be established its physiological impact is probably negligible. By comparison the only other study that examined the effect of IL-6 on MDA-MB-231 breast cancer cells, did not observe any change in cell proliferation, as measured by cell count of viable cells using Trypan Blue staining[2], after treatment with 100 ng/ml IL-6 for 24 h or 6 days (Asgeirsson *et al.*, 1998). Thus there is probably no effect of IL-6 on cell proliferation in MDA-MB-231 breast cancer cells.

In MCF-10A breast epithelial cells, IL-6 treatment increased proliferation after 48 h treatment (Figure 6-2, B), suggesting a proliferative effect of IL-6 on these cells. In one study, MCF-10A breast epithelial cells were grown in "conditioning medium", *i.e.* medium in which breast cancer cells had been growing previously and which was now used to cultivate MCF-10A breast epithelial cells (Lieblein *et al.*, 2008). Cell proliferation was then assessed using the MTT-assay and was found to be increased. Additional incubation of MCF-10A cells with a specific IL-6 antibody inhibited this observed increase in proliferation, suggesting that breast cancer cell derived IL-6 acted mitogenic on MCF-10A breast epithelial cells (Lieblein *et al.*, 2008). Furthermore these

[2] Trypan Blue is a dye staining only the nuclear membrane, not the cell membrane. It can also not diffuse past the cell membrane. Consequently cells that appear blue after staining possess a corrupt cell membrane, indicative of dead or necrotic cells. Therefore these cells are discounted as non-viable and only non-stained cells are defined as viable.

authors observed an increase in IL-6 secretion from MCF-10A cells after incubation in conditioning medium (Lieblein *et al.*, 2008) (see sections 1.5.3.4 and 8.5). Additionally, using MTT analysis a 64% increase in cell proliferation of MCF-10A breast epithelial cells with 10 ng/ml IL-6 for 5 days was observed (Basolo *et al.*, 1993). These authors also observed IL-6 receptor expression in MCF-10A breast cancer cells. These authors did however not comment on the significance of this finding and it could not be assessed, if the authors had performed a statistical test on these results. This is understandable as the aims of their study were to assess the impact of IL-6 on cyclin D expression primarily and cell proliferation changes of normal MCF-10A cells were used only as control (Basolo *et al.*, 1993). Thus it might well be that IL-6 treatment had a significant effect on cell proliferation in this study and these results would thus support the results observed in the study presented here. IL-6 may therefore be an interesting candidate for the connection of obesity and breast cancer aetiology.

In SK-BR-3 breast cancer cells, IL-6 treatment did not change cell proliferation after 24 h or 48 h treatment (Figure 6-2, C). There are no comparable studies, describing changes of cell proliferation in SK-BR-2 breast cancer cells[3]. Thus there seems to be no indication that IL-6 may be involved in promoting cell proliferation in SK-BR-3 breast cancer cells.

There are indications that IL-6 activates the PI3-kinase and MAP-kinase cell signalling pathways in T47D cells (Badache and Hynes, 2001). Thus it would be interesting to

[3] Only one study was obtained treating SK-BR-3 breast cancer cells with 10 ng/ml IL-6 (Honma *et al.*, 2002). However these authors did not examine cell proliferation and only reported on an increase in aromatase activity after treatment.

investigate the activation of cell signalling pathways in response to adiponectin or IL-6 exposure. Time-constraints have however not permitted to investigate such a connection.

In the study presented here, TNF-α was preferred over IL-6 for further investigation as IL-6 lacked the ability to increase proliferation in either breast cancer cells. IL-6 however is the only factor that has been observed to induce an autocrine loop of cell proliferation[4] (Sansone *et al.*, 2007). In this study, MCF-7 breast cancer cells, which did not secrete IL-6 at baseline, were treated with 10 ng/ml IL-6 for 24 h, after which the cytokine was withdrawn and the cells continued to grow. Two weeks after treatment, MCF-7 cells produced IL-6 themselves and displayed a highly invasive phenotype. If the cells were treated with an IL-6 antibody, this effect was not observed, indicating that this increase in invasiveness was IL-6 dependent. Thus a short-term challenge with IL-6 triggered a sustained IL-6 driven autocrine loop in these cells, which greatly increased their malignancy (Sansone *et al.*, 2007).

6.4.3 Rationale for the approach

Together with oestrogen, insulin, leptin and TNF-α, adiponectin and IL-6 are the other factors identified as most likely to mediate the obesity-postmenopausal breast cancer link (Housa *et al.*, 2006; Lorincz and Sukumar, 2006). Therefore the influence of these two adipokines on cell proliferation in MDA-MB-231 breast cancer cells, MCF-10A breast epithelial cells and SK-BR-3 breast cancer cells was examined. Different from the other adipokines used in the study presented here, adiponectin concentrations

[4] The idea of a self-sustained autocrinic loop is hypothesised in section 8.5

(250 ng/ml and 500 ng/ml) were below the physiological concentrations identified (3 μg/ml-30 μg/ml; Heliovaara *et al.*, 2006). The chosen adiponectin concentrations were chosen as they were found to decrease cell proliferation in MCF-7, T47D and SK-BR-3 breast cancer cells (Dieudonne *et al.*, 2006; Arditi *et al.*, 2007, Grossmann *et al.*, 2008). Subsequently Dos Santos and colleagues (2008) observed that adiponectin decreased cell proliferation in MDA-MB-231 breast cancer cells incubated in serum-free medium only at concentrations <25 ng/ml. Higher concentrations led to a decrease in adiponectin receptor protein expression and the authors suggested that consequently adiponectin signalling was inhibited (Dos Santos *et al*., 2008).

Serum IL-6 concentrations in obese (mean BMI=35.6 kg/m^2) patients with metabolic syndrome (according to IDF criteria) was 3.2 pg/ml (Gnacinska *et al*., 2010). IL-6 concentrations used in the study presented here (10 ng/ml) were about 312-times higher than physiological concentrations, which was in accordance with the used insulin concentrations. The chosen IL-6 concentration was used in one study, where cell proliferation in T47D breast cancer cells decreased (Badache and Hynes, 2001).

Chapter 7
Insulin part 2

7 INSULIN PART 2

In this chapter the results of additional experiments after 100 nM insulin treatment are presented. Several additional methods to examine cell proliferation and cell signalling pathway activation were attempted to confirm findings presented in chapter 3. Furthermore several additional experiments, *i.e.* measurement of apoptosis and gene expression changes, are presented. It was aimed to increase the understanding of the contribution insulin may make on the link between obesity and postmenopausal breast cancer. Therefore the effect of high insulin concentrations on cell proliferation, cell signalling pathway activation, apoptosis and gene expression changes in MDA-MB-231 breast cancer cells, MCF-10A breast epithelial cells and SK-BR-3 breast cancer cells was examined.

7.1 Introduction

In chapter 3 it was suggested that high insulin concentration may affect breast cancer progression or aetiology and thus play a role in the molecular mechanism linking obesity and postmenopausal breast cancer (see section 3.4). It was observed that only in non-cancerous MCF-10A breast epithelial cells, insulin treatment increased cell proliferation, when measured using the BrdU incorporation assay. In order to validate these findings, several additional methods to estimate changes in cell proliferation were employed in the study presented here. The first of which was employment of the MTT-assay, which assesses the metabolic rate of cells measuring the turn-over rate of MTT, by mitochondrial reductases[1]. A comparison study evaluating the BrdU- and MTT-assays found high correlation between the two assays (Wagner *et al.*, 1999),

[1] See materials and methods section 2.2.2 for further particulars on this assay.

despite the similarity in these results and the similarity in the methodological approach however, it should be emphasised that what is measured in these assays is very different (DNA-replication vs. mitochondrial metabolism). The second additional method measured the protein expression of two protein markers, whose expression is positively correlated to cell proliferation. Proliferating cell nuclear antigen (PCNA) is a protein involved in DNA replication, linking DNA polymerases to a DNA strand. PCNA is expressed in proliferating cells only, thus its expression can be used to assess cell proliferation in response to insulin treatment. Similarly a newly synthesised mouse monoclonal antibody, JC-1, recognises a nuclear antigen present only in proliferating cells and absent in resting cells (Garrido *et al.*, 1992). Monoclonal antibodies against PCNA and the JC-1 antibody, as part of Western Blotting, were used to assess proliferation after insulin treatment in the cell culture model.

In chapter 1 it was hypothesised that any intra-cellular signalling initiated by insulin in breast cancer cells will utilise the MAP-kinase cell signalling pathway in order to exert a proliferative response, which could be the underlying mechanism in the link between obesity and breast cancer (see section 1.6.2 and 1.7). In chapter 3 it was observed that insulin treatment increased phosphorylation of ERK1/2, a member of the MAP-kinase cell signalling pathway, in MDA-MB-231 breast cancer cells and MCF-10A breast epithelial cells, but decreased ERK1/2-phosphorylation in SK-BR-3 breast cancer cells (section 3.3.3.1). Phosphorylation of ERK1/2 in response to insulin treatment was therefore also assessed using antibodies specific for phosphorylated ERK1/2 and total ERK1/2 in MDA-MB-231 breast cancer cells after treatment with 100 nM insulin.

Apoptosis is the process to initiate cell death after recognition of an apoptotic signal, either extra- or intra-cellular. Recognition of irreparable DNA damage for example is one of the intra-cellular signals inducing apoptosis. Aberrant genome restructuring is a notable event in cancer progression and in order to sustain cancer survival, circumvention of apoptosis-initiating and -mediating factors has to be accomplished. Certain proteins involved in initiating and mediating apoptosis are known as tumour suppressors, the most well-known of these is probably TP53, which has been observed to be inactivated in almost 50% of all cancers (Harris and Hollstein, 1993). Other than disrupting the apoptotic signalling, cancers may evade apoptosis by increased expression and activation of negative regulators of apoptotic signalling, *i.e.* BCL-2. Using this latter mechanism apoptosis can be suppressed by endocrine factors such as insulin (Gupta *et al.*, 2002). Thus an additional mechanism by which insulin may promote cancer aetiology and progression is suppression of apoptosis.

In order to assess the change in apoptosis in the cell culture model after insulin treatment, two investigative methods were employed. The first used flow cytometry to detect phosphatidylserine (PS) on the cell surface. In healthy cells, PS is located at the cytosolic side of the cell membrane. During apoptosis initiation, this asymmetry is lost and PS is exposed on both sides of the cell membrane. Annexin-V is a phospholipid binding protein with high affinity to PS. In the utilised assay, Annexin-V is conjugated with FITC a fluorescent dye. In addition the used assay also contains 7-AAD dye, a fluorescent dye, which stains the nuclear membrane, but not the cell membrane (similarly to Trypan Blue, as explained in footnote 2 in chapter 6). Thus only cells with compromised cell membranes, as present in late apoptotic and necrotic cells, will

contain high 7-AAD staining. Detection of these two fluorochromes allows assessment of early apoptotic (Annexin-V) and late apoptotic or necrotic cells (7-AAD). The second method examines the gene expression of BCL-2, an anti-apoptotic protein. The exact mechanism of BCL-2's anti-apoptotic properties is still being evaluated, a role in the activity of the mitochondrial apoptosis-induced channel however has been suggested (Dejean *et al.*, 2006). Given its negative correlation with apoptosis an increase in BCL-2 expression would correlate to a decrease in apoptotic rate.

The downstream events of both PI3-kinase and MAP-kinase cell signalling pathways include the activation of transcription factors. While the role of AKT and PI3-kinase in the activation of transcription has not been fully evaluated (Scheid and Woodgett, 2001b), ERK1/2 itself is a transcription factor. In addition ERK1/2-phosphorylation also activates several other transcription factors, including ribosomal protein S6 (Lenormand *et al.*, 1996) and CREB (Sato *et al.*, 1997). As such it would be expected that activation of these pathways should result in changes in gene expression and these changes may be responsible for mediating any potential observations of increased cell proliferation, inhibition of apoptosis or cell cycle progression. A microarray analysis allows to examine the expression of a number of genes in a given cell sample. Here the results of a microarray analysis after treatment with 100 nM insulin are presented. The chosen microarray examined the expression of 114 genes, thought to be involved in progression and aetiology of cancer. As such the manufacturer (SABiosciences) called this particular array their Human Cancer PathwayFinder™ array. The manufacturer groups the examined genes in six functional groupings. The groups describe genes involved in: 1) cell cycle control and DNA damage repair, 2) apoptosis and cell

senescence, 3) signal transduction molecules and transcription factors, 4) adhesion, 5) angiogenesis and 6) invasion and metastasis. While being cost and time effective in allowing assessment of a large number of genes, the array experiments were nonetheless fiscally challenging to the project. It was consequently decided not to repeat the presented experiment, thus only allowing for a qualitative overview of the gene expression changes and the results should therefore be taken as indicators of gene expression changes rather than as quantitative measurements. As such the main findings reported in this chapter are indicated by arrows rather than fold-changes.

In chapter 3 results on the effect of insulin on cell cycle progression were presented (section 3.3.5). The utilised method examined DNA content to evaluate the distribution of the cell population sample across the cell cycle stages. Complementarily the gene expression of two proteins, cyclin D and cyclin E, involved in regulating cell cycle progression is reported in this chapter. Complexion of cyclin D with cyclin dependent kinase 4 (CDK4) and cyclin E with CDK2 is necessary for cell cycle progression from G1-phase to S-phase (Grana and Reddy, 1995). The cyclin D/CDK4 complex is thought to promote the actual G1-phase to S-phase transition, while cyclin E/CDK2 sustains progression through S-phase (Sanchez and Dynlacht, 2005). Another function of the cyclin E/CDK2 complex is thought to be initiation of the progression of cell cycle from G0- to S-phase (Welcker and Clurman, 2005). Thus gene expression of cyclin D and cyclin E can provide insights into the cell cycle progression in the cell culture model. Additionally, gene expression of cyclin D and cyclin E is also measured in the array experiment. Thus these additional experiments on cyclin D and cyclin E gene expression may act as measures of the validity of the microarray results.

7.2 Materials and Methods

7.2.1 Cell lines and insulin treatment

MDA-MB-231, MCF-10A and SK-BR-3 cells were cultured and treated as described in Materials and Methods sections 2.1.1 and 2.1.2. Formulation of insulin treatment medium and final insulin treatment concentration is described in Materials and Methods section 2.1.3.1.

7.2.2 Cell proliferation

7.2.2.1 MTT-assay

In addition to detecting cell proliferation by measuring incorporation of BrdU, MDA-MB-231's ability to metabolise MTT in response to insulin treatment was assessed. MDA-MB-231 cells were plated in 96-well at a density of 5000 cells/well and incubated for 24 h at 37°C. After this initial incubation all medium was removed and cells were washed in PBS. Then cells were re-supplemented with serum-free medium and incubated for an additional 24 h at 37°C. After incubation in serum-free medium, medium was removed from all wells and cells were washed in PBS. Control wells were re-supplemented with serum-free medium or growth medium. Treatment wells were re-supplemented with treatment medium or 100 nM insulin in growth medium. Plates were then incubated for either 20 h or 42 h. After this MTT (final concentration 1 mg/ml) was added to wells and plates were incubated for an additional 4 h, without removal of treatment medium, resulting in a total treatment time of 24 h and 48 h, respectively. Then Formazan production was assessed as described in Materials and Methods section 2.2.2. Three experiments were performed for MDA-MB-231 cells for 24 h and three for 48 h treatment. Each experiment contained six wells for each treatment (*i.e.* six for control, six for "FCS", and six for "insulin", six for "insulin + FCS").

7.2.2.2 Expression of proteins related to cell proliferation

In addition to examining cell proliferation using BrdU incorporation as described in chapter 3, cell proliferation changes after insulin treatment were also examined using protein expression changes of cell proliferation marker PCNA and the target for JC-1.

MDA-MB-231 breast cancer cells or MCF-10A breast epithelial cells were plated in 60 mm^2 dishes at a density of 1 x 10^6 cells/dish and incubated for 24 h at 37°C. After this, cells were washed once in PBS and re-supplemented with serum-free medium. Incubation in serum-free medium lasted for 24 h. After incubation in serum-free medium, medium was replaced with 100 nM insulin treatment medium and cells were treated for 24 h or 48 h. After treatment, protein was extracted and Western Analysis was performed as described in section 2.6. Three experiments examining expression of PCNA and two experiments examining the expression of the target for JC-1 were performed.

7.2.3 Cell signalling pathway activation

In addition to examining MAP-kinase pathway activation in response to insulin treatment using cell signalling pathway ELISA assays (see section 2.3), ERK1/2-phosphorylation was also examined using western blotting as described in sections 7.2.2.2 and 2.6. Three experiments were performed with insulin incubations between 15 min, 30 min and 60 min. At each time point, proteins were extracted from two dishes, thus creating two replicates for each time point. Additionally proteins from two dishes were extracted after incubation in serum-free medium and acted as control.

7.2.4 Apoptosis

7.2.4.1 Flow cytometry

Cells were plated in 6-well plates at 75 x 10^4 cells/well and incubated for 24 h at 37°C. After the initial incubation, medium was removed, cells were washed with PBS and re-supplemented with serum-free medium. Incubation in serum-free medium lasted for 24 h. After incubation in serum-free medium, medium was removed, cells were washed in PBS and re-supplemented with 100 nM insulin treatment medium. Treatment time was either 5 h or 24 h. After treatment, cells were collected and analysed as described in section 2.8.2. Three experiments were performed for each timepoint. Each experiment had two wells (samples) treated identically, two wells with growth medium (+FCS), two with serum-free medium (NO FCS) and two with treatment medium (Insulin). Each replicate was analysed once on the flow cytometer, resulting in six measurements for each treatment. Each control and treatment was re-expressed as percentage of the averaged control value of that experiment.

7.2.4.2 Gene expression of BCL-2

MDA-MB-231 breast cancer cells and MCF-10A breast epithelial cells were plated at a density of 1 x 10^6 cells/dish in 60 mm^2 dishes and incubated for 24 h. After this initial incubation, medium was removed, cells were washed once in PBS re-supplemented with serum-free medium. Cells were incubated in serum-free medium for 24 h before being treated for between 15 min and 120 min with 100 nM insulin. RNA was extracted as described in section 2.7.2.1 and RT-PCR was performed as described in section 2.7.2.2.

7.2.5 Expression changes of CancerPathway™ genes

A profile of changes in gene expression after treatment with high concentrations of insulin was examined using a microarray assay. The procedure of this analysis is described in detail in Materials and Methods section 2.7.1. A single experiment was performed for MDA-MB-231 cells and MCF-10A cells. For each cell line two arrays were analysed, one control, one after 60 min treatment with 100 nM insulin. The array controls for each gene by hybridising in four distinct spots. Analysis was performed as a single densitometry value from four replicates. Internal gene expression controls, examining the expression of "housekeeping" genes were used to adjust for background and normalise the "control" and "treatment" array. As only one array was performed, no statistical test was performed to analyse the significance of the observed expression changes.

7.2.6 Gene expression of cyclin D and cyclin E

In addition to changes in distribution of cell population across cell cycle stages using flow cytometry, cell cycle changes were also assessed by examining the change in expression of cell cycle genes cyclin D and cyclin E in response to high concentrations of insulin. MDA-MB-231 breast cancer cells, MCF-10A breast epithelial cells and SK-BR-3 breast cancer cells were plated, incubated in serum-free medium and treated as described in section 7.2.4.2, section 2.7.2.1 and 2.7.2.2.

7.3 Results

7.3.1 Additional measurements of the effects of insulin on cell proliferation

7.3.1.1 Changes in cell proliferation by MTT-analysis

Assessment of changes in cell proliferation using MTT metabolism was assessed only in MDA-MB-231 breast cancer cells. Differently from previously presented data, MTT metabolism was assessed after treatment with different concentrations of insulin (50 nM, 100 nM, 150 nM). Different from assessment with BrdU-incorporation assay, cells were also treated in growth medium (+FCS) supplemented with insulin at the indicated concentrations[2]. The rate of MTT metabolism was assessed after 24 h or 48 h of treatment (Figure 7-1).

Formazan formation in MDA-MB-231 breast cancer cells did not change after 24 h of insulin treatment in serum-free medium with any examined insulin concentration (Figure 7-1, A). Treatment with insulin in growth medium increased Formazan production by 11% (p=0.041) and 15% (p=0.004) after treatment with 100 nM insulin and 150 nM insulin for 24 h, respectively (Figure 7-1, B). Formazan production did not change after treatment with increasing insulin concentrations for 48 h in either serum-free medium or growth medium (Figure 7-1, C, and D).

[2] See Appendix section 9.1.2 for a comparison of cells incubated in serum-supplemented vs. serum-free medium.

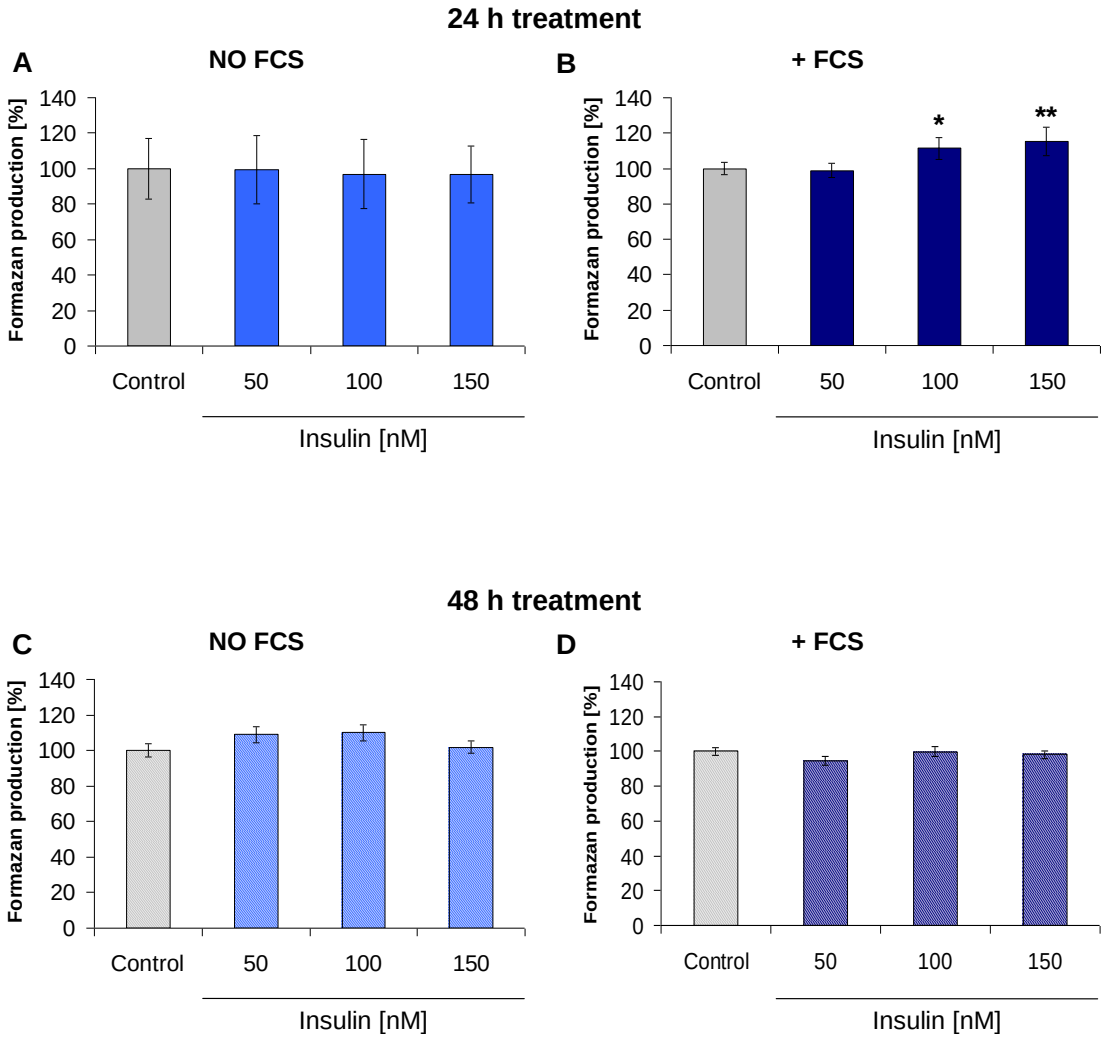

Figure 7-1: Changes in cell proliferation in MDA-MB-231 breast cancer cells after treatment with insulin for 24 h (A, B) or 48 h (C, D) as assessed with the MTT-assay. Treatment medium consisted either of insulin-supplemented serum-free medium (NO FCS, A, C, blue) or of insulin-supplemented growth medium (+ FCS, B, D, dark blue), the composition of which can be found in section 2.1.2 and 2.1.3. Bars represent Formazan production in relation to the respective control and expressed as a percentage thereof. Error bars represent ± SEM of three experiments, with six replicates, *i.e.* 18 data points for each bar.
*significance value compared to control, obtained using Dunnett's post-hoc t-test following univariate analysis of variance (*0.05>p>0.01; **0.01>p>0.001)

7.3.1.2 Effect of insulin on expression of cell proliferation protein markers

The expression of markers of cell proliferation PCNA and JC-1 in response to insulin treatment was assessed in MDA-MB-231 breast cancer cells. PCNA expression was also assessed in MCF-10A cells in response to insulin treatment. Protein expression was assessed using Western Blotting Analysis.

In MDA-MB-231 breast cancer cells, PCNA expression increased significantly by 53% after 30 min ($p=0.016$) treatment with 100 nM insulin. No significant change was observed after treatment for 60 min ($p=0.086$) or 120 min ($p=0.411$, Figure 7-2, A). In MCF-10A breast epithelial cells PCNA expression did not significantly change after 30 min ($p=0.57$), 60 min ($p=0.991$) or 120 min ($p=0.997$) of treatment with 100 nM insulin (Figure 7-2, B). Representative raw data and antibody specificity control can be found in Appendix section 9.4.1.

In MDA-MB-231 breast cancer cells, JC-1 expression did not significantly change after treatment for 30 min ($p=0.808$), 60 min ($p=0.283$) or 120 min ($p=0.787$) with 100 nM insulin (Figure 7-3). Representative raw data and antibody specificity control can be found in Appendix section 9.4.2.

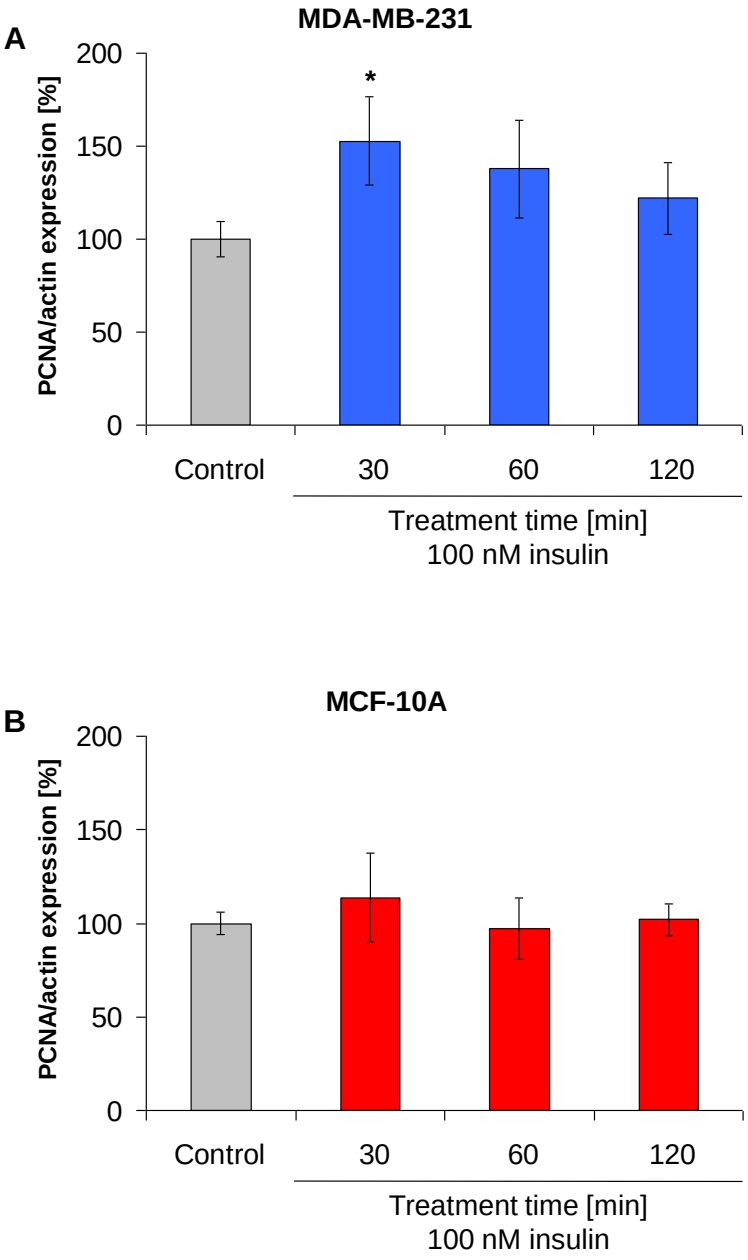

Figure 7-2: Changes in expression of PCNA after treatment with 100 nM insulin in A) MDA-MB-231 breast cancer cells and B) MCF-10A breast epithelial cells. Bars represent protein expression of PCNA in relation to control and expressed as a percentage thereof. Error bars represent ± SEM of three experiments with two replicates, *i.e.* six data points for each bar. *Significance value compared to control, obtained using Dunnett's post-hoc t-test following univariate analysis of variance (*0.05>p>0.01)

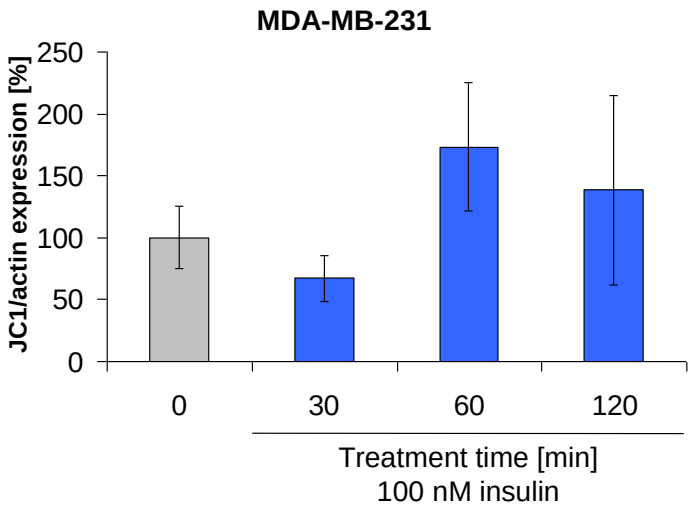

Figure 7-3: Changes in expression of JC-1 after treatment with 100 nM insulin in MDA-MB-231 breast cancer cells. Bars represent protein expression of JC-1 in relation to control and expressed as a percentage thereof. Error bars represent ± SEM of two experiments with two replicates, *i.e.* four data points for each bar. Results were compared to control, using Dunnett's post-hoc t-test following univariate analysis of variance.

7.3.2 Effect of insulin on activation of MAP-kinase cell signalling pathway in breast cancer cell determined by western analysis

In MDA-MB-231 breast cancer cells, phosphorylation of ERK1/2, as measurement of activation of the MAP-kinase cell signalling pathway, was assessed using western blotting in addition to its assessment described in section 3.3.3.

In MDA-MB-231 breast cancer cell, ERK1/2-phosphorylation increased non-significantly by 54% after 15 min (p=0.439) treatment, by 17% after 30 min (p=0.953) treatment and decreased non-significantly after 60 min (p=0.993) treatment with 100 nM insulin (Figure 7-4). Representative raw data and antibody specificity control can be found in Appendix section 9.4.3.

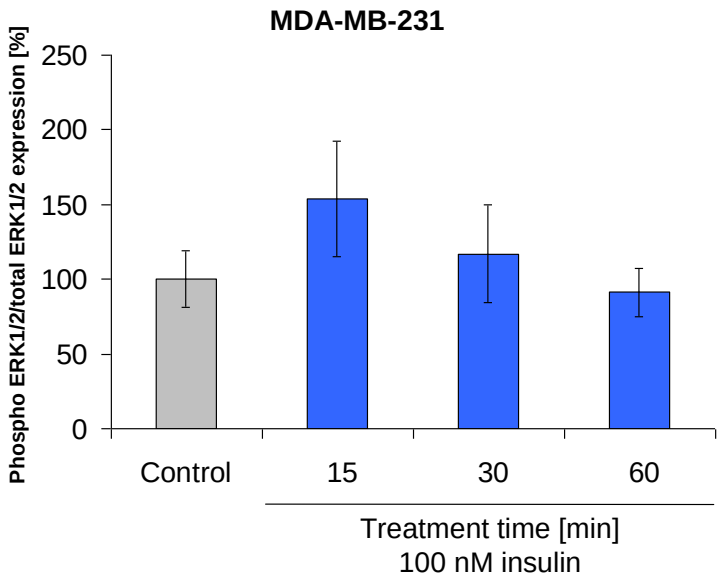

Figure 7-4: Changes in ERK1/2-phosphorylation after 100 nM insulin treatment in MDA-MB-231 breast cancer cells using western analysis. Bars represent percentage of ERK1/2-phosphorylation in relation to control and expressed as a percentage thereof. ERK1/2-phosphorylation was normalised against total ERK1/2 expression. Error bars represent ± SEM of three experiments with two replicates, *i.e.* six data points for each bar. Results were compared to control, using Dunnett's post-hoc t-test following univariate analysis of variance.

7.3.3 The effect of insulin on apoptosis in breast cancer

7.3.3.1 Detection of Annexin-V in early apoptotic and 7AAD-staining in late apoptotic cells by flow cytometry

Data was acquired by subjectively determining an Annexin-V or 7-AAD low cut-off point in the untreated cell distribution (Control)[3]. Only cells for which Annexin-V detection or 7-AAD staining was above this point were counted. The flow cytometry software then provided a percentage of the total cell sample (10000 cells were counted in each sample), which were above the cut-off point. The percentage of cells obtained for control was determined as standard from which percentage change of Annexin-V detection and 7-AAD staining were calculated. Cut-off values, once established, were retained for all three experiments. Further controls, cells treated with 3% Formaldehyde for 30 min and cell grown in growth medium for the duration of the treatment (see Appendix section 9.3.3.2), were included in aid of determining the cut-off point.

In MDA-MB-231 breast cancer cells, treatment with 100 nM insulin did not significantly change Annexin-V detection or 7-AAD staining after 5 h or 24 h treatment (Figure 7-5, A). Annexin-V detection decreased non-significantly by 4% after 5 h ($p=0.661$) and by 6% after 24 h ($p=0.298$) with 100 nM insulin. The amount of cells with high levels of 7-AAD staining decreased non-significantly by 8% after 5 h ($p=0.441$) and increased non-significantly by 6% after 24 h ($p=0.136$) treatment with 100 nM insulin (Figure 7-5, B).

In MCF-10A breast epithelial cells, Annexin-V detection did not change significantly after 5 h ($p=0.963$) and decreased significantly by 13% after 24 h ($p=0.037$) treatment

[3] See Appendix section 9.3.2 for a representative raw-data output file.

with 100 nM insulin (Figure 7-5, C). The amount of cells with high level of 7-AAD staining did not change significantly after 5 h (p=0.48) or 24 h (p=0.95) treatment with 100 nM insulin (Figure 7-5, D).

In SK-BR-3 breast cancer cells, Annexin-V detection did not change significantly after 5 h (p=0.99) or 24 h (p=0.096) treatment with 100 nM insulin (Figure 7-5, E). The percentage of cells with high levels of 7-AAD staining did not change after 5 h (p=0.552) and increased significantly by 9% after 24 h (p=0.024) of 100 nM insulin treatment (Figure 7-5, F).

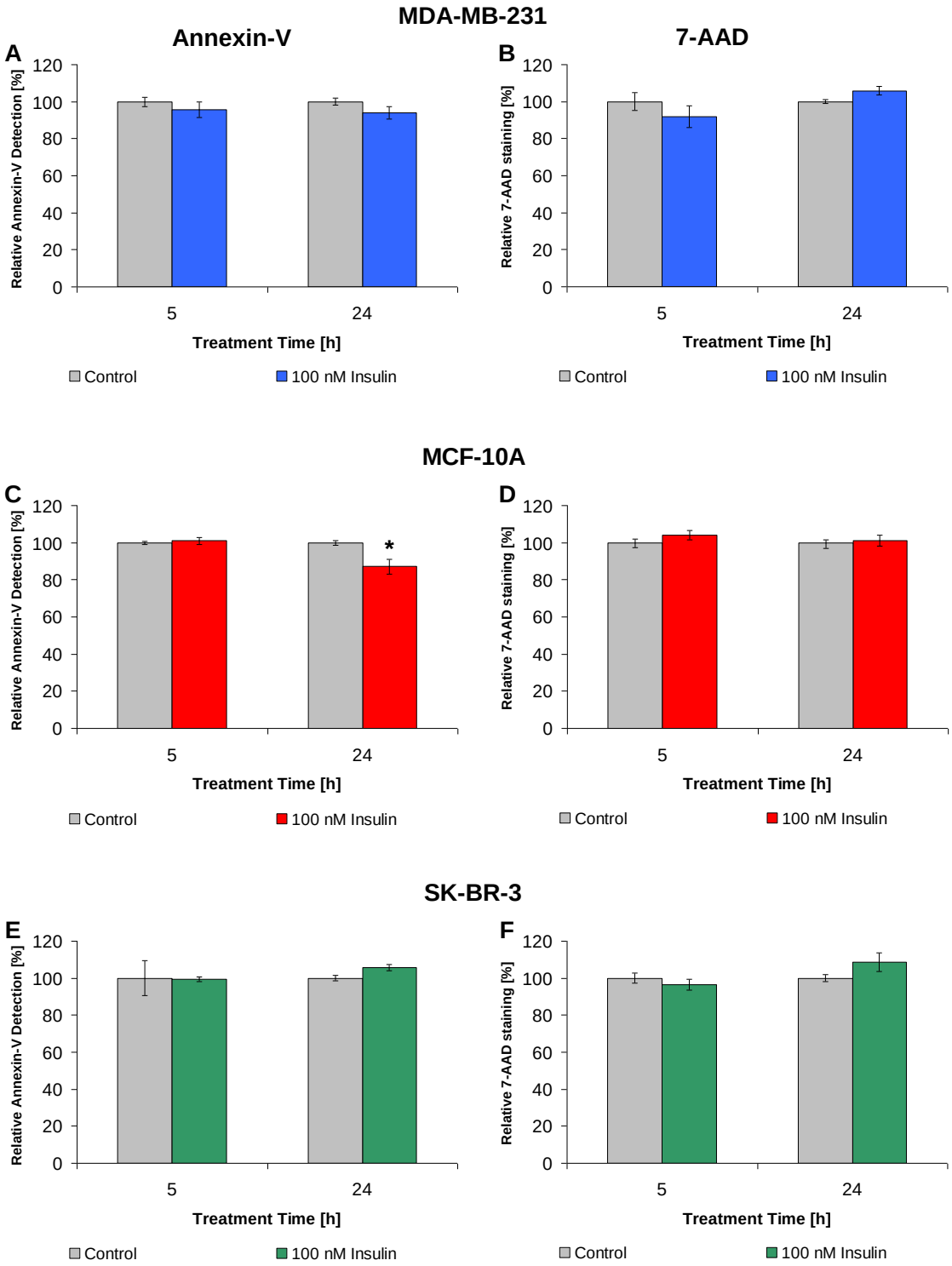

Figure 7-5: Changes in Annexin-V detection (A, C, E) and 7-AAD staining (B, D, F) after 100 nM insulin treatment for 5 h or 24 h in A, B) MDA-MB-231 breast cancer cells, C, D) MCF-10A breast epithelial cells and E, F) SK-BR-3 breast cancer cells. Bars represent percentage of cells above lower cut-off point in flow cytometry histogram (see Appendix section 9.3.2) in relation to control and expressed as a percentage thereof. Error bars represent ± SEM of three experiments with two replicates, *i.e.* six data points for each bar.
*Significance value compared to control of the same treatment time, obtained using Dunnett's post-hoc t-test following univariate analysis of variance (*0.05>p>0.01)

7.3.3.2 Effect of insulin treatment in gene expression of BCL-2 as a measure of apoptosis in breast cancer

In MDA-MB-321 breast cancer cells and MCF-10A breast epithelial cells the expression of BCL-2 after treatment with 100 nM insulin was assessed. BCL-2 is an anti-apoptotic marker, which would increase, if insulin decreases apoptosis. Thus it could provide additional information in combination with apoptosis flow-cytometry results.

In MDA-MB-231 breast cancer cells, BCL-2 gene expression increased non-significantly by 16% after 30 min (p=0.322) insulin treatment and by 18% after 60 min (p=0.233) treatment with 100 nM insulin (Figure 7-6, A). In MCF-10A breast epithelial cells, BCL-2 gene expression did not change after 15 min (p=0.99) treatment, 30 min (p=0.765) treatment, 60 min (p=0.676) treatment or 120 min (p=0.917) treatment with 100 nM insulin (Figure 7-6, B). A non-significant trend suggests a maximum increase in BCL-2 gene expression after 60 min of insulin treatment. A representative gel-picture of the BCL-2 PCR-product can be found in Appendix section 9.5.1.

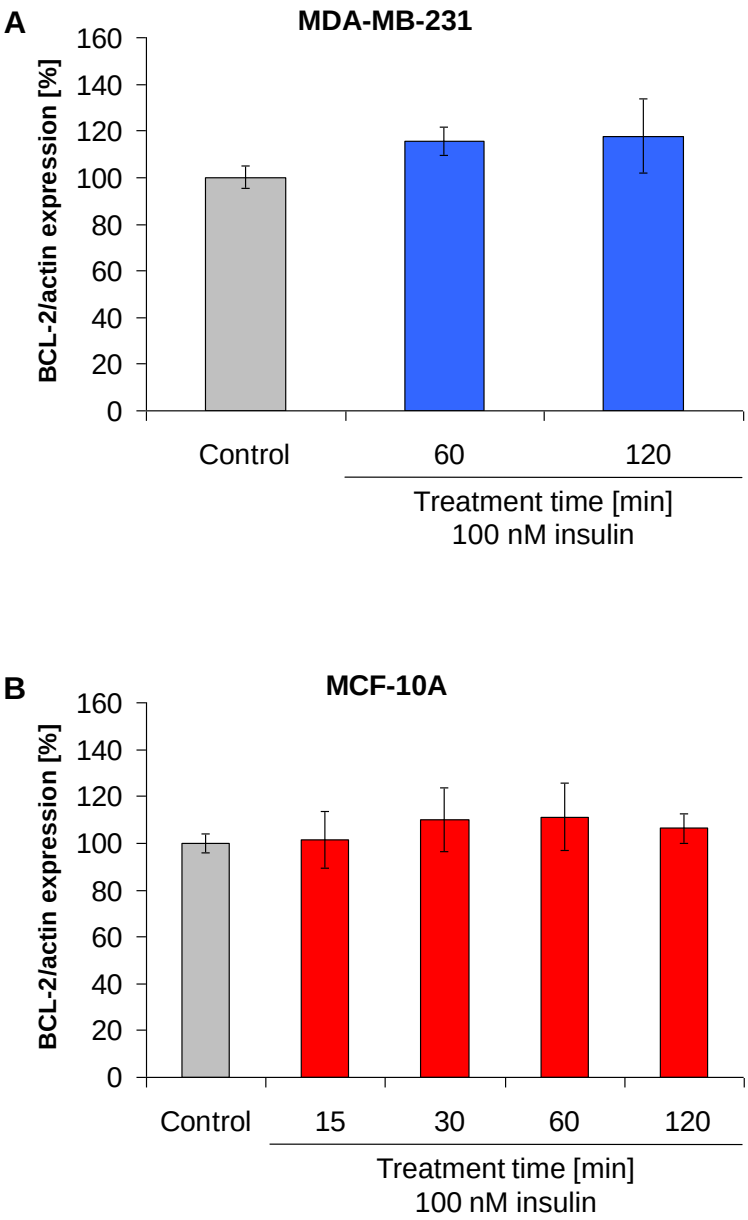

Figure 7-6: Changes in BCL-2 gene expression after treatment with 100 nM insulin in A) MDA-MB-231 breast cancer cell and B) MCF-10A breast epithelial cells. Bars represent percentage of BCL-2 expression in relation to control and expressed as a percentage thereof. Error bars represent ± SEM of three experiments with two replicates, *i.e.* six data points for each bar. Results were compared to control, using Dunnett's post-hoc t-test following univariate analysis of variance.

7.3.4 Effect of insulin on CancerPathway gene expression

Microarray analysis showed changes in gene expression after treatment with 100 nM insulin for 60 min. The gene expression changes of 114 genes were examined in MDA-MB-231 breast cancer cells and MCF-10A breast epithelial cells. The microarray also examined thirteen control genes (see Table 9-2 in the Appendix), which were used to normalise control membranes and treatment membranes. The examined genes were grouped by the microarray manufacturer (SABiosciences) according to function. The main changes in gene expression are summarised in Table 7-1 grouped according to the six functional groupings suggested by the microarray manufacturer. These genes were selected, if at least a two-fold increase in either cell line was observed. Arrows were used to visualise gene expression changes to emphasise that the obtained results were not subject to statistical evaluation. A full list of all genes examined with their raw data, as obtained after densitometry can be found in Appendix section 9.6.

Table 7-1: Main changes in gene expression after treatment for 60 min with 100 nM insulin in MDA-MB-231 breast cancer cells and MCF-10A breast epithelial cells compared to control, analysed by CancerPathway microarray

↓/↑ decrease/increase after insulin treatment
↔ no change after insulin treatment
ND no expression detected in control and treatment array

Cell Cycle control & DNA Damage Repair		
Gene description	**MDA-MB-231**	**MCF-10A**
Breast cancer 1, early onset	↑	ND
Breast cancer 2, early onset	↓	↔
Cyclin E1	↑	↔
Cell division cycle 25 homolog A (S. pombe)	↔	↑
Cyclin-dependent kinase 2	↓	↔
Cyclin-dependent kinase 4	↔	↑
Retinoblastoma 1 (including osteosarcoma)	↑	↑

Apoptosis and Cell Senescence		
Gene description	**MDA-MB-231**	**MCF-10A**
Apoptotic peptidase activating factor 1	↓	ND
BCL2-antagonist of cell death	↓	ND
B-cell CLL/lymphoma 2	↑	↔
BCL2-like 1	↑	ND
Baculoviral IAP repeat-containing 5 (survivin)	↔	↑
Caspase 8, apoptosis-related cysteine peptidase	↓	ND
Tumor necrosis factor receptor superfamily, member 1A	↔	↓
Tumor necrosis factor receptor superfamily, member 25	ND	↑

Signal Transduction Molecules and Transcription Factors		
Gene description	**MDA-MB-231**	**MCF-10A**
V-akt murine thymoma viral oncogene homolog 1	↓	↑
V-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)	↔	↑
V-Ets erythroblastosis virus E26 oncogene homolog 2 (avian)	↑	ND
V-fos FBJ murine osteosarcoma viral oncogene homolog	↔	↑
Jun oncogene	↑	↑
Mitogen-activated protein kinase kinase 1	↓	↔
Phosphoinositide-3-kinase, regulatory subunit 1 (alpha)	↔	↑
Synuclein, gamma (breast cancer-specific protein 1)	↔	↑

Adhesion		
Gene description	**MDA-MB-231**	**MCF-10A**
CD44 molecule (Indian blood group)	↑	↔
Integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor)	↔	↑
Integrin, beta 5	↑	↑
Melanoma cell adhesion molecule	↓	ND

Angiogenesis		
Gene description	**MDA-MB-231**	**MCF-10A**
Angiopoietin 1	↓	ND
Brain-specific angiogenesis inhibitor 1	↓	ND
Collagen, type XVIII, alpha 1	↑	ND
Epidermal growth factor (beta-urogastrone)	↓	ND
Epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian)	↑	↑
Interferon, alpha 1	↓	↔
Interleukin 8	↓	↔
Platelet-derived growth factor beta polypeptide (simian sarcoma viral (v-sis) oncogene homolog)	↓	↔
Thrombospondin 2	ND	↓

Invasion and Metastasis		
Gene description	**MDA-MB-231**	**MCF-10A**
Matrix metallopeptidase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)	↓	↑
Non-metastatic cells 1, protein (NM23A) expressed in	↔	↑
Non-metastatic cells 4, protein expressed in	↓	↓
Plasminogen activator, urokinase receptor	↔	↑
Serpin peptidase inhibitor, clade B (ovalbumin), member 2	↔	↑
TIMP metallopeptidase inhibitor 3 (Sorsby fundus dystrophy, pseudoinflammatory)	↑	↔

Uncategorised Genes		
Gene description	**MDA-MB-231**	**MCF-10A**
Ribosomal protein S27a	↔	↑
HIV-1 Tat interactive protein 2, 30kDa	↔	↑
Pinin, desmosome associated protein	↑	↔

7.3.5 Effect of insulin on cyclin D and cyclin E gene expression in breast cancer

In MDA-MB-231 breast cancer cells, cyclin D gene expression did not significantly change after 15 min (p=0.782), 30 min (p=0.081), 60 min (p=0.332) or 120 min (p=0.999) treatment with 100 nM insulin compared to untreated control (Figure 7-7, A). Cyclin E gene expression did not significantly change after 15 min (p=0.999), 30 min (p=0.972), 60 min (p=0.530) or 120 min (p=0.864) treatment with 100 nM insulin compared to untreated control (Figure 7-7, B). A representative gel-picture of the cyclin D and cyclin E PCR product can be found in Appendix sections 9.5.2 and 9.5.3, respectively.

In MCF-10A breast epithelial cells cyclin D gene expression did not change after 15 min (p=0.963), 30 min (p=0.409), 60 min (p=0.999) or 120 min (p=0.052) after treatment with 100 nM insulin compared to untreated control (Figure 7-7, C). Cyclin E gene expression did not change after 15 min (p=0.999), 30 min (p=0.992), 60 min (p=0.718) or 120 min (p=0.652) after treatment with 100 nm insulin compared to untreated control (Figure 7-7, D).

In SK-BR-3 breast cancer cells, cyclin D gene expression increased by 18% after 15 min (p=0.005) of treatment with 100 nM treatment compared to untreated control. Cyclin D gene expression did not significantly change after 30 min (p=0.895), 60 min (p=0.161) or 120 min (p=0.999) treatment with 100 nM treatment compared to untreated control (Figure 7-7, E). Cyclin E gene expression increased by 22% after 15 min (p<0.001), by 19% after 30 min (p=0.001), by 17% after 60 min (p=0.003) and

by 15% after 120 min (p=0.012) treatment with 100 nM insulin compared with untreated control (Figure 7-7, F).

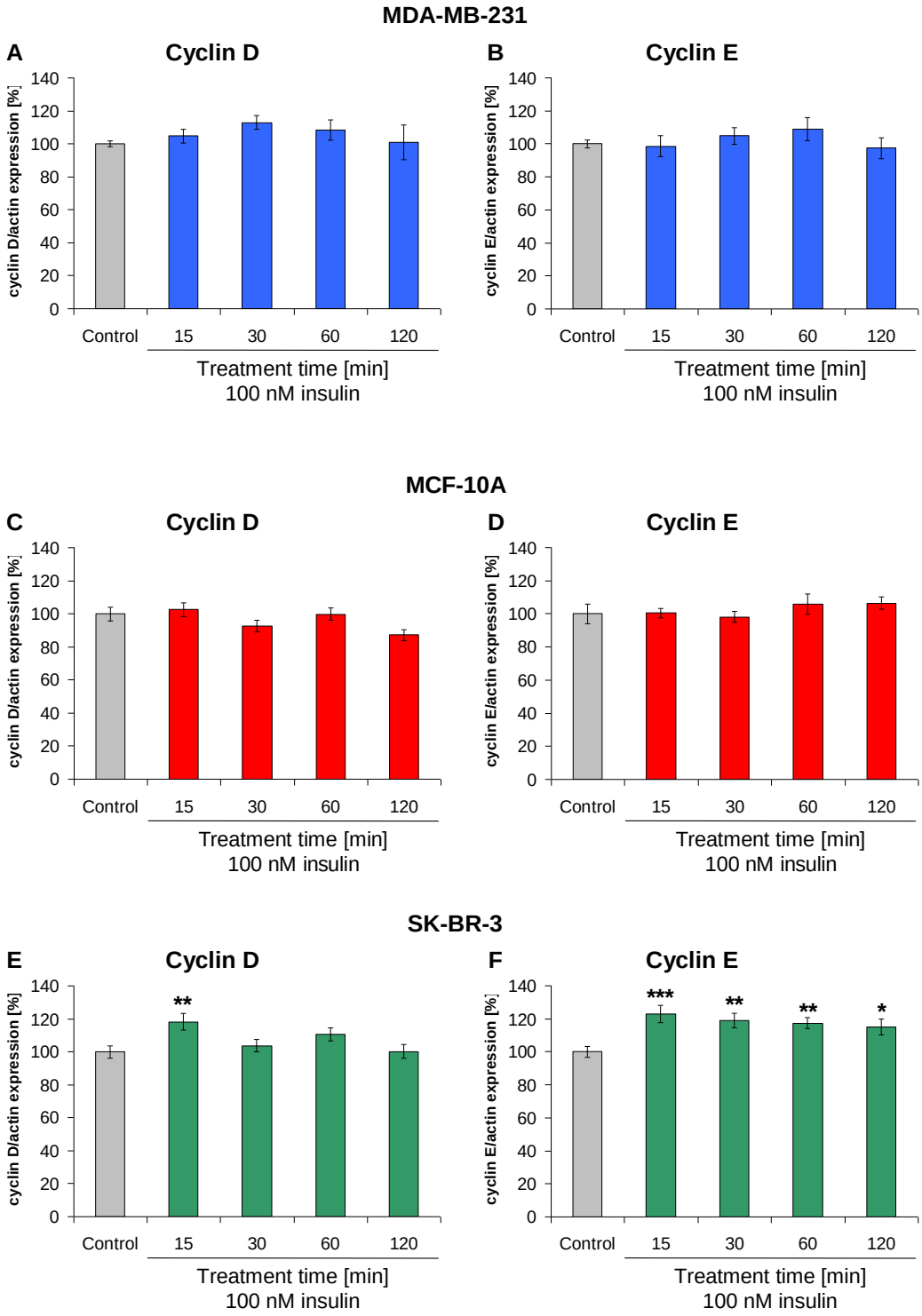

Figure 7-7: Changes in gene expression of cyclin D (A, C, E) and cyclin E (B, D, F) after treatment with 100 nM insulin in A, B) MDA-MB-231 breast cancer cell, C, D) MCF-10A breast epithelial cells and E, F) SK-BR-3 breast cancer cells. Bars represent percentage of cyclin D and cyclin E gene expression in relation to control and expressed as a percentage thereof. Error bars represent ± SEM of three experiments with two replicates, *i.e.* six data points for each bar.
* Significance value compared to control, obtained using Dunnett's post-hoc t-test following univariate analysis of variance. (*0.05>p>0.01; **0.01>p>0.001; ***p<0.001),

7.4 Discussion

7.4.1 Additional effects of insulin treatment on human MDA-MB-231 breast cancer cells

A number of additional experiments were performed using MDA-MB-231 breast cancer cells. In addition to analysing cell proliferation using BrdU-incorporation measurement of three other markers of cell proliferation was investigated, *i.e.* rate of metabolism of MTT and protein expression of cell proliferation marker PCNA and of the JC-1 antigen. Cell proliferation measured using the MTT-assay increased with 100 nM and 150 nM insulin treatment after 24 h in growth medium. No increase was observed with the standard treatment medium, following 24 h incubation in serum-free medium. No increase was observed after 48 h treatment in either medium (Figure 7-1). In chapter 3 and references cited therein, it has been shown that the overall findings do not suggest an influence of insulin on MDA-MB-231 breast cancer cells. Using cells incubated in serum-free medium for 24 h (NO FCS), which is the same approach as used for the BrdU assay, no increase in proliferation, using the MTT-assay, was observed (Figure 7-1). Performing the same experiment in cells retained in serum-supplemented medium for the duration of the experiment (+FCS) however increased cell proliferation. Incubation in serum-free medium was used to synchronise cell cycle of the examined cells, as cells incubated in serum-free medium arrest their cell cycle at the G1 stage (see Appendix section 9.3.3.1). Furthermore, incubation in serum-free medium decreased the exposure of the examined cells to other growth factors usually found in bovine serum. Thus any observed change can confidently be attributed to insulin action. The observation that insulin increases cell proliferation in serum-supplemented treatment medium may suggest a synergistic signalling mechanism of insulin and growth factors in the bovine serum supplement to stimulate growth. While this approach lacks the

possibility of a scientific explanation, as it increases variables, which cannot be controlled for, it also indicates that insulin may play a part in cell cycle stimulation in combination with other factors. In addition a brief analysis of the foetal calf serum (FCS) using a clinical analyser to examine insulin concentration, showed no detectable traces of insulin (C. Rolland, personal communication). Thus it could be speculated that insulin alone is not able to increase cell proliferation, in combination with FCS growth factors however cell proliferation occurs. The effect was only observed after 24 h treatment and not after 48 h treatment. Additionally the actual increases were only 11% and 15% for 24 h with 100 nM and 150 nM insulin treatment, respectively, which might not reflect an effect that has significant physiological impact.

Analysis of MDA-MB-231 cell proliferation using the BrdU assay showed a significant decrease in cell proliferation after 48 h of 100 nM insulin treatment (Figure 3-4). A similar result was not obtained using the MTT assay. No significant difference in cell proliferation after 48 h of 100 nM insulin treatment was observed (Figure 7-1). Thus the controversial finding that insulin may decrease cell proliferation in MDA-MB-231 breast cancer cells was not supported by the MTT-analysis and further analysis may clarify the difference in the obtained results.

In MDA-MB-231 breast cancer cells PCNA expression increased significantly after 30 min treatment with 100 nM insulin. This experiment was performed using the usual approach of incubation in serum-free medium. It is the only experiment, whose results may be interpreted to indicate an increase in cell proliferation with insulin treatment alone in MDA-MB-231 breast cancer cells. All the other results examining cell

proliferation (BrdU, MTT, and JC-1) did not indicate an increase. It was observed that MDA-MB-231 cells were affected by insulin treatment in several ways (AKT-phosphorylation, ERK1/2-phosphorylation, IR-phosphorylation, cell cycle changes, all in chapter 3). All these changes should be indicative of increased proliferation, yet, using several method to assess cell proliferation, this feature was not observed. Thus PCNA expression changes may fall into the same category of molecular changes with insulin that do not seem to have a physiologically proliferative effect.

In MDA-MB-231 breast cancer cells, expression of the antigen detected by JC-1 did not change with 100 nM insulin treatment. The expression of the antigen detected by JC-1 was very low in MDA-MB-231 breast cancer cells, sometimes barely above background values and as such the quantification process was challenging. This low expression may also account for the increased error, which, despite changes of almost 90% after 60 min of treatment, is the reason for the non-significance of the obtained findings (Figure 7-3). If detection of the proliferin antigen by JC-1 is desired a further optimisation of the western blotting procedure may yield quantifiable results. In addition protein expression of an additional cell proliferation marker, minichromosome maintenance protein 2 (MCM2), was assessed. The MCM-protein family are regulators, involved in DNA replication and their concentration increases during cell growth making them viable markers for cell proliferation (Cho Mar *et al.*, 2006). Additionally gene expression of MCM2 in MDA-MB-231 breast cancer cells had been detected previously (Moggs *et al.*, 2005). Using western blotting, no protein expression of MCM2 in MDA-MB-231 breast cancer cells was detected in the study presented here in treated or untreated MDA-MB-231 breast cancer cells.

In MDA-MB-231 breast cancer cells no significant change in ERK1/2-phosphorylation after treatment with 100 nM insulin was observed (Figure 7-4). In chapter 3 significant increases in ERK1/2-phosphorylation after treatment with insulin were observed (section 3.3.3.1). The timeframe in that experiment however examined phosphorylation between 5 min and 20 min, while here phosphorylation of ERK1/2 was examined between 15 min to 60 min treatment. Despite the non-significant observation, total numbers indicate an increase after 15 min and a return to baseline after 60 min of treatment. Phosphorylation of ERK1/2 after 15 min of 100 nM insulin treatment was measured with both methods. The observed increased in ERK1/2-phosphorylation of 95% was significant using the *in vitro* assay and was slightly lower at 54% increase using western blotting analysis, which did not reach significance. Taking the large error of the western blotting analysis into account, it may be concluded that a similar increase may have been observed. If the results of the two experiments are viewed in conjunction a time-dependent increase, with the maximum between 20 min and 30 min, in ERK1/2-phosphorylation, followed by a return to baseline after 60 min of 100 nM treatment, is observed. As such the western data on ERK1/2-phosphorylation changes with insulin treatment do not contradict the findings of the *in vitro* analysis. As the observed increase in the western blotting analysis is however not significantly different from control, the western blotting results fail to supplement the significant findings of the ELISA assay results.

In MDA-MB-231 breast cancer cells, no significant changes in cyclin D or cyclin E expression after treatment between 15 min and 120 min with 100 nM insulin were observed (Figure 7-7). These experiments were primarily used to supplement the

findings of the cell cycle analysis presented in section 3.3.5. Those results showed an increase in S-phase population after treatment with 100 nM insulin for 24 h (Figure 3-11). Gene expression of cyclin D shows a maximum after 30 min of treatment, the increase however is not significant. Similarly cyclin E shows a non-significant increase in gene expression after 60 min of treatment. In MDA-MB-231 breast cancer cells there is therefore no indication that insulin treatment may increase gene expression of cyclin D or cyclin E significantly. Progression from G1-phase to S-phase is cyclin D dependent with progression through S-phase also being dependent on cyclin E (Welcker and Clurman, 2005). Thus, assuming incubation in serum-free medium induced cell cycle arrest at the G1-phase and insulin being able to induce progression from G1-phase to S-phase (see Figure 7-8), one would expect an increase in cyclin D first as cell cycle progresses from G1-phase to S-phase, followed by an increase in cyclin E, the cell progresses through S-phase. Not having observed a significant increase in either cyclin expression one would expect that one of the assumptions is wrong, most likely that insulin does not increase progression from G1 to S-phase[4]. In the study presented here, changes in mRNA expression are measured, which may not necessarily translate equally to changes on the protein level. In section 3.3.5, it was observed that insulin increased S-phase population in MDA-MB-231 breast cancer cells, which was interpreted as constituting increased progression from G1-phase into S-phase. Thus the non-significant findings from the cyclin gene expression changes do not contradict these findings, but not necessarily provide further support of this finding.

[4] Discussion on what happens, if the other assumption, i.e. incubation in serum-free medium failing to arrest cell cycle at G1-phase is attempted in section 8.8.2.

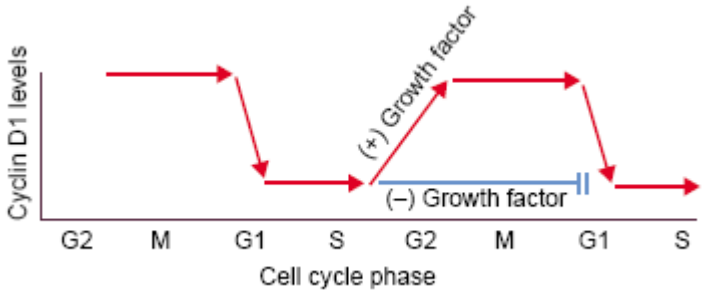

Figure 7-8: Function of oscillating cyclin D concentration as regulatory switch of cell cycle progression. After progression for G1-phase into S-phase, cyclin D protein concentrations begin to decrease and stay low until G2-phase. In the presence of growth factors, such as potentially insulin, cyclin D concentrations start to increase and promote normal progression of the cell cycle into the next S-phase. In the absence of growth factors, such as after incubation in serum-free medium, cyclin D concentrations do not increase in G2-phase and as a consequence the cell cycle arrests at the next transition from G1-phase to S-phase (adapted from Stacey, 2003).

In MDA-MB-231 breast cancer cells, treatment with 100 nM insulin did not significantly change detection of Annexin-V or 7AAD-staining after 5 h or 24 h treatments. This suggests that insulin does not affect the apoptotic rate of MDA-MB-231 breast cancer cells. None of the other studies examining the effect of insulin on these cells have investigated its effect on apoptosis, thus there is no validation from other studies for this finding. This observation however fits with results shown in chapter 3 and in this chapter, which demonstrated that insulin treatment may not significantly affect cell proliferation in these cells.

BCL-2 is a survival protein, inhibiting apoptotic signalling. Thus an increase in BCL-2 expression corresponds to a decrease in apoptosis and its increased gene expression may provide insight to the effect insulin may have on these breast cancer cells. In MDA-MB-231 breast cancer cells BCL-2 gene expression did not significantly change after insulin treatment compared to untreated control (Figure 7-6). Thus an influence of insulin on apoptosis is increasingly unlikely. Furthermore there are no available

publications reporting on the effect that insulin may have on apoptosis in MDA-MB-231 breast cancer cells. This could be because no one has yet examined this effect, or because similar negative findings have been observed but not been reported on[5].

The microarray analysis indicated a number of gene expression changes after 60 min treatment with 100 nM insulin in MDA-MB-231 breast cancer cells (Table 7-1 and Appendix Table 9-1). Surprisingly BCL-2 expression was decreased and cyclin E expression was increased, which had not been observed measuring mRNA concentrations using RT-PCR. Likewise the expression of several genes, which are key indicators of cancer malignancy, was decreased, such as CDK-2, AKT, MEK1/2 and EGF. CDK-2 needs to complex with cyclin E to promote cell cycle progression through S-phase. It was also observed that AKT and MEK1/2 gene expression was also decreased after insulin treatment. These two kinases are crucially involved in PI3-kinase and MAP-kinase cell signalling. A major affector of cell proliferation, the epidermal growth factor (EGF), was also decreased. This may indicate that insulin could inhibit potential autocrine signalling of EGF on these cells, MDA-MB-231 breast cancer cells however do not overexpress HER2, so this idea is not particularly valuable. Expression of the epidermal growth factor receptor on the other hand was increased. This decrease

[5] Another apoptosis marker, caspase 3 and cleaved caspase 3, was examined after insulin treatment. Caspase 3 is one of the key players in initiating apoptosis. It needs to be cleaved in order to become activated. It was aimed to detect total caspase 3 and cleaved caspase 3 by western blotting in insulin treated and untreated samples to estimate the rate of caspase 3 cleavage to gain insight to the effects of insulin on apoptotic rate of MDA-MB-231 breast cancer cells. No cleaved caspase 3 was detected after treatment in either sample. These western experiments were performed at the same time as detection of MCM-2 was pursued, which equally was not detectable and western blotting experiments were ceased at this point, as no results were obtained and reproducibility of existing results was poor. However further investigation of these markers may be worthwhile, using a more systematic optimising procedure, before quantifiable results are obtained.

in expression of a number of key cancer promoting genes is unexpected. It may again be emphasised at this point that these results were not available to be subject to statistical scrutiny. These changes however do not seem as surprising in the light of no change of cell proliferation or apoptosis with simultaneous increases in AKT- and ERK1/2-phosphorylation in MDA-MB-231 breast cancer cells. Thus far from mediating a proliferative response, insulin may act as proliferation inhibitor in these cells, reducing several key genes involved in cancer progression, and thus maybe inhibiting crucial autocrine signalling in these cells. In fact one may be reminded of the observed decrease in proliferation observed after 48 h insulin treatment using BrdU incorporation (see section 3.3.1), which may add some credibility to a decrease in growth promotion of insulin on these cells.

Combining the observations described in chapter 3 and in this chapter, insulin convincingly increases PI3-kinase and MAP-kinase cell signalling and may increase cell cycle progression and mediate a suppressive cancer pathway gene expression response in MDA-MB-231 breast cancer cells. Cell proliferation analysis resulted in conflicting findings. Using BrdU incorporation analysis, cell proliferation decreased after 48 h treatment (see section 3.3.1). Using the MTT assay cell proliferation increased, when cells were treated in full growth medium (+FCS) with 100 nM or 150 nM insulin for 24h (see section 7.3.1.1). Additionally PCNA protein expression was increased after 30 min of treatment with 100 nM insulin (see section 7.3.1.2). Thus there were some conflicting results after treatment with insulin in MDA-MB-231 breast cancer cells, suggesting both growth promoting and growth inhibiting properties of insulin on these cells.

7.4.2 Additional effects of insulin treatment on human MCF-10A breast epithelial cells

Several additional methods testing the effect of high insulin concentrations on MCF-10A breast epithelial cells were performed. Cell proliferation was assessed by examining expression of PCNA after insulin treatment. In MCF-10A breast epithelial cells, PCNA expression did not change after treatment with 100 nM insulin between 30 min and 120 min (Figure 7-2). Cell proliferation increased by 184% after 24 h treatment using the BrdU incorporation assay (see section 3.3.1). Two hours may probably be too short a treatment time to significantly increase expression of PCNA. Alternatively insulin may increase cell proliferation in MCF-10A breast epithelial cells in a PCNA independent manner. Thus the presented PCNA analysis does not provide any additional confirmation to the effect of insulin on cell proliferation in MCF-10A breast cancer cells. Extending the currently employed treatment time of 120 min to 24 h may provide better estimation to the effect of PCNA on insulin-induced cell proliferation.

In MCF-10A breast epithelial cells, cyclin D and cyclin E expression did not change after treatment with 100 nM insulin (Figure 7-7). Taken together with the flow cytometry results, which did not indicate an effect of insulin on the velocity of cell cycle progression (see section 3.3.5), there is no indication that insulin effects cell cycle progression in MCF-10A breast epithelial cells.

In MCF-10A breast epithelial cells, detection of bound Annexin-V significantly decreased after 24 h treatment with 100 nM insulin, while 7AAD-staining was not affected (Figure 7-5). This suggests the conclusion that insulin may decrease early

apoptosis in MCF-10A breast epithelial cells incubated in serum-free medium. The result of decreased apoptosis in MCF-10A cells support the findings from the BrdU-incorporation assay in that the effect of insulin on promoting cell growth in MCF-10A cell is two-fold, by increasing cell proliferation and decreasing apoptotic rate. Both are key features of cancer cells, thus further suggesting that insulin may play a role in the possible carcinogenic transformation of MCF-10A cells. There was no change in the amount of cells with high 7-AAD staining, suggesting that insulin does not affect late apoptosis or necrosis in MCF-10A cells after 24 h of treatment. One may be interested in extending the treatment time to evaluate, if the insulin effect further translates when more cells enter late stages of apoptosis with increased incubation in serum-free medium time.

In MCF-10A breast epithelial cells, BCL-2 expression did not change with 100 nM insulin treatment for between 15 min and 120 min (Figure 7-6, B). Thus initially the BCL-2 results do not support the results obtained from the Annexin-V expression analysis. Annexin-V expression however was not affected by insulin treatment after 5 h treatment (Figure 7-5), which was even longer than the maximum of 120 min treatment for BCL-2 gene expression. Thus potentially longer treatments may still provide an increase in BCL-2 that could support the anti-apoptotic effect of insulin on MCF-10A breast cancer cells.

The CancerPathway microarray resulted in an increase in expression of seventeen genes compared to a decrease in expression of three genes after insulin treatment was observed (in MDA-MB-231 cells an increase in expression thirteen genes compared to a

decrease in expression of sixteen genes). Among the notable gene expression increases was an increase in TNF-R, CDK-4, CDC-25 and EGF-R. This further suggests a more proliferative effect of insulin on MCF-10A breast epithelial cells compared to MDA-MB-231 breast cancer cells.

Combining the observations documented in chapter 3 and this chapter, MCF-10A breast epithelial cells are highly responsive to a challenge by high insulin concentrations. High concentrations of insulin increased cell proliferation, increased PI3-kinase and MAP-kinase cell signalling. Insulin may also promote an increase in cancer pathway gene expression and decrease apoptosis. In MCF-10A breast epithelial cells, high insulin concentrations did not have effect on cell cycle progression.

7.4.3 Additional effects of insulin treatment on human SK-BR-3 breast cancer cells

In SK-BR-3 breast cancer cells, Annexin-V expression and 7-AAD staining did not change after 5 h of 100 nM insulin treatment. Treatment for 24 h however increased detection of bound Annexin-V and 7-AAD staining, with the increase in 7-AAD staining being statistical significant. This may suggest that treatment with insulin for 24 h may increase late apoptosis and necrosis in SK-BR-3 breast cancer cells. In the other results of the study presented here, insulin did not appear to have a growth promoting action on SK-BR-3 breast cancer cells. Cell proliferation and cell cycle progression were no affected by insulin treatment (see Figure 3-4 and Figure 3-11), while ERK1/2-phosphorylation was significantly decreased. Thus the combined findings would indicate a growth arresting and even apoptotic effect of insulin on SK-BR-3 breast cancer cells. Time constraints did not allow examining BCL-2 gene

expression in SK-BR-3 breast cancer cells to back-up these observations with an additional method. It would indeed be worth pursuing, if these cells respond consistently with increased apoptotic rate to insulin treatment. The possibility for SK-BR-3 breast cancer cells to decrease growth promotion after insulin treatment may be explained by insulin disrupting an autocrine signalling loop in these cells. This may however not necessarily translate into insulin being protective of breast cancer, but merely that the high insulin concentrations used in the study presented here may have this particular effect on one single breast cancer cell line.

In SK-BR-3 cells, gene expression of cyclin D was significantly increased after 15 min treatment with 100 nM insulin (Figure 7-7). Cyclin D is an activator of non-mitotic phases of the cell cycle. As such an increase in its expression could indicate a potential for insulin to increase cell cycle progression in SK-BR-3 breast cancer cells. On the other hand cell cycle analysis by flow cytometry did not indicate increased cell cycle progression with insulin treatment after 24 h (see section 3.2.5). It may however be difficult to conclude a connection of an increase in gene expression after 15 min treatment to cell cycle changes after 24 h treatment especially in the light that cyclin D expression did not change at any other examined treatment time. Similar to cyclin D gene expression increases, cyclin E gene expression increased in SK-BR-3 breast cancer cells between 15 min and 120 min treatment with 100 nM insulin. Cyclin E, when complexed with CDK-2, promotes cell cycle progression through S-phase. Surprisingly, even though the increase is non-significant, cell cycle analysis in chapter 3 showed a slight increase in G1-phase population with insulin treatment (Figure 3-11). Thus the increase in cyclin E and the cell cycle analysis are not conclusively promoting cell cycle

progression. The difference in treatment time however may play an important role in these findings. Cyclin E expression is highest after 15 min of treatment and steadily declines up to 120 min of treatment. Similarly cyclin D expression was only significantly increased after 15 min of treatment. Thus insulin may act to increase cell cycle progression in the short-term in SK-BR-3 breast cancer cells. Hence cell cycle analysis at shorter treatment times may provide additional information to the short acting properties of insulin on cell cycle in SK-BR-3 breast cancer cells. An evaluation experiment after three and six hours of insulin treatment however did not indicate a significant change. On the other hand all changes that were observed in a number of experiments to this point did not show several-fold changes, but rather subtle ones. Thus further analysis is needed to determine changes in cell cycle after shorter treatment times in SK-BR-3 breast cancer cells.

SK-BR-3 breast cancer cells were included in this study to provide some evaluation of the influence of the MAP-kinase pathway member mutation in MDA-MB-231 breast cancer cells. Many of the aspects of SK-BR-3's different behaviour to MDA-MB-231 cells following treatment with insulin however may not exclusively be explained by differences in MAP-kinase cell signalling, but may be caused by other differences in these cells, most notably the difference in expression of the erbb2-gene product, HER2 (MDA-MB-231 low, SK-BR-3 high) Combining the observations described in chapter 3 and this chapter high insulin concentrations increase PI3-kinase but decrease MAP-kinase cell signalling pathways. Furthermore insulin may play a part in increasing cell cycle progression in these cells. Insulin did not have an influence on cell proliferation or apoptosis in these cells. As such their physiological response to insulin is similar to that

of MDA-MB-231 breast cancer cells, some of the morphological changes are however different, most notably the opposite reaction of MAP-kinase pathway activation with insulin treatment (MDA-MB-231 cells increase, SK-BR-3 decrease), which may be explained by the MAP-kinase pathway member mutations in MDA-MB-231 breast cancer cells.

7.5 Summary

The study presented here, along with previous studies, suggests involvement of insulin resistance in breast cancer aetiology and progression. All cell lines were affected by insulin treatment. Insulin specifically increased growth of MCF-10A breast epithelial cells, by increasing cell proliferation and decreasing apoptosis. MDA-MB-231 breast cancer cells showed an increase in IR-phosphorylation and PI3-kinase and MAP-kinase cell signalling. In SK-BR-3 breast cancer cells insulin treatment significantly increased cyclin D and cyclin E gene expression. Thus there is much evidence to suggest that insulin alone is a key mediator of the molecular link between obesity and postmenopausal breast cancer.

Chapter 8

Discussion

8 DISCUSSION

8.1 Chapter outline

In the previous chapters, the necessity for further exploration of the molecular connections between obesity and postmenopausal breast cancer was established (chapter 1). Then a description of the experimental approach was given (chapter 2). In the following the different results from the experimental approaches were described and briefly interpreted (chapter 3 to 7). In this chapter the data will be summarised and differences between cell lines and treatments pointed out to provide a most comprehensive overview of the results. An in-depth comparative review of the obtained results with findings in the literature will be omitted; key issues arising from the literature however will be discussed. These two points will be used to examine the investigated factors for their involvement in potentially mediating the increased risk of developing breast cancer in obese postmenopausal women. A potential mechanism, by which obesity may initiate and promote progression of breast tumours, will be hypothesised. Lastly the strength and weaknesses of the used experimental approach as far as not covered in the previous chapters will be demonstrated and unanswered or new arisen research questions will be identified and formulated.

8.2 Comparison of effect of insulin, leptin, TNF-*α*, IL-6 and adiponectin all cell lines

In this section the results outlined in chapters 3 to 7 are compared to identify the obesity-related factor that is most influential in mediating cell proliferation, activation of cell signalling pathways, cell cycle progression and inhibition of apoptosis.

8.2.1 Cell proliferation

In MDA-MB-231 breast cancer cells, cell proliferation decreased after treatment with 100 nM insulin after 48 h treatment (see Figure 3-4) and increased after treatment with 100 nM leptin for 24 h treatment (see Figure 4-1). Both changes are rather subtle with 18% decrease with insulin treatment and 21% increase with leptin. All other treatments (TNF-α, IL-6 (see footnote 1), adiponectin) did not change cell proliferation in MDA-MB-231 breast cancer cells. In MCF-10A normal breast epithelial cells, cell proliferation increased with insulin, leptin, TNF-α and IL-6 treatment. In terms of effectiveness of cell proliferation increases, insulin increased cell proliferation the most (184% increase), followed by TNF-α (26% increase) after 24 h of treatment. Leptin and IL-6 did not have an effect on cell proliferation after 24 h treatment. After 48 h of treatment insulin, TNF-α and IL-6 increased cell proliferation equally (34% increase for insulin and 38% increase for both TNF-α and IL-6). Leptin increased cell proliferation by 14% after 48 h treatment. All treatments increased cell proliferation (see Table 8-1). Adiponectin did not change cell proliferation significantly after 24 h or 48 h treatment. SK-BR-3 breast cancer cells showed a maximum increase in cell proliferation after treatment with 6.25 nM leptin treatment for 24 h and similarly for 48 h. In SK-BR-3 breast cancer cells, cell proliferation increased by 31% and 59% after TNF-α treatment. Neither insulin nor IL-6 increased cell proliferation significantly in these cells.

Adiponectin decreased cell proliferation at both concentrations tested (250 ng/ml and 500 ng/ml), but only after 24 h treatment (Table 8-1).

Table 8-1: Summary of all changes in cell proliferation for all treatments and all methods. ↑ increase, ↓ decrease, ↔ no change, NA not analysed

	Insulin				Leptin	TNF-α	Adiponectin	IL-6
Cell proliferation	BrdU	MTT	MTT (+FCS)	PCNA	BrdU	BrdU	BrdU	BrdU
MDA-MB-231	↓	↔	↑	↑	↑	↔	↔	↑[1]
MCF-10A	↑	NA	NA	↔	↑	↑	↔	↑
SK-BR-3	↔	NA	NA	NA	↑[2]	↑	↓	↔

Thus there is no strong trend that would favour a particular adipokine or insulin as the main proliferative signal that could solely explain the link between obesity and breast cancer. Insulin increased proliferation, measured by BrdU-incorporation, only in MCF-10A cells. Additional experiments however suggest that insulin may also increase proliferation in MDA-MB-231 cells. Leptin and IL-6 both increased proliferation in MDA-MB-231 cells and MCF-10A cells, but not in SK-BR-3 cells. Conversely TNF-α increased cell proliferation in MCF-10A and SK-BR-3 cells, but not MDA-MB-231 cells. Adiponectin decreased cell proliferation only in SK-BR-3 cells. Thus different cells are differently affected by different adipokines and insulin, suggesting an individualised mechanism, by which obesity affects postmenopausal breast cancer, rather than a single common link. It also suggests that all examined factors may play a role in cell proliferation in these cells.

[1] Treatment of MDA-MB-231 breast cancer cells with 10 ng/ml IL-6 increased cell proliferation by 4% after 24 h treatment, which was statistical significant. However the low total increase was determined to not represent a physiological significant increase in proliferation (section 6.4.2).

[2] Cell proliferation increased only at low leptin concentrations than used for the majority of the other experiments. The maximum increase in cell proliferation was observed at 6.25 nM leptin treatment after 24 h and 48 h treatment. Statistically significant increases in cell proliferation were also observed for other leptin concentrations.

8.2.2 Phosphoinositide-3 kinase pathway

Phosphosinositide-3 kinase cell signalling pathway was activated by insulin, but not by leptin or TNF-α in MDA-MB-231 breast cancer cells. Activation of the PI3-kinase cell signalling pathway in MCF-10A cells increased after treatment with insulin, but not with leptin or TNF-α treatment. In SK-BR-3 breast cancer cells AKT-phosphorylation is significantly increased after treatment with insulin and TNF-α. The increase in AKT-phosphorylation was similar, with insulin treatment increasing AKT-phosphorylation by 67% after 5 min compared to control (Figure 3-6), while the AKT-phosphorylation increase after TNF-α treatment was 66% after 5 min (Figure 5-2). AKT-phosphorylation however was still significantly increased after 10 min and 20 min with insulin treatment.

Table 8-2: Summary of all changes in AKT-phosphorylation for all treatments. ↑ increase, ↓ decrease, ↔ no change

AKT-phosphorylation	Insulin	Leptin	TNF-α
MDA-MB-231	↑	↔	↔
MCF-10A	↑	↔	↔
SK-BR-3	↑	↔	↑

Thus activation of the PI3-kinase pathway seem specific for insulin treatment in all cell lines, the degree of increase however was highest in MDA-MB-231 breast cancer cells (294% after 5 min), followed by MCF-10A (75% after 5 min) and SK-BR-3 (67% after 5 min). This reflects the same patterns as observed for the IR-phosphorylation (Figure 3-5), suggesting that IR-phosphorylation translates directly to increases in AKT-phosphorylation. Leptin did not affect AKT-phosphorylation in any cell lines, thus the PI3-kinase pathway may not be a target for leptin signalling in these cells. AKT-phosphorylation was also significantly increased after TNF-α treatment in

SK-BR-3 breast cancer cells and may mediate some of its effects, e.g. increase cell proliferation. A correlation between increased cell proliferation and increased AKT-phosphorylation, which would suggest PI3-kinase mediated cell proliferation, is observed in insulin treated MCF-10A cells and in TNF-α treated SK-BR-3 cells. Both treatments however also increased ERK1/2-phosphorylation, thus suggesting that, at least in part, their signal may be mediated through the MAP-kinase cell signalling pathway.

8.2.3 Mitogen-activated protein kinase pathway

In MDA-MB-231 breast cancer cells, ERK1/2-phosphorylation increased by 174% with insulin treatment after 20 min (section 3.3.3.1), but decreased by 20% with leptin treatment after 20 min (section 4.3.2) and by 22% with TNF-α treatment after 5 min (section 5.3.2). Using Western Blotting, ERK1/2-phosphorylation increased non-significantly by 54% after 30 min of insulin treatment (section 7.3.2). In MCF-10A breast epithelial cells, phosphorylation of ERK1/2 increased after treatment with insulin, leptin and TNF-α. In terms of amount of increase in activation, TNF-α increased ERK1/2-phosphorylation the most (144% increase; see section 5.3.2), followed by leptin (78% increase; see section 4.3.2) and insulin (29% increase; see section 3.3.3.1). In SK-BR-3 breast cancer cells, ERK1/2-phosphorylation increased by 47% after 5 min TNF-α treatment. Insulin treatment (24% decrease after 15 min) and leptin treatment (34% decrease after 15 min) decreased ERK1/2-phosphorylation significantly (summarised in Table 8-3).

Table 8-3: Summary of all changes in ERK1/2-phosphorylation for all treatments and all methods. ↑ increase, ↓ decrease, ↔ no change, NA not analysed

	Insulin		Leptin	TNF-α
ERK1/2-phosphorylation	ELISA	Western	ELISA	ELISA
MDA-MB-231	↑	↔	↓	↓
MCF-10A	↑	NA	↑	↑
SK-BR-3	↓	NA	↓	↑

Thus there is a trend for all adipokines and insulin to increase phosphorylation of ERK1/2 in at least one cell line. Except for leptin-mediated increase in cell proliferation, all other observed increases in ERK1/2-phosphorylation correlate with an increase in cell proliferation, suggesting MAP-kinase cell signalling pathway activation to be responsible for the majority of adipokine and insulin induced increases in cell proliferation in these cell lines. There was however one anomaly in MDA-MB-231 breast cancer cells, where increased ERK1/2-phosphorylation did not result in increased cell proliferation. Leptin treatment increased cell proliferation, yet ERK1/2-phosphorylation was decreased, suggesting that, as also AKT-phosphorylation was not affected, leptin induced cell proliferation through activation of a different pathway (see also section 4.4.1).

8.2.4 Cell cycle

In MDA-MB-231 breast cancer cells, cell cycle profiles changed with insulin treatment and TNF-α treatment, but not with leptin treatment. Insulin treatment increased S-phase population by 13% after 24 h treatment (see section 3.3.5), while 24 h TNF-α treatment decreased G1-phase population by 6% and S-phase population by 15% with a simultaneous 27% increase in the amount of cells with subG1 DNA content (section 5.3.3). Cell cycle analysis of MDA-MB-231 breast cancer cells after TNF-α treatment

also suggested an increase in apoptosis after treatment, as the subG1 population increased significantly (see section 8.2.5 and 5.4.1). In MCF-10A breast epithelial cells no significant changes in distribution of cell population across cell cycle stages were observed with any of the examined treatments. In SK-BR-3 breast cancer cells insulin and did not significantly alter distribution of the cell population across cell cycle stages. Insulin however increased gene expression of cyclin D by 18% and cyclin E by 22% after 15 min of treatment (see section 7.3.5). Treatment with TNF-α significantly decreased S-phase population by 25% and G2-phase population by 24% in favour of a non-significant increase in subG1-phase population (see section 5.4.1)

Table 8-4: Summary of all changes in cell cycle for all treatments and all methods. ↑ increase, ↓ decrease, ↔ no change

	Insulin			Leptin	TNF-α
Cell cycle	PI-staining	cyclin D	cyclin E	PI-staining	PI-staining
MDA-MB-231	↑ S-phase	↔	↔	↔	↑ subG1 ↑ G1-phase ↓ S-phase
MCF-10A	↔	↔	↔	↔	↔
SK-BR-3	↔	↑	↑	↔	↓ S-phase ↓ G2-phase

From these results it can be seen that the cell cycle of breast cancer cell lines is affected with treatment (Table 8-4). Leptin treatment did not change cell cycle in any of the cells. The other treatments, insulin and TNF-α, seemed to have opposite effects on cell cycle. Insulin increased S-phase population in MDA-MB-231 breast cancer cells and cyclin gene expression in SK-BR-3 breast cancer cells, indicating growth promoting properties on both cells. TNF-α on the other hand, seemed to inhibit cell cycle progression and, at least in MDA-MB-231 breast cancer cells, may also have increased apoptosis. Of all adipokines and insulin tested, TNF-α had the highest effect on cell

cycle progression. Taking into account the large, but just above significant level (p=0.051), decrease in G1-phase in MCF-10A cells into account, TNF-α had opposing effects on cell cycle in breast epithelial and breast cancer cells.

8.2.5 Apoptosis

In MDA-MB-231 breast cancer cells, 24 h treatment with TNF-α increased the amount of cells, whose DNA amount was below that of the G1-phase significantly by 27% (see section 5.3.3). In MCF-10A breast epithelial cells, 24 h treatment with insulin decreased detection of bound Annexin-V by 13% (see section 7.3.3.1). In SK-BR-3 breast cancer cells, 24 h treatment with insulin increased the amount of cells with high 7-AAD-staining by 9% (see section 7.3.3.1).

Table 8-5: Summary of all changes in apoptotic rate for all treatments and all methods.
↑ increase, ↓ decrease, ↔ no change, NA not analysed

	Insulin				Leptin	TNF-α
Apoptosis	Annexin-V	7-AAD	BCL-2	subG1	subG1	subG1
MDA-MB-231	↔	↔	↔	↔	↔	↑
MCF-10A	↓	↔	↔	↔	↔	↔
SK-BR-3	↔	↑	NA	↔	↔	↔

Using the methods summarised in Table 8-5, it was observed that insulin and TNF-α, but not leptin had an effect on apoptosis. All cell lines were affected however by at least one treatment. Insulin decreased early apoptosis in MCF-10A cells and increased late apoptosis in SK-BR-3 cells. Leptin did not affect apoptosis, while TNF-α treatment may increase apoptosis in MDA-MB-231 breast cancer cells.

8.3 Insulin, leptin and TNF-α as mediators of increased breast cancer risk in obese postmenopausal women

MDA-MB-231 breast cancer cells were the prime example of ER-negative breast cancer cells to examine the impact of insulin, leptin and TNF-α in an oestrogen independent manner. In MDA-MB-231 breast cancer cells only leptin induced cell proliferation (see footnote 1). The increase in leptin-induced proliferation in MDA-MB-231 cells is low compared to insulin-induced cell proliferation increases in MCF-10A cells (21% with leptin vs. 184% with insulin), increasing speculations on the physiological impact on this cell line, especially as other leptin concentrations (25 nM and 50 nM) showed a decrease in proliferation after 48 h treatment. The leptin induced increase in cell proliferation is most likely not mediated by the PI3-kinase or MAP-kinase cell signalling pathway, as no increase in AKT- or ERK1/2-phosphorylation with leptin treatment was observed, making the JAK2/STAT3 pathway the most likely candidate for this connection, which should be assessed in further experiments (see sections 4.4.1 and 8.10). Cell proliferation after insulin treatment in MDA-MB-231 cells was assessed with a variety of techniques, returning conflicting results. Thus the impact of insulin on cell proliferation in MDA-MB-231 cells is inconclusive. Some discrepancies were observed with insulin treatment, which resulted in much higher increases of IR-phosphorylation, AKT-phosphorylation and ERK1/2-phosphorylation compared to the other two cell lines. None of these changes however convincingly increased cell proliferation or reduced apoptosis and the impact of the increase in cell cycle progression (S-phase population was increased) may be negligible. TNF-α exerted generally cell inhibitory effects on MDA-MB-231 breast cancer cells and may not increase proliferation or growth in these cells. Thus obesity-associated factors insulin, leptin and TNF-α may have some effects on MDA-MB-231 breast cancer cells. Many of

the observed changes however were not conclusive and their actual impact on these breast cancer cells may be negligible. Other adipokines, which were not yet tested on these cells, may also contribute to the link between obesity and ER-negative advanced breast cancer cells.

All adipokines thought to increase proliferation were found to do so in MCF-10A breast epithelial cells[3]. This is an interesting and promising observation in linking these adipokines to higher risk of developing breast cancer in postmenopausal obese women. Diagnostic or treatment benefits may however be difficult to achieve, as many different factors are involved in mediating cell proliferation in MCF-10A cells. Insulin may be the best marker; this cell model has however some shortcomings regarding insulin (see section 8.8.1). It may be of great interest to examine cell proliferation after a combination of two or more adipokines and insulin to examine the total effect adipokines have on promoting cell proliferation in MCF-10A cells. A combination of several factors may better reflect the physiological environment in the obese. Furthermore examination of several adipokines and insulin may provide insights into whether these factor act additive, synergistic or inhibitory in combination. Finding these links may provide targets for disrupting adipokine cross-communications, unknown to date[4]. In summary, MCF-10A breast epithelial cells increase in cell proliferation after treatment with insulin, leptin, TNF-α and IL-6. Compared to the other treatments, TNF-α increased phosphorylation of ERK1/2 the most (144% increase for TNF-α, 78%

[3] The only adipokine not to increase cell proliferation in MCF-10A breast epithelial cells was adiponectin (see section 6.3.1), however adiponectin was hypothesised to decrease or to not change cell proliferation.

[4] A summary of known and hypothesised cross-talks of adipokines with other cytokines is attempted in section 8.6.

for leptin, 29% for insulin). The resulting increase in cell proliferation however is below that observed for insulin, indicating no correlation between ERK1/2-phosphorylation and cell proliferation. Thus all treatments, for which ERK1/2-phosphorylation was assessed, increased ERK1/2-phosphorylation in MCF-10A breast epithelial cells. As all treatments also increased cell proliferation it may be suggested that ERK1/2-phosphorylation may be responsible for mediating the majority of induced increases in cell proliferation. Other cell signalling pathways however may also be involved in mediating this proliferative effect, as the amount of ERK1/2-phosphorylation increase did not correlate to the amount of change in cell proliferation.

SK-BR-3 breast cancer cells were added to the study as a second ER-negative breast cancer cell line, primarily to examine the impact of the RAS and RAF mutations in MDA-MB-231 on insulin, leptin and TNF-α mediated changes in cell proliferation and especially in MAP-kinase cell signalling pathway activation with these obesity associated factors. Cell proliferation was strongly and significantly increased with lower leptin concentrations than used for the other experiments. The maximum increase in cell proliferation was around 6.25 nM leptin. No increase in cell proliferation however was observed at 100 nM leptin, which was the concentration used for the other experiments and may thus explain why leptin treatment did not have any other effect on this cell line. Cell proliferation also increased with TNF-α treatment, which can conclusively be backed up with an increase in ERK1/2-phosphorylation and the cell cycle data to support a significant growth promoting signal by TNF-α on these cells.

In order to investigate the influence of RAS and RAF mutations on these factors one may compare ERK1/2-phosphorylation between MDA-MB-231 cells and SK-BR-3 breast cancer cells by referring to Table 8-3. Insulin treatment increased ERK1/2-phosphorylation in MDA-MB-231 breast cancer cells, while it was decreased in SK-BR-3 breast cancer cells. TNF-α treatment increased ERK1/2-phosphorylation in SK-BR-3 breast cancer cells, while it was reduced in MDA-MB-231 breast cancer cells. Leptin treatment decreased ERK1/2-phosphorylation in both cell lines. It is highly unlikely that any of these observations could be explained by RAS and RAF mutations in MDA-MB-231 breast cancer cells and are more likely caused by specific actions of the treatment. Furthermore the excessive increase in ERK1/2-phosphorylation after insulin treatment in MDA-MB-231 breast cancer cells (easily the highest increase in ERK1/2-phosphorylation observed in the study presented here) after insulin treatment may not be caused by these mutations either, but rather be a reflection of an equally high increase in IR phosphorylation and AKT-phosphorylation, which in turn may be caused by the previously observed IR gene amplification in MDA-MB-231 breast cancer cells (Milazzo *et al.*, 1992). Results from SK-BR-3 breast cancer cells imply that TNF-α may be an important obesity associated factor that could affect proliferation and progression of this type of breast cancer.

8.4 Additional mediators

8.4.1 Oestrogen

Besides hyperinsulinaemia and impaired adipokine secretion, increased postmenopausal oestrogen exposure was suggested as a possible link between obesity and breast cancer (Lorincz and Sukumar, 2006). Obesity affects oestrogen exposure dependent on

menopausal status. Prior to menopause, obese women are exposed to lower concentrations of oestrogen compared to normal weight women. This may be caused by an interference of obesity with ovarian oestrogen production, most notably by a strong association of obesity with polycystic ovarian syndrome (PCOS). After menopause however oestrogen concentrations in normal weight women decrease, while adipose tissue in obese women continues to produce oestrogen and oestrogen concentrations are now higher than in normal weight women. This increases lifetime exposure to oestrogen, which is a contributing factor of increased breast cancer risk (Lippman *et al.*, 2001). Thus oestrogen is another hypothetical factor in mediating the obesity breast cancer link. With oestrogen being closely linked to the presence of its own receptor its effect is limited to a particular kind of cancer, unless oestrogen independent growth is a development of all breast cancers and not an *ab initio* determined feature. Even then, oestrogen would have no influence on breast cancer once it had developed ER negativity, *i.e.* in the advance stages of breast cancer development, which thus far have greatly eluded explanations for their independent growth mechanism.

8.4.2 IGF-1

The research field investigated in this study has long struggled with the paradox that epidemiological studies continuously link hyperinsulinaemia and type-2 diabetes with increased breast cancer risk and mortality and the apparent lack of insulin to stimulate breast cancer growth in *in vitro* and xenograft studies. In an attempt to address the issue, an axis from obesity to insulin resistance to increased insulin-like growth factor concentrations to breast cancer has been proposed (Kaaks and Lukanova, 2001; Kazer, 1995). The attraction to propose this connection derives from the observation that

insulin stimulates hepatic IGF-1 secretion (Boni-Schnetzler *et al.*, 1991). Thus, with higher insulin concentrations present in the obese, the suggestion of increased IGF-1 concentrations with obesity-related hyperinsulinaemia seems viable. Additionally, IGF-1 is a potent mitogen for cell growth and has been observed to increase cell proliferation of breast cancer cells (Rosen *et al.*, 1991). Furthermore increased IGF-1 concentrations have been identified as increasing risk for breast cancer development (Hankinson *et al.*, 1998). Indeed many breast tumours and other cancers may be susceptible to inhibiting IGF-1 signalling (Sachdev and Yee, 2007). Furthermore studies indicate possible interactions between IGF-1 and oestrogen (Lanzino *et al.*, 2008; Parisot *et al.*, 1999) and IGF-1 and HER2 (Esparis-Ogando *et al.*, 2008), suggesting the possibility of IGF-1 signalling inhibition in order to increase efficacy of therapies targeting ER or HER2. Thus IGF-1 plays an important role in breast cancer development and progression and increased IGF-1 circulating concentrations should be taken as indicators of increased breast cancer risk. Likewise IGF-1 signalling inhibition is a most promising field of breast cancer therapy and could be exploited to decrease resistance to non-toxic breast cancer therapies like tamoxifen and herceptin. Indeed the therapeutic potential of targeting IGF-1 signalling seems a far more promising target than insulin or any of the examined adipokines. Thus theoretically, there seems to be good evidence connecting insulin resistance with IGF-1 expression and IGF-1 concentrations with breast cancer.

A connection between IGF-1 concentrations and breast cancer has however only been observed for pre-menopausal breast cancer risk (Schernhammer *et al.*, 2006). Thus there should be some concern to use IGF-1 as a contributing factor for postmenopausal breast

cancer risk. Furthermore there is considerable evidence that obesity and IGF-1 concentrations do not correlate linearly (Frystyk *et al.*, 1995; Frystyk *et al.*, 1999; Frystyk *et al.*, 2009; Renehan *et al.*, 2006). These authors hypothesised that IGF-1 concentrations would decrease as it has been known that obesity decreases GH secretion, the major stimulant for IGF-1 secretion. They were surprised by their observation that IGF-1 concentrations did not significantly decrease in moderately obese individuals (30 kg/m^2<BMI<35 kg/m^2) but only in the severely obese (BMI>35 kg/m^2). They concluded that obesity-related hyperinsulinaemia can compensate partly for the obesity-related GH decrease, with insulin being however a less potent stimulant than GH for IGF-1 secretion, hyperinsulinaemia cannot compensate for the obesity-induced decrease in GH secretion. This is reflected in the observation that IGF-1 concentrations were highest in overweight women with BMIs between 26-27.9 kg/m^2 and lower for both lean (BMI<20 kg/m^2) and obese women (BMI>30 kg/m^2, Allen *et al.*, 2003). Additionally a recent study observed increased IGF-1 concentrations after weight-loss in obese men, which suggested decreased IGF-1 concentrations in obesity (Belobrajdic *et al.*, 2010)[5]. Furthermore trying to evaluate the underlying factors of the obesity-breast cancer connection, Gunter and colleagues (2009) observed no correlation between BMI and IGF-1 concentrations in women that had never used hormone replacement therapy (HRT). The authors concluded that hyperinsulinaemia itself is responsible for mediating the obesity-breast cancer connection, rather than hyperinsulinaemia causing increasing IGF-1 concentrations,

[5] The study subjects were 76 men with a mean BMI of 32.8 kg/m^2 prior to intervention. Weight loss and increase in IGF-1 concentrations were independent of dietetic approach (high carbohydrates (HC) vs. high protein (HP). Mean weight loss was 8.2 kg (HC) and 8.5 kg (HP). Total IGF-1 increased by 18% (HC) and by 23% (HP); bioactive (free) IGF-1 increased by 15% (HC) and 18% (HP).

which in turn increases breast cancer risk. Thus there are considerable doubts about the significance of the obesity-IGF-1 connection. This leaves to draw two conclusions. First despite being a promising target to explain the obesity-postmenopausal breast cancer connection, IGF-1 does not correlate with either of them. Further investigation may provide however a link involving IGF-1 in the obesity-breast cancer scenario, *e.g.* in the adipocyte-tumour cell microenvironment of the breast or the life-time exposure to IGF-1, which was suggested by Rollison and colleague (2006[6]). Additionally, the possibility of an oestrogen-IGF-1 connection could be important in that obesity increases bio available oestrogen, which in turn could increase IGF-1 signalling (see section 8.6). Second, IGF-1 is an important risk marker for breast cancer and inhibition of IGF-1 signalling is a possible treatment option for pre-menopausal breast cancer. Thus its apparent non-involvement in the obesity-breast cancer connection should not distract from its important role in breast cancer. Indeed early clinical trials in cancer patients suggest benefits by inhibition of IGF-1 receptors alone or in combination therapies (Weroha and Haluska, 2008).

8.5 Insulin and adipokines as breast cancer initiators and inducers of autocrine self-sustenance: a hypothesis

In the study presented here, the effects of one isolated factor on breast cancer at a time were considered. It is of no doubt that in an individual all the examined factors, in addition to others, will have an influence on breast cancer aetiology and progression. This effect might be additive, synergistic, competitive, or mutually inhibitory. With an array of different growth promoting factors at its disposal, it might be conceivable that

[6] The authors of this case-control study measured IGF-1 concentrations in pre-menopause and examined its correlation with post-menopausal breast cancer. However no significant connection was observed (Rollison *et al.*, 2006).

the individualised breast tumour will exploit one, or perhaps a selected group of obesity related factors, in order to achieve and maintain malignant growth. The development of ER-positive breast tumours may act as an example. Tumours achieve increased and sustained growth by increasing the amount of ER and exploiting its proliferative signal through modifications of cell signalling pathways, gene expression or suppression of apoptosis or a combination of all. In the obese patient, displaying signs of Metabolic Syndrome, a multitude of cytokines, hormones and other obesity related factors are in disarray. Several of these factors have increased circulatory concentrations in the obese, such as insulin, leptin, TNF-α and IL-6. The here presented results along with previous findings from other researchers indicate that many of these factors induce molecular changes in breast cancer cells and breast epithelial cells indicative of increased malignancy.

While not directly assessed, one is inclined to speculate on the possible way, by which these increased circulatory factors in the obese postmenopausal women may promote and sustain malignant growth of the breast epithelium. In the obese setting a constant exposure to the obesity-associated growth promoting factors may increase cell proliferation in ductal or lobular breast endothelial cells. Evidence for this has been observed in the study presented here, where insulin (section 3.3.1), leptin (section 4.3.1), TNF-α (section 5.3.1) and IL-6 (section 6.3.2) increased cell proliferation of MCF-10A breast epithelial cells. This increased proliferation may be balanced by other outside regulating factors that counteract the increased growth promoting signal, or may be balanced within the healthy cell to remain within consistent proliferation. The epithelial cells will however remain under this increased proliferative pressure exerted

by increased adipokine availability. DNA mutation events occur continuously over life-time and may affect at random a gene or the regulation of its expression. Mutations in those genes whose products have thus far inhibited the increased proliferative pressure may alter this regulatory mechanism and combined with other factors may tip a healthy epithelial cell towards malignant growth and carcinogenesis (Calvin, 1975). Some of these mutations can for example be seen in one of the breast cancer cell lines used in the cell model of the study presented here, without being necessarily involved in the carcinogenic process. MDA-MB-231 breast cancer cells have mutations in two of the kinases (KRAS and BRAF) involved in mediating cell signalling within the MAP-kinase cell signalling pathway (Hollestelle *et al.*, 2007). These mutations are generally not found in healthy cells.

One may also speculate on whether the increased proliferative pressure exerted by the obesity-associated factors may increase the likelihood of any such mutations occurring, *i.e.* increased proliferative pressure itself acting mutagenic. While not inconceivable, the connection of forced DNA replication errors as a result of increased proliferative pressure seems unlikely. Thus one will proceed with the idea that a mutational chance event, independent of obesity, may manifest itself more readily in the obese than the normal weight women on the observed fact of increased proliferative signalling, and not on a potentially increased mutagenic pressure exerted by obesity associated factors. For example, obesity increases oestrogen availability in the obese postmenopausal woman. This does not lead *per se* to an increase in carcinogenesis or proliferation, if regulatory mechanisms, *e.g.* decrease in ER expression are in place in the healthy cell. A randomly occurring mutagenic event however decreases this regulatory mechanism and ER

expression is increased, which aids in carcinogenesis of the cell[7]. In the normal weight postmenopausal woman this event is not as dramatic as in the obese postmenopausal woman, since only the obese woman has increased oestrogen concentrations, which now exert an increased proliferative signal on those cells. Similar events are conceivable for insulin, leptin, TNF-α and other adipokines.

Taking into account the rarity of mutagenic events, it seems conceivable that of the multitude of available obesity associated growth promoting factors, it may, in the most simplified case, only be one of those factors that now exerts a growth promoting signal in the newly formed cancer cell. Thus in keeping in line with the previous example, a cancer may grow only on the proliferative signal of *e.g.* oestrogen, if a mutation has increased the amount of ER expression for example. This consideration derives its validity from the observation that early stage breast tumours, which display this increased ER expression, are susceptible to treatment such as tamoxifen. If other factors were in play, tamoxifen treatment would not be beneficial. This partly explains the benefits of early detection and indeed many tumours show favourable outcomes, if detected and treated early.

Other tumours however may not be detected and may develop oestrogen-independent growth. If oestrogen-independent growth occurs during tamoxifen treatment, the tumour develops tamoxifen resistance. There is also the possibility of the existence of *ab initio* oestrogen-independent cells within the tumour for which tamoxifen positively selects,

[7] Carcinogenesis involves in fact a number of mutations to develop a cancer cell. However for the here presented speculation only one will be assumed as necessary.

or which eventually become the dominant cell type of the tumour[8]. How can a tumour sustain oestrogen-independent growth? Going back to the initial situation of increased circulatory concentrations of a multitude of obesity associated growth promoting factors in the obese postmenopausal woman, it may be conceivable that the tumour or some of the tumour cells have developed a second way to promote growth by exploiting the growth promoting ability of *e.g.* leptin. Leptin receptor expression has been found in MDA-MB-231 breast cancer cells, which have achieved oestrogen-independent growth (Garofalo *et al.*, 2006). There is also an observation that leptin receptor expression is more common in metastasised, *i.e.* more advanced, breast cancer (Ishikawa *et al.*, 2004). The development of this phenomenon may again not be triggered by obesity, but if such an event were to happen, the obese environment would more readily manifest its physiological impact. Leptin may promote cancer growth on its own or employ other growth promoting receptors to increase malignancy (see section 8.6).

Lastly the breast tumour may develop growth independent of exogenous leptin, by increasing expression of leptin itself and thereby increasing independent growth. Thus obesity may affect the development and progression of breast cancer by increasing the effect of random carcinogenic mutational events, by facilitating the development of hormone independent growth and, as a result, promote adipokine autocrine self sustained growth. Indeed leptin expression has been observed in breast tumour samples (Ishikawa *et al*., 2004) and several breast cancer cell lines (O'brien *et al*., 1999).

[8] There is some awareness among researchers in the possibility that tumours quickly develop heterogeneity in their early development. Thus a resistance to a certain treatment may not develop during the treatment, even though this possibility is not excluded, but through treatment specifically selecting for tumour cells that were already treatment-resistance before the treatment commenced. If that be the case on a large scale than pre-screening of tumours becomes even more important and would give rise to a whole new treatment approach. However at the moment there are no facilities to investigate this interesting idea.

8.6 Cross-talk of adipokines and insulin with each other and other receptors as additional links in the obesity-postmenopausal breast cancer connection

There are some indications that obesity associated factors may promote cancer growth by activating not only their own receptor, but also other receptors and thus exploit additional growth promoting facilities. In MCF-7 breast cancer cells, leptin increased phosphorylation of HER2 to a similar degree as EGF did (Fiorio *et al.*, 2008). Furthermore Ob-R co-localised with HER2 suggesting physical interaction of these two receptors. Increased HER2 activity was suggested to increase cell proliferation in these cells (Fiorio *et al.*, 2008). Thus besides acting through its own receptor to increase growth, leptin mediates its proliferative effect through cross activation of HER2. Additionally a possible cross talk between Ob-R and IGF-R has been reported (Ozbay and Nahta, 2008; Saxena *et al.*, 2008). Both studies examined the effect of leptin and/or IGF-1 on MDA-MB-231 breast cancer cells amongst others. Both studies observed an increase in activation of Ob-R by IGF-1, only Saxena and colleagues (2008) however also observed an increase in IGF-1R phosphorylation after leptin treatment. Both studies also observed the highest increase in cell proliferation after co-treatment with leptin and IGF-1 (Ozbay and Nahta, 2008; Saxena *et al.*, 2008). There may also be a cross talk between insulin and IGF-1 as both IR and IGF-1R consist of dimers and, if both receptors are expressed in the same cell line may form hybrid receptors, one subunit from the IR and one subunit from the IGF-1R (Pandini *et al.*, 1999), which increases intra-cellular response to IGF-1 signalling and to a lesser extent insulin signalling (Pandini *et al.*, 2002). While cross-talk of Ob-R with other receptors has not been explored extensively yet, there is considerable knowledge of a cross-talk between IGF-1 and ER (Fagan and Yee, 2008). This cross talk may however not necessarily be

involved in the involvement of obesity in breast cancer as it involves the previously mentioned problem between IGF-1 and obesity.

8.7 Obesity as diagnostic marker for breast cancer risk

In this section it will be explored how findings of this study may aid the general and specialised physicians in identifying patients at-risk of obesity associated breast cancer. A female patient of peri-menopausal age presents at a clinic or GP. Measurements of body height, weight, waist circumference are used to identify total and abdominal obesity. Total and abdominal obesity are then part of the other usual characteristics of increased breast cancer risks, *i.e.*, current age, family history of breast cancer, age at menarche, age at first full-time pregnancy, HRT-use, alcohol consumption, breast parity. These are inexpensive examinations that could identify at-risk patients without expensive, uncomfortable or invasive screening procedures. An at-risk patient would then be identified by having several risk-factors, or by having one risk-factor at the extreme, *i.e.* having one or more first-degree relatives with breast cancer or having severe (BMI>35 kg/m^2) or morbid (BMI>40 kg/m^2) obesity. For these patients, mammography will exclude the existence of breast cancer. For patients with first-degree relatives with breast cancer, mutational analysis for BRCA1-2, will exclude them from the at-risk patient group. Analysis of adipokine, insulin, IGF-1 and oestrogen concentrations may allow exclusion of patients from the at-risk group. All non-excluded patients may be offered preventive treatments. For those with several risk-factors, life-style modifications[9], where possible, may reduce their risk, *e.g.* cessation of HRT-use, decrease alcohol consumption, increased physical activity, increased consumption of

[9] A full list of suggested life-style choices for the prevention of cancer has been published by the AICR (2007).

anti-oxidant-rich foods, etc. For patients with BRCA1-2 mutations increased screening should be applied and the possibility of preventive mastectomy should be offered[10] (Metcalfe, 2009). For severely and morbidly obese patients with increased serological risk-factors the possibility of bariatric surgery could be explored (McCawley *et al.*, 2009)[11]. Should that proof unavailable, a reduction in the serological risk-factors should be attempted. Body-weight reductions of only a few kilograms significantly improve the serological profile in obese patients, and may reduce breast cancer risk (Anderson and Caswell, 2009). Furthermore pharmacological intervention with glitazones, PPAR-γ agonists, which improves serological markers and metformin, an AMPK agonist, reduces hepatic gluconeogenesis, thus decreasing blood glucose and subsequently insulin secretion could be explored[12]. Preventive treatment with tamoxifen may also be indicated (Castrellon and Gluck, 2008). Thus, using obesity as a diagnostic marker, the identification of at-risk patients and the possibility of early intervention could be implemented for peri-menopausal women in an attempt to decrease breast cancer incidences.

8.8 Study shortcomings and strengths

8.8.1 Utilisation of a cell culture model

The utilisation of a cell culture model has several advantages compared to mouse models or indeed experiments on humans. A cell culture is easily maintained, if

[10] One may also be reminded that patients with BRCA1/2 mutations also carry an increased risk of ovarian cancer development and need to be advised on preventive options.

[11] Their study suggests that bariatric surgery may be used to prevent cancer development in obese women.

[12] In the last months several publications observed a decrease in breast cancer incidences in metformin users (Bodmer *et al.*, 2010; Gonzalez-Angulo and Meric-Bernstam, 2010). Furthermore metformin treatment of breast cancer cells *in vitro* decreased cell proliferation and cell cycle progression (Alimova *et al.*, 2009; Liu *et al.*, 2009).

facilities exist and an established cell culture provides sufficient cells for multiple experiments and repeatability. Furthermore experimentation on cultured cells does not require ethical approval from outside the research institute and thus increases availability to more researchers and increases research output. Comparison to data in the literature is possible, if long established cells are used, such as MDA-MB-231 breast cancer cells. The availability of cells with specific requirements is possible, such as ER-negative breast cancer cells, *e.g.* MDA-MB-231, and ER-positive breast cancer cells, MCF-7, allowing to create a cell culture model specific to the requirements of the research question. Long established cell lines have been examined for several of their properties[13].

There was a determined intention to address the influence of obesity on breast cancer aetiology and understand the cell mechanisms that may be affected. This aspect of the connection between increased body weight and postmenopausal breast cancer had not been explored in the available literature. In order to address this issue, MCF-10A, a non-cancerous immortal breast epithelial cell line was studied to examine the effects of insulin and certain adipokines. This cell line had the advantage to grow at a similar rate as MDA-MB-231 breast cancer cells, thus allowing experiments to be carried out simultaneously on both cell lines, sometimes on all three cell lines, when SK-BR-3 cells were available. Furthermore the cell line grows adherent, thus the procedure of maintaining and assay analysis was the same between the three cell lines. Of major concern using this cell line was its insulin dependence. Normal growth medium was supplemented with 10 µg/ml insulin. This corresponds to approximately 1.7 µM

[13] See section 2.1.1 for a description of previously published characteristics of the cell lines used in this study.

(molecular weight for insulin is 5808 Da). Since cell growth is dependent on insulin supplementation, the hypothesis that insulin will increase growth is essentially answered. This does not exclude this model however from being useful in this study's analysis. The dependence of MCF-10A cells on insulin is rather another indication of the importance insulin plays in the promotion of breast cancer. Furthermore the growth promoting effects of insulin on MCF-10A cells may seem obvious from their growth medium composition, the exact molecular mechanisms of insulin's action on these cells has not been examined and may provide insight on "where to look" in subsequent studies involving additional cell lines, mouse or patient samples. Thus even if the used insulin concentration was below the normal growth medium, this cell system provided the possibility to study the molecular mechanism involved in insulin signalling in breast epithelial cells. The utilisation of this cell line was therefore warranted to investigate the link between obesity and postmenopausal breast cancer.

Obesity is affecting breast cancer risk according to menopausal status. Epidemiological studies indicate no correlation, or a slightly inverse correlation, between premenopausal breast cancer and BMI, thus increased body weight may be protective of premenopausal breast cancer. There is however a strong correlation between BMI and postmenopausal breast cancer. Interestingly premenopausal breast cancer, though far less common than postmenopausal breast cancer, is more often aggressive and with less favourable outcomes (Vetto *et al.*, 2009). According to the supplied data sheet, MDA-MB-231 breast cancer cells were harvested from a 51 year old woman. Her menopausal status is not given and the derived MDA-MB-231 breast cancer cells may represent a pre-menopausal cancer. Likewise the observation of MDA-MB-231 breast cancer cells

being tripe-negative (ER, PR, HER2 negative) is a feature more often observed in pre-menopausal breast tumours. Thus the choice of MDA-MB-231 breast cancer cell line may be controversial as it may represent a pre-menopausal breast cancer, which are not influenced or indeed inhibited by obesity. Even if MDA-MB-231 breast cancer cells were derived from a post-menopausal woman, their triple-negative status does reflect only a small number of post-menopausal breast cancer cases.

8.8.2 Incubation in serum-free medium

One of the assumptions that arose from the utilisation of a cell culture model is that during normal culture growth, the culture will be heterogenic with regard to their cell cycle stage. Thus the cells are generally proliferating in no distinct pattern. In this heterogenic setting any treatment would affect the cells differently thus providing a different cellular response and bias the true result of the treatment. To overcome this problem, before each treatment the cells were incubated in serum-free medium, which did not contain serum supplements[14]. Incubation in serum-free medium decreases the availability of growth factors and hormones usually found in serum, thus decreasing the growth stimuli to the cells. As a result cell growth is inhibited. The stage at which cell growth can be significantly halted is the G1 stage[15]. At this stage treatment should exert a uniform effect on the cells. Thus successful incubation in serum-free medium should increase the G1-phase cell population and decrease the remaining S-phase and G2-phase populations. The effect of incubation in serum-free medium is crucial to the subsequent treatment. If incubation in serum-free medium does not induce a significant inhibition

[14] See section 2.1.2 for a description of serum-free medium. MCF-10A serum-free medium had also all additional supplements (insulin, hydrocortisone. EGF, cholera toxin) removed.

[15] If the cells remain in G1-phase significantly longer than the normal cell cycle, cells may enter a dormant stage known as G0.

of cell cycle progression the results may be biased or have decreased reproducibility, especially in short treatment times, where the diverse cell population may react increasingly different, according to how the cell population is distributed across the cell cycle stages. Only if a significant inhibition of cell progression can be achieved by incubation in serum-free medium will it be possible to reproduce a similar result after treatment.

There are some indications that in MDA-MB-231 breast cancer cells that incubation in serum-free medium did not have the desired effect. In early BrdU-assays there was no significant difference between cells in growth medium and cells incubated in serum-free medium [16] (incubation time 24 h). Both samples also synthesised high amounts of Formazan. Similarly early western blotting analysis examining ERK1/2-phosphorylation found phosphorylated ERK1/2 in cells incubated in serum-free medium for 24 h (see Appendix section 9.4.3), which should not be observable, as all exogenous growth factors had been removed by incubation in serum-free medium[17]. With no growth factors present, no activation of the cell signalling pathways was expected. Lastly comparing cell cycle in cells incubated in serum-free medium and serum-supplemented cells resulted in no significant difference in amount of cell in the G1-phase (Appendix section 9.3.3.1). Thus incubation in serum-free medium did not seem to arrest cell cycle in G1-phase.

[16] Total absorption values for serum supplemented cells were 1.83 vs. 1.68 for cells incubated in serum-free medium. By comparison the same values for MCF-10A cells were 1.77 and 0.3 for serum-supplemented and cells incubated in serum-free medium, respectively.

[17] For comparison, one may refer to Frankenberry and colleague's (2006) publication (Figure 4, p.989), where, using western blotting analysis, no phosphorylated ERK1/2 was observed after 20 h incubation in serum-free medium in MDA-MB-231 breast cancer cells.

Similarly measuring apoptosis by Annexin-V detection relied on the ability of incubation in serum-free medium to increase apoptotic rate. From the cell cycle data it may however be concluded that the subG1 DNA content, a measure of apoptotic cells, in MDA-MB-231 breast cancer cell showed a significant reduction in subG1 cells after incubation in serum-free medium compared to cells in serum-supplemented growth medium (Appendix section 9.3.3.2). Similarly detection of bound Annexin-V and 7-AAD staining was significantly increased in cells in growth medium, compared to cells incubated in serum-free medium (see Appendix 9.3.3.2). Thus incubation in serum-free medium did not induce apoptosis and may have actually decreased apoptosis, compared to cell in serum-supplemented medium.

In MCF-10A breast epithelial cells incubation in serum-free medium[18] did reduce BrdU-incorporation (see footnote 16). Analysis of cell cycle stages did however not show an increase in G1-phase population after incubation in serum-free medium (Figure 3-11). All other cell cycle phases were affected by incubation in serum-free medium. Most notably is the increase in sub-G1 content, which comes at the expense of S-phase and G2-phase populations. A DNA-content below G1-content, *i.e.* a DNA-content that reflects cells containing less than one complete set of chromosomes, indicates DNA-degradation, which in turn is indicative of increased apoptosis. This observation was different than the result for MDA-MB-231 breast cancer cells, where the sub-G1 population decreased after incubation in serum-free medium. In support of this observation, a similar result was observed using the Annexin-V expression analysis (see

[18] Reference to "incubation in serum-free medium" of MCF-10A cells shall be understood as incubation in medium containing no serum supplementation, and also no additional supplements (insulin, EGF, hydrocortisone, cholera toxin). Composition of MCF-10A serum-free medium can be found in section 2.1.2.

Appendix section 9.3.3.2). Annexin-V detection decreased after incubation in serum-free medium in MDA-MB-231 cells, but increased after incubation in serum-free medium in MCF-10A cells. Thus incubation in serum-free medium produced opposing effects in MDA-MB-231 and MCF-10A cells. This may have an effect on the results in the here presented study that were all observed after incubation in serum-free medium.

In SK-BR-3 breast cancer cells BrdU-incorporation was not assessed after growth in serum-supplemented medium. DNA-content and Annexin-V however were analysed after serum-supplemented growth. Interestingly SK-BR-3 cells were the only examined cell line that did not change their cell cycle profile after incubation in serum-free medium. While there were notable changes in sub-G1 population in MDA-MB-231 (decrease) and MCF-10A (decrease) cells, sub-G1 population did not change in SK-BR-3 cells (Figure 3-11). Simultaneously there is no increase in G1-phase population. A change in Annexin-V expression between cells grown in serum-supplemented medium and after incubation in serum-free medium was similarly not observed (Appendix section 9.3.3.2). Thus incubation in serum-free medium in SK-BR-3 breast cancer cells did not have the intended effects of increasing G1-phase population and so inhibiting cell cycle progression for the purpose of cell cycle synchronisation. Furthermore both insulin and leptin treatment decreased ERK1/2-phosphorylation. Thus after the initial 24 h incubation in serum-free medium, SK-BR-3 breast cancer cells may still carry consecutively phosphorylated ERK1/2. This might be explained by these cells overexpressing the HER2/neu receptor, which in turn may provide a continuous signal for ERK1/2-phosphorylation, even in the absence of extracellular signals. Thus the high concentrations of insulin and leptin used in the here presented experiments may be able

to interfere with this stimulation and subsequently reduce ERK1/2-phosphorylation. Thus this may be interpreted as another failure of incubation in serum-free medium to exert the desired effects of decreasing cell signalling in these cells.

It can be concluded that incubation in serum-free medium had different and indeed opposite effects on the three examined cell lines. The use of incubation in serum-free medium is wide-spread in the published results examining the effect of endogenous factors, such as insulin, leptin, etc. on breast cancer. Thus incubation in serum-free medium does not uniformly produce the intended result and as cells incubated in serum-free medium are used as a starting point for any investigation in the here presented study, results may be affected by the difference in incubation in serum-free medium. This does not decrease any confidence in the obtained results. The application of these results will however have to be validated in model systems that are able to closer mirror the actual breast environment.

8.9 Originality of study

This study is set in the emerging field of research on the co-morbidities of obesity. Many of these diseases are well known to the physician and researcher (heart disease, diabetes, cancer). The connection of obesity on several debilitating diseases however has grown in importance with the emergence of obesity as a global epidemic. Identification of molecular connections between obesity and its associated diseases has therefore equally gained momentum. This study provides new insights into the possibility of insulin as a possible connective factor. Insulin had been suggested as an important factor of breast cancer growth, molecular studies were however unable to

identify insulin as a mitogenic factor. Here insulin was shown to stimulate growth of breast epithelial cells and activate cell signalling pathways associates with cancer growth in breast cancer and breast epithelial cells. Furthermore insulin was shown to reduce apoptosis and increase cell cycle progression in breast cancer cells. Similar observations have not been documented previously. Additionally this study added to the knowledge of leptin as a molecular marker connecting obesity and breast cancer. Similar to previous findings, this study observed increases in cell proliferation of breast cancer and breast epithelial cells with leptin treatment. Furthermore this study validated published findings on activation of cell signalling pathways in breast cancer and breast epithelial cells with leptin treatment. Thus this study added to the knowledge of leptin as a major molecular marker connecting obesity and breast cancer. This study identified TNF-α as adipokine involved in the obesity breast cancer connection, by observing increased cell proliferation and increased activation of cell signalling pathways in breast cancer cells (SK-BR-3 cells only) and breast epithelial cells. As a result of this study, TNF-α may be added to the obesity induced factors that could affect breast cancer development and progression. Additionally this study added to the understanding of the influence of two further adipokines (IL-6 and adiponectin) on cell proliferation in breast cancer cells and breast epithelial cells. Thus this study uncovered and supported new possibilities for the connection of obesity and breast cancer, which may add to the overall understanding of this connection and could aid in the identification of at-risk patients, the development of advanced screening methods and provide indications for pharmaceutical targets to disrupt the obesity breast cancer connection.

8.10 Expanding the project

There are a number of further investigations that could be explored directly within the current setting of the study. Some were planned to be performed as part of the study, but were left in favour of additional insulin experiments, some derived as a result of the observations after treatment.

It would be interesting to employ Wortmannin and PD98059 in cell proliferation analysis after treatments with leptin and TNF-α. This would provide some indications on the utilisation of these pathways by leptin and TNF-α to promote proliferation. Furthermore it seems evident to examine changes in apoptosis with these treatments.

The findings from the microarray are to be followed-up and validated. This would further increase the insights into the exact molecular changes that insulin causes in breast cancer cells and breast epithelial cells. It may also help to investigate the physiological changes in MDA-MB-231 breast cancer cells. These cells exhibit an excessive increase in IR phosphorylation combined with higher increases, than the other cell lines, in PI3-kinase and MAP-kinase cell signalling activity. Cell proliferation, apoptosis or cell cycle did however not change. Thus it remains unanswered, where this significant molecular change manifests in physiological terms. Changes in gene expression seem evident to be the next step to be examined. Furthermore changes in metabolism, possibly with re-employment of the MTT assay may be attempted afterward.

One may also want to increase experimentation on IL-6, for which only cell proliferation has been assessed. Also as more adipokines emerge, it will be interesting to assess the impact of some of the newly emerged ones. At the moment there does not seem to be a single adipokine that could convincingly be ascribed to promote the obesity breast cancer connection, although leptin has a frontrunner role within the literature at the time of writing. Thus further analysis of the impact of additional adipokines such as resistin, appelin, visfatin, omentin, PA-1, MCP-1 amongst others, on breast cancer cells may provide additional insights into the obesity postmenopausal breast cancer connection.

Furthermore a targeted approach of combining adipokines and insulin in co-treatments to better reflect the physiological conditions may provide further insights, especially in the light of possible cross-links and synergistic effects.

There is one further experiment that may carry high significance for the hypothesis presented in section 8.5, which is the evaluation of an autocrine feedback loop of leptin in MDA-MB-231 breast cancer cells. MDA-MB-231 breast cancer cells need to be incubated in serum-free medium for 24 h as previously and then treated with leptin antagonists or OB-R monoclonal antibodies to disrupt leptin signalling. After that cell proliferation and ERK1/2-phosphorylation is to be tested to estimate the effect of autocrine leptin signalling in these cells and possibly validate the speculations attempted in section 8.5.

There is the possibility of measuring cell proliferation, apoptosis and cell signalling pathway activation after treatment with insulin, leptin or TNF-α in primary breast cancer cells derived from breast cancer patients. Comparison would then be done according to obesity and diabetic status of the patient examined. Thus there would be four groups of breast cancer patients, non-insulin resistant normal weight, insulin resistant normal weight, non-insulin resistant obese and insulin resistant obese. This should provide insights into whether breast cancer in obese and/or insulin resistant patients are utilising insulin or any of the adipokines to increase cell proliferation. It would also provide insights into any differential effect obesity or insulin resistance may have on their respective breast cancer and whether there are common links between breast cancer from these different groups that might be exploitable in future breast cancer treatment.

Additionally it would be interesting to investigate tumour growth in insulin resistant mouse models. Several mice model that mimic insulin resistance have been described (Nandi *et al.*, 2004). Similarly tumours may be grown in *db/db* mice, which are OB-R deficient and therefore have increase circulatory leptin. Thus tumours would grow in an obese/hyperinsulinaemic environment *in vivo* and its effect could be closer reflected to the situation in humans. The current insulin resistant and *db/db* mice models however are not genetically immunocompromised, thus cannot act as xenografts. Furthermore control mice would have to come from a different mouse strain, thus there would be difficulties in the analysis. Ideally one would want a nude mouse model, which also displays insulin and/or leptin resistance.

8.11 Conclusion

The study presented here aimed to provide insights into the role that obesity and its closely associated condition insulin resistance may have on postmenopausal breast cancer risk and mortality. The results indicate that high concentrations of insulin, leptin and TNF-α had differential effects on breast cancer cells and breast epithelial cells. Especially the effects on breast epithelial cells are pronounced and indicate an involvement of obesity in breast cancer carcinogenesis. This finding has not been examined previously and will need to be evaluated by others to identify its significance. Leptin has emerged as an interesting adipokine, which probably exerts its effects most unanimously in breast cancer cells and breast epithelial cells. It may thus be an interesting target for intervention in upcoming studies. Thus this study has succeeded in uncovering some of the molecular effects that obesity-associated factors may have on breast cancer risk and mortality and may contribute in identifying intervention targets for therapeutic prevention and/or treatment of postmenopausal breast cancer.

Appendix

9 APPENDIX

9.1 Comparison of cell proliferation in serum-supplemented and cells incubated in serum-free medium

9.1.1 BrdU-incorporation

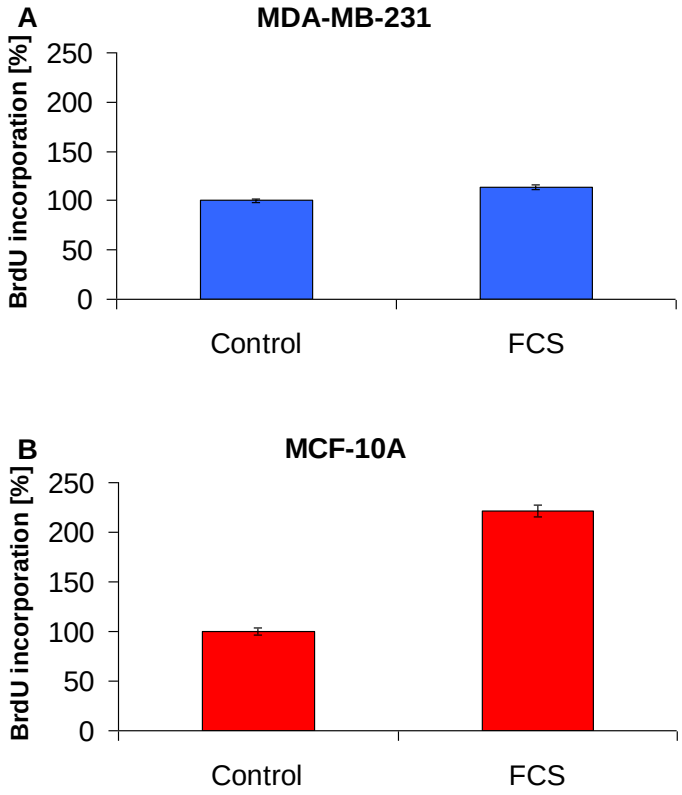

Figure 9-1: Changes in BrdU-incorporation in A) MDA-MB-231 breast cancer cells and B) MCF-10A breast epithelial cells after growth in serum-free medium (Control) or supplemented growth medium (FCS). Graphs represent optimisation results and were not assessed by statistical analysis.

9.1.2 MTT-assay

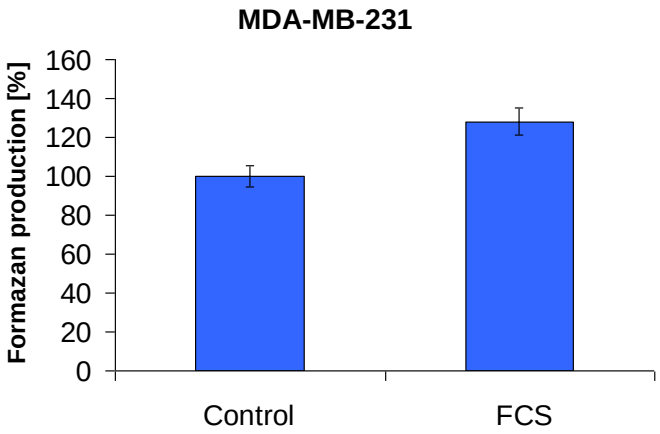

Figure 9-2: Changes in Formazan production in MDA-MB-231 breast cancer cells after growth in serum-free medium (Control) or supplemented growth medium (FCS). Values were assessed in different experiments and as such were not accessible to determine statistical differences.

9.2 Cell proliferation after 48 h insulin treatment in the presence of BrdU for 48 h

One should expect to observe at least the same increase in proliferation after 48 h treatment than after 24 h treatment. Any change could then be attributed to the second 24 h treatment period. In fact there is a methodological explanation for this observation. Analysis of cell proliferation after insulin treatment was performed to a slightly different protocol than after treatment with any of the adipokines. For cell proliferation analysis after 48 h insulin treatment, BrdU was only added after an initial 24 h treatment. Thus the value observed for the 48 h treatment is in fact only the value for the second 24 h treatment period. Thus in order to estimate the total increase in cell proliferation after 48 h treatment, one may be inclined to add the values obtained for 24 h and 48 h treatment. For all the cell proliferation experiments after adipokine treatment BrdU was added for the entire treatment time, *i.e.* 24 h or 48 h. In doing so, the protocol established in the study presented here deviates significantly from the established protocol, which is also widely used among researchers. In this established protocol, BrdU would only be added for the last 4 h of any given treatment. This allows establishment of the status of cell proliferation after a given treatment time, but not during the entire treatment. On the other hand leaving BrdU for the entirety of the treatment may have the disadvantage that highly proliferating cells may reach the maximum linear absorbance values during the spectrophotometric analysis. According to the manufacturer the maximal linear absorption value for the utilised spectrophotometer was 3.5. The highest absorption value obtained in MDA-MB-231 breast cancer cells, grown in fully supplemented growth medium with BrdU for 48 h was 2.7 (see also Figure 9-1, A). Thus the analysis supplementing BrdU for the duration of the treatment was determined to be available for the study presented here. As such it

was also hoped to capture any early change in cell proliferation, in the initial stages of the treatment, which would be lost, if not sustained, if BrdU was only added for the final 4 h of treatment. In order to assess this assumption one additional experiment, leaving BrdU for the entire 48 h insulin treatment, was performed, where this assumption was confirmed.

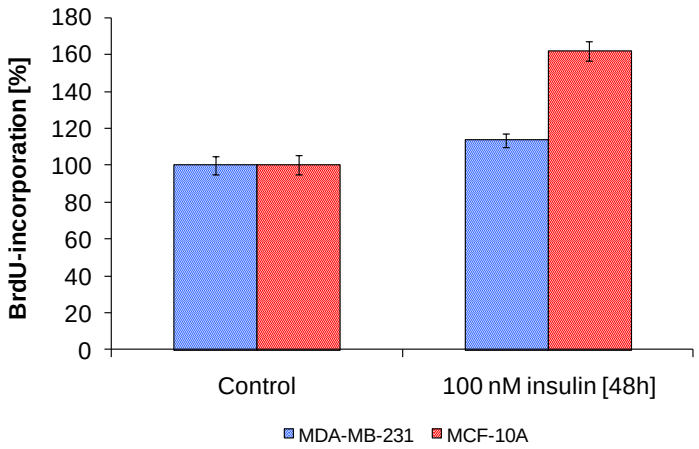

Figure 9-3: Changes in cell proliferation after treatment with 100 nM insulin for 48 h in MDA-MB-231 breast cancer cells (blue) and MCF-10A breast epithelial cells (red). In this experiment, BrdU was added at the beginning of the 48 h incubation. For the presented results, BrdU was only added for the last 24 h of a 48 h experiment (see footnote 11 in chapter 3).

9.3 Flow cytometry examination

9.3.1 Raw histogram for Cell Cycle flow cytometry

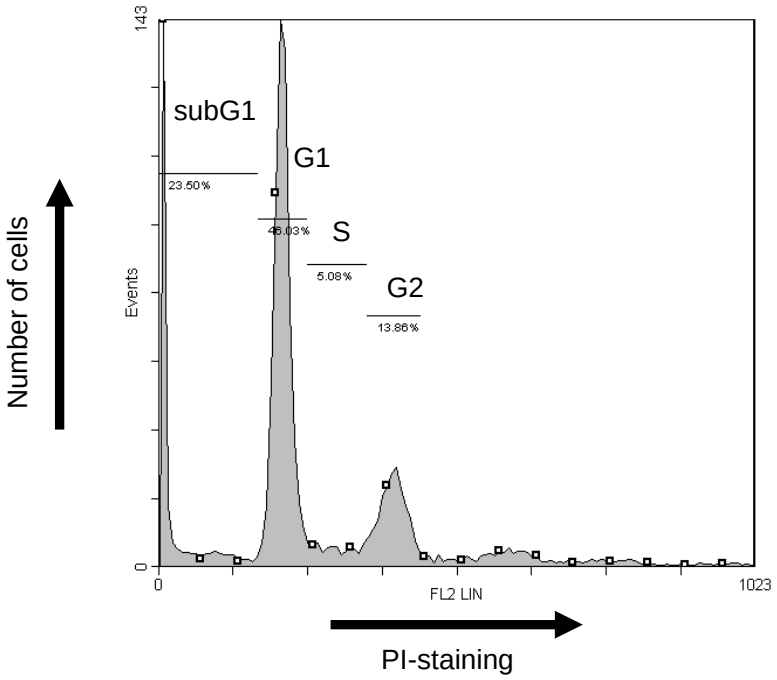

Figure 9-4: Histogram of PI-staining in MDA-MB-231 breast cancer cells grown in fully supplemented medium. This graph plots the number of cells (y-axis) against the level of PI-staining (x-axis). The two dominant observable peaks correspond to a doubling in PI-intensity, thus are representative of G1-phase and G2-phase in the cell cycle. The area between those two peaks represents cells transitioning from G1-phase to G2-phase, *i.e.* cells in S-phase. Staining below the G1-peak indicates cells without a full set of chromosomes, *i.e.* cells in apoptosis. According to treatment, the number of cells in the different phases would shift and change the peak height, which can then be detected. Two smaller peaks may be identified at around 3-times and 4-times the intensity of the G-1 peak, which are indicative of two cells simultaneously entering the detection mechanism and producing a higher result. In the study presented here those peaks were low throughout the experiments.

9.3.2 Raw histogram for Apoptosis flow cytometry

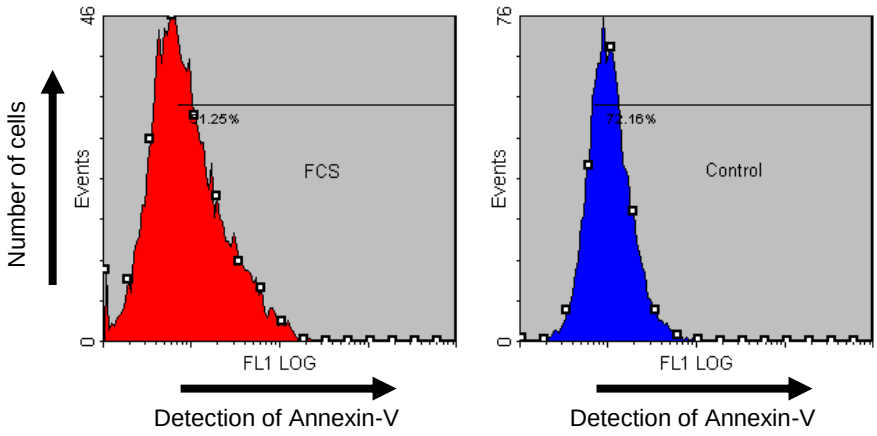

Figure 9-5: Histogram data of Annexin-V detection in MCF-10A cells. These graphs plot the number of cells (y-axis) against the level of fluorescence detected (FITC-coupled bound Annexin-V, x-axis), corresponding to the distribution in apoptotic rate in the examined cell sample (10000 cells). Shown are histograms of cells in fully supplemented medium (FCS, red) and cells incubated in serum-free medium (Control, blue). Gate was placed so that approximately 50% of FCS cells were detected, corresponding to cells with high amounts of bound Annexin-V. The gate was identical for each treatment sample, thus detection of changes in bound Annexin-V were possible. Similarly 7-AAD staining was detected.

9.3.3 Flow cytometry controls

9.3.3.1 Comparison of controls for cell cycle analysis

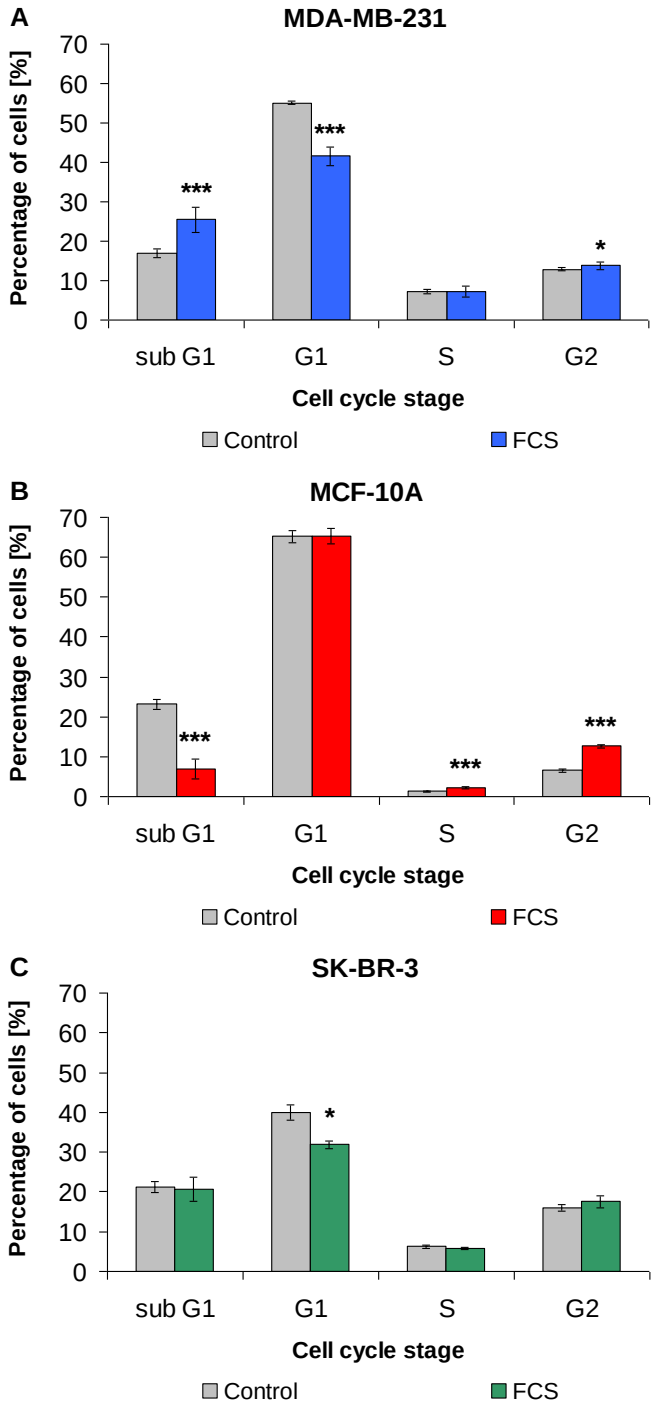

Figure 9-6: Changes of cell population distribution across cell cycle stages after growth in serum-free medium (Control) or supplemented medium (FCS) in A) MDA-MB-231 cells, B) MCF-10A cells and C) SK-BR-3 cells. Bars represent percentage of cells as obtained from a 10000 cell sample. Error bars represent ± SEM of three experiments with two replicates for each treatment, *i.e.* 6 data points for each bar.
* Significance value compared to control (grey) at that stage, obtained using Dunnett's t-test following univariate analysis of variance, (* 0.05>p>0.01; *** p<0.001)

9.3.3.2 Comparison of controls for Annexin-V apoptosis analysis

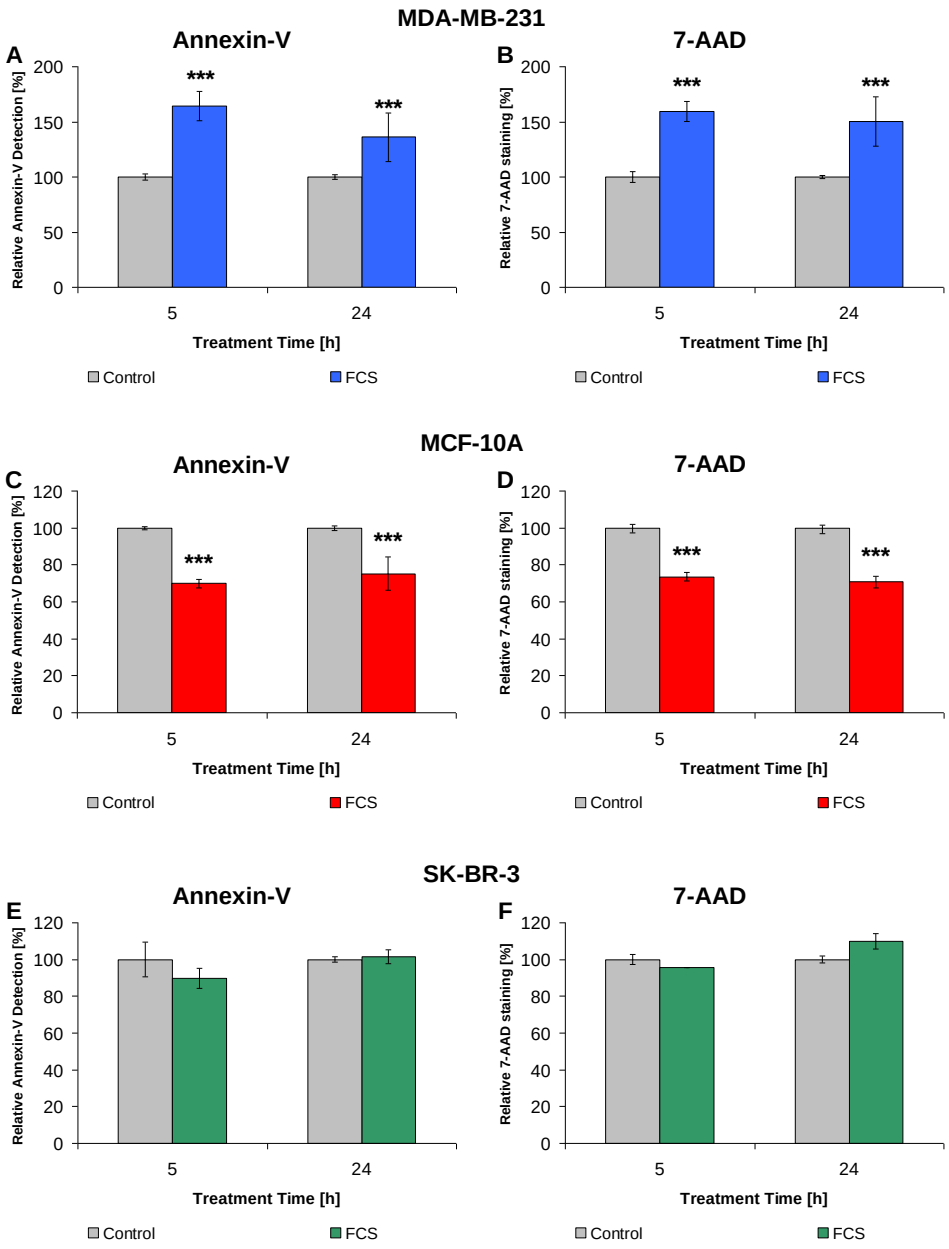

Figure 9-7: Changes in Annexin-V detection (A, C, E) and 7-AAD staining (B, D, F) after growth in serum-free medium (Control) or fully supplemented medium (FCS) for 5 h or 24 h in A, B) MDA-MB-231 breast cancer cells, C, D) MCF-10A breast epithelial cells and E, F) SK-BR-3 breast cancer cells. Bars represent percentage of cells above lower cut-off point in flow cytometry histogram (see Appendix section 9.3.2) in relation to control and expressed as a percentage thereof. Error bars represent ± SEM of three experiments with two replicates, *i.e.* six data points for each bar.
* Significance value compared to control of the same treatment time, obtained using Dunnett's post-hoc t-test following univariate analysis of variance (***p>0.001).

9.4 Raw Data of Western Blotting for Antibody Specificity

9.4.1 PCNA expression

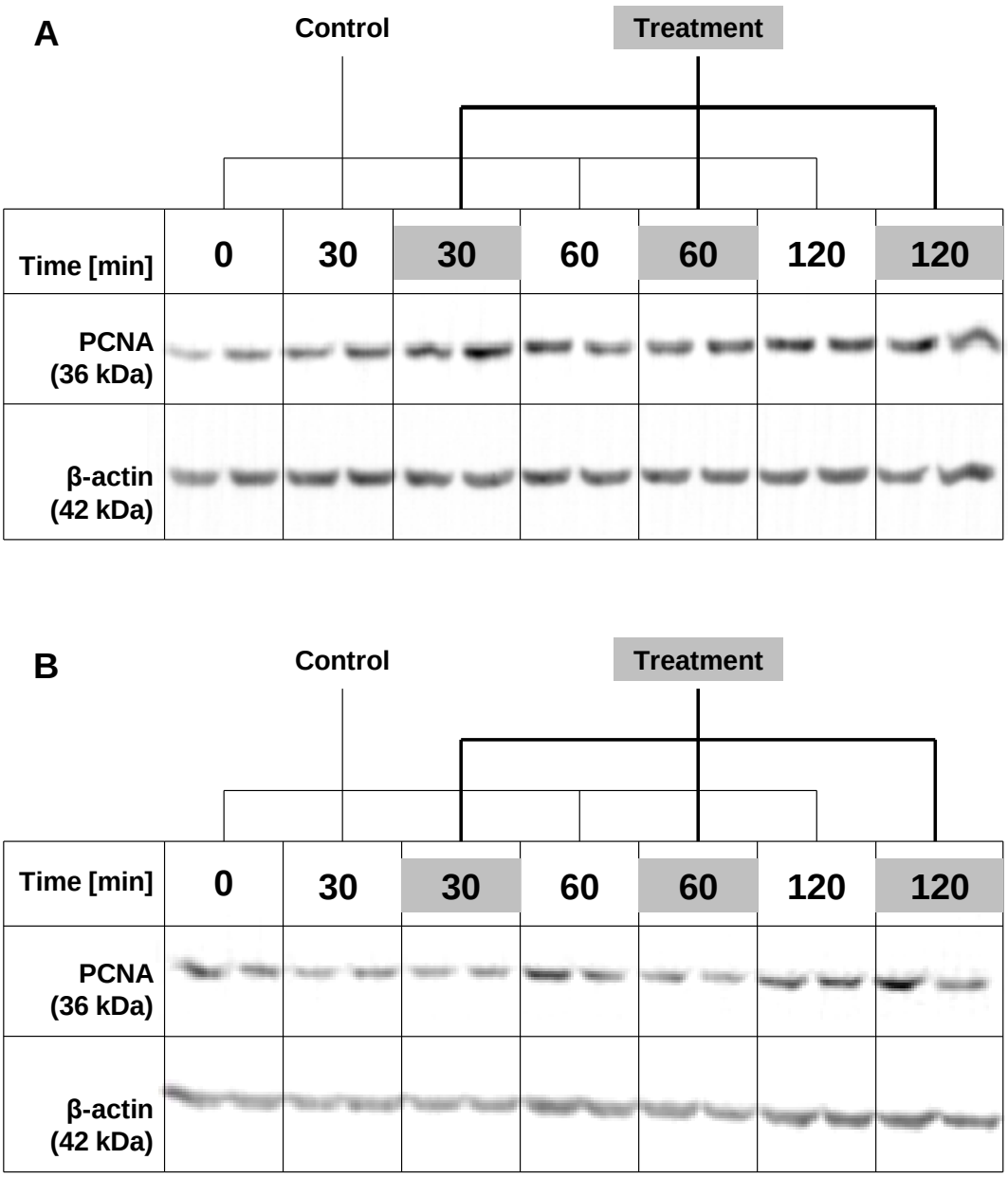

Figure 9-8: Specificity of PCNA and actin antibody for results presented in section 7.3.1.2. Picture is raw western data, representative of three independent experiments in A) MDA-MB-231 breast cancer cells and B) MCF-10A breast epithelial cells. Picture is adapted to increase contrast.

9.4.2 JC-1 expression

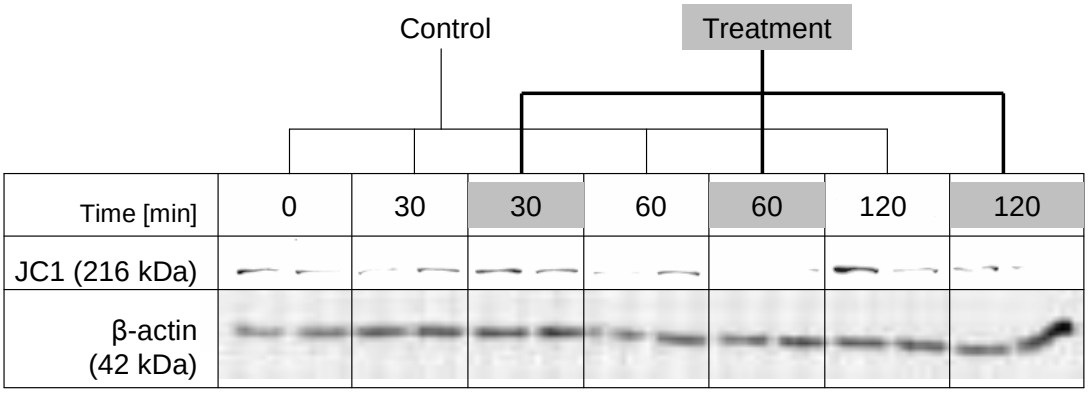

Figure 9-9: Specificity of JC-1 and actin antibody for results presented in section 7.3.1.2. Picture is raw western data, representative of three independent experiments in MDA-MB-231 breast cancer cells. Picture is adapted to increase contrast.

9.4.3 ERK1/2-phosphorylation and expression

Detection of ERK1/2-phosphorylation, using the approach outlined in Materials and Methods section 2.6, proved challenging. For consistency it was attempted to detect phosphorylated and total ERK1/2 on the same membrane, requiring the membrane to be "stripped" of their initial antibody detection, either phosphorylated or total protein, to be re-probed with the other. The second antibody detection was very inconsistent, possibly due to differences the "stripping" made to the protein epitope, immobilised to the membrane. This resulted in high variability after quantification and resulted in the shown large errors. Further experiments may explore the possibility of keeping analysis of phosphorylated and total protein on separate membranes, thus negating the necessity to "strip" the membrane and reducing the error. As a result the experiment would be open to increased operator error, as the same amount of protein has to be loaded on the gels. Thus the phosphorylation analysis using the ELISA kit (see section 2.3) should be favoured.

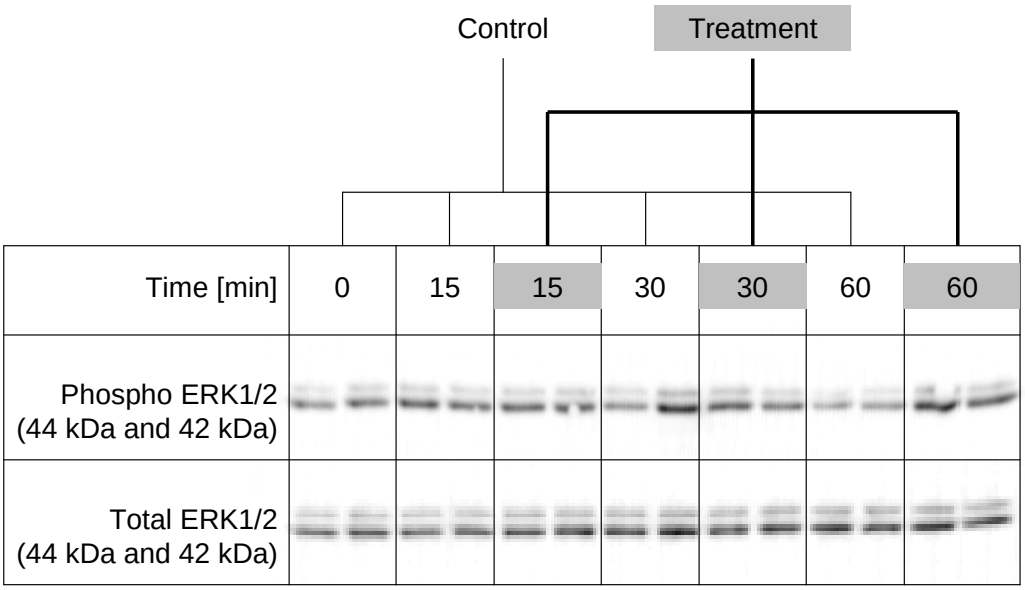

Figure 9-10: Specificity of phosphorylated and total ERK1/2 antibody for results presented in section 7.3.2. Picture is raw western data, representative of three independent experiments in MDA-MB-231 breast cancer cells. Picture is adapted to increase contrast.

9.5 Gene expression results

9.5.1 PCR-products of BCL-2 gene expression analysis

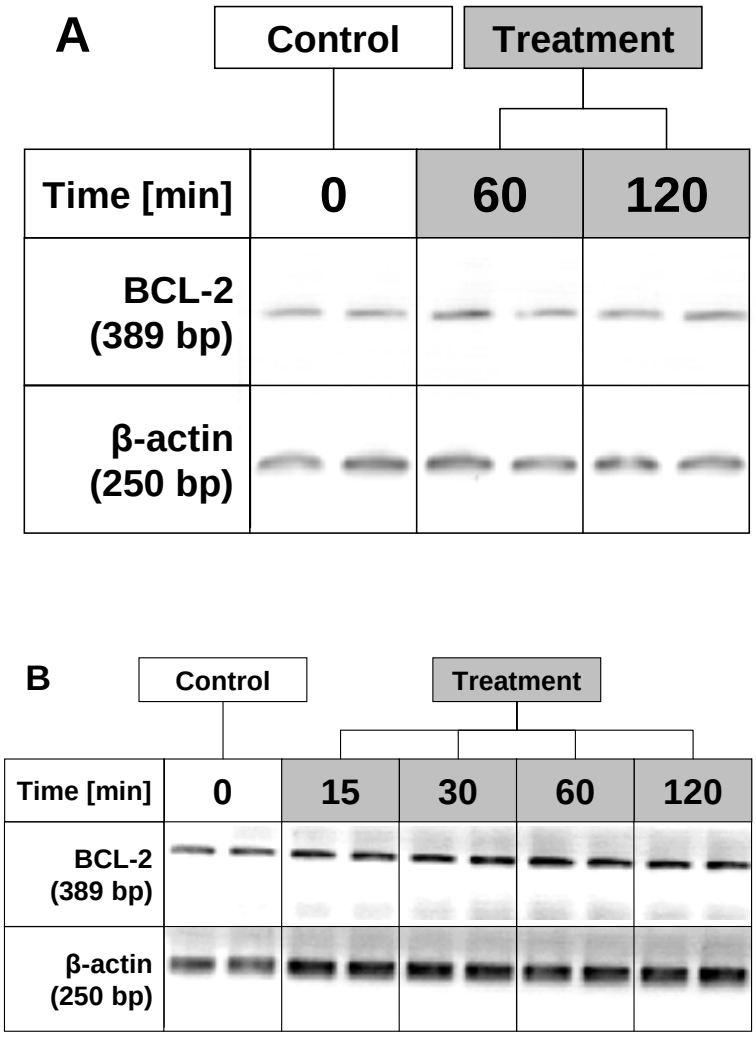

Figure 9-11: Size of BCL-2 PCR-product and actin control. Picture is representative of three independent experiments in A) MDA-MB-231 breast cancer cells and B) MCF-10A breast epithelial cells. Picture is adapted to increase contrast.

9.5.2 PCR-products for cyclin D gene expression analysis

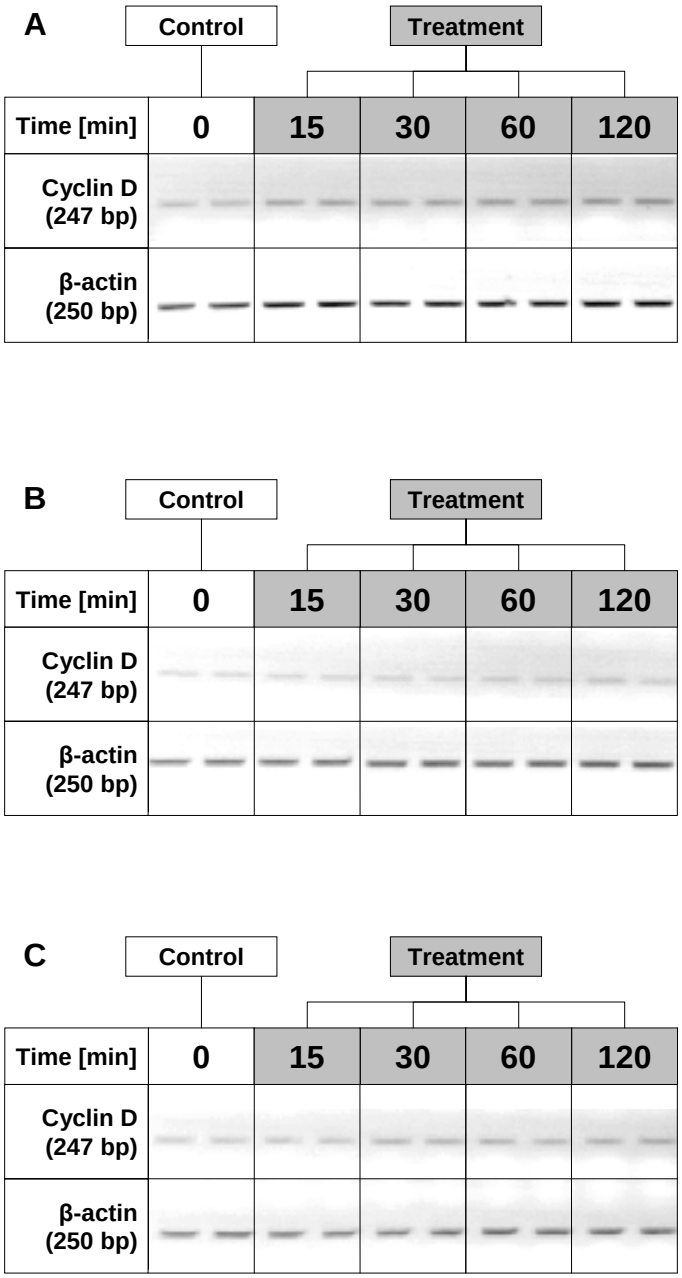

Figure 9-12: Size of cyclin D PCR-product and actin control. Picture is representative of three independent experiments in A) MDA-MB-231 breast cancer cells and B) MCF-10A breast epithelial cells and C) SK-BR-3 breast cancer cells. Picture is adapted to increase contrast.

9.5.3 PCR-products for cyclin E gene expression analysis

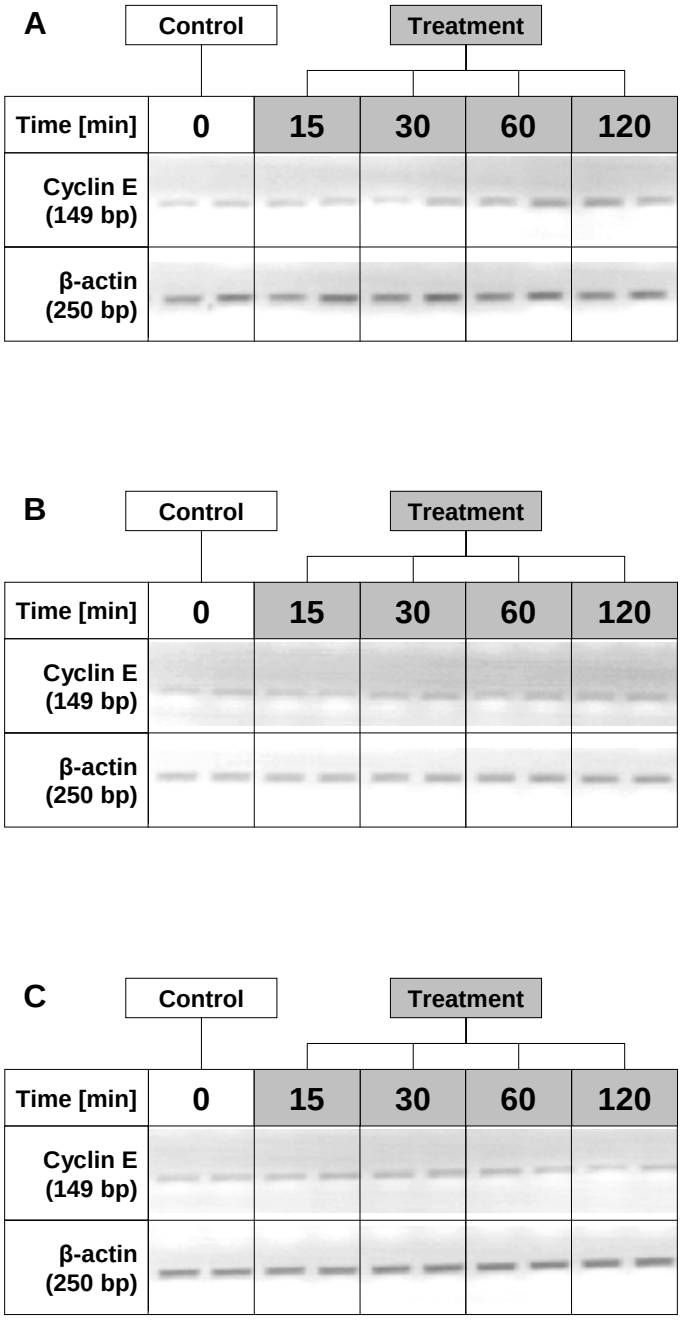

Figure 9-13: Size of cyclin E PCR-product and actin control. Picture is representative of three independent experiments in A) MDA-MB-231 breast cancer cells and B) MCF-10A breast epithelial cells and C) SK-BR-3 breast cancer cells. Picture is adapted to increase contrast.

9.6 Microarray analysis

Table 9-1: Raw data table with results from microarray analysis as described in section 7.3.4. All data is unaltered as obtained after densitometry for all four microarrays performed. Data was obtained as intensity per square millimetre. All genes examined are in order found on the array, see Table 2-4.

	MDA-MB-231 Control	MDA-MB-231 Insulin	MCF-10A Control	MCF-10A Insulin
Gene description	**Density [INT/mm2]**	**Density [INT/mm2]**	**Density [INT/mm2]**	**Density [INT/mm2]**
Ribosomal protein S27a	352.56	348.04	448.68	660.68
V-akt murine thymoma viral oncogene homolog 1	377.81	307.92	427.76	421.43
Angiopoietin 1	334.32	257.06	423.71	404.17
Angiopoietin 2	333.13	255.11	428.52	403.00
Apoptotic peptidase activating factor 1	338.88	272.47	429.07	406.92
Ataxia telangiectasia mutated	318.83	273.17	427.75	410.64
BCL2-antagonist of cell death	310.03	271.94	423.68	403.18
Brain-specific angiogenesis inhibitor 1	298.81	260.19	426.77	403.23
BCL2-associated X protein	327.61	274.21	429.10	417.25
B-cell CLL/lymphoma 2	335.77	272.90	426.80	411.91
BCL2-like 1	356.01	299.56	426.73	408.78
Baculoviral IAP repeat-containing 5 (survivin)	557.69	618.75	437.02	423.16
Breast cancer 1, early onset	319.57	282.12	428.90	405.56
Breast cancer 2, early onset	313.26	283.16	428.90	415.37
Caspase 8, apoptosis-related cysteine peptidase	296.32	261.39	422.17	408.74
Caspase 9, apoptosis-related cysteine peptidase	314.19	258.34	430.02	414.15
Cyclin D1	314.34	278.11	424.68	416.61
Cyclin E1	343.12	312.97	431.10	419.02
CD44 molecule (Indian blood group)	333.63	307.55	432.29	416.90

	MDA-MB-231 Control	MDA-MB-231 Insulin	MCF-10A Control	MCF-10A Insulin
Gene description	**Density [INT/mm2]**	**Density [INT/mm2]**	**Density [INT/mm2]**	**Density [INT/mm2]**
Cell division cycle 25 homolog A (S. pombe)	355.28	343.10	453.17	484.95
Cadherin 1, type 1, E-cadherin (epithelial)	296.17	262.00	428.33	404.37
Cyclin-dependent kinase 2	326.18	283.08	428.43	409.03
Cyclin-dependent kinase 4	914.87	952.61	636.50	1036.21
Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	1334.01	1214.62	1310.23	1385.98
Cyclin-dependent kinase inhibitor 1B (p27, Kip1)	314.64	279.23	432.62	419.92
Cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)	311.83	275.22	428.92	413.44
CASP8 and FADD-like apoptosis regulator	305.59	276.53	428.61	410.89
CHK2 checkpoint homolog (S. pombe)	300.07	270.66	429.43	406.48
Collagen, type XVIII, alpha 1	328.32	296.65	425.83	411.22
Catenin (cadherin-associated protein), beta 1, 88kDa	305.37	252.84	423.29	405.66
E2F transcription factor 1	424.17	356.16	430.00	407.83
Epidermal growth factor (beta-urogastrone)	321.52	243.49	428.22	411.14
Epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian)	326.01	310.97	439.67	452.29
V-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)	345.09	329.02	453.75	467.79
V-Ets erythroblastosis virus E26 oncogene homolog 2 (avian)	326.38	299.27	429.99	418.80
Fibroblast growth factor 2 (basic)	297.53	249.26	428.15	407.45

	MDA-MB-231 Control	MDA-MB-231 Insulin	MCF-10A Control	MCF-10A Insulin
Gene description	**Density [INT/mm2]**	**Density [INT/mm2]**	**Density [INT/mm2]**	**Density [INT/mm2]**
Fibroblast growth factor receptor 2 (bacteria-expressed kinase, keratinocyte growth factor receptor, craniofacial dysostosis 1, Crouzon syndrome, Pfeiffer syndrome, Jackson-Weiss syndrome)	300.61	243.36	429.71	412.49
Fms-related tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor receptor)	312.47	241.01	428.70	406.72
V-fos FBJ murine osteosarcoma viral oncogene homolog	319.59	245.39	497.87	497.31
Granzyme A (granzyme 1, cytotoxic T-lymphocyte-associated serine esterase 3)	312.79	241.49	428.92	400.11
Hepatocyte growth factor (hepapoietin A; scatter factor)	290.87	268.17	421.52	412.70
HIV-1 Tat interactive protein 2, 30kDa	336.21	302.90	463.18	484.16
Intercellular adhesion molecule 1 (CD54), human rhinovirus receptor	314.19	248.64	423.10	406.79
Interferon, alpha 1	321.42	258.64	438.70	437.18
Interferon, beta 1, fibroblast	330.05	252.89	433.29	413.10
Insulin-like growth factor 1 (somatomedin C)	324.16	249.07	424.63	401.78
Interleukin 8	569.06	429.12	425.88	410.58
Integrin, alpha 1	299.92	251.18	430.36	400.69
Integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)	301.08	259.54	423.49	413.15

	MDA-MB-231 Control	MDA-MB-231 Insulin	MCF-10A Control	MCF-10A Insulin
Gene description	**Density [INT/mm2]**	**Density [INT/mm2]**	**Density [INT/mm2]**	**Density [INT/mm2]**
Integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor)	671.81	642.82	557.61	594.34
Integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor)	333.45	264.35	425.30	414.99
Integrin, alpha 5 (fibronectin receptor, alpha polypeptide)	311.85	259.06	423.09	403.84
Integrin, alpha 6	308.72	268.44	425.02	403.88
Integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51)	301.99	266.28	428.45	403.92
Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	1105.72	1002.09	1069.78	1111.56
Integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)	291.09	243.81	426.30	405.06
Integrin, beta 5	376.56	344.41	454.79	457.75
Jun oncogene	351.60	309.92	445.30	464.26
CD82 molecule	323.22	279.15	422.11	408.35
KiSS-1 metastasis-suppressor	309.16	277.28	424.17	407.98
Mitogen-activated protein kinase kinase 1	338.38	315.19	432.66	421.29
Mitogen-activated protein kinase 14	325.83	254.28	423.50	401.80
Melanoma cell adhesion molecule	322.60	264.06	424.33	401.68
Mdm2, transformed 3T3 cell double minute 2, p53 binding protein (mouse)	306.75	228.29	423.27	401.05

	MDA-MB-231 Control	MDA-MB-231 Insulin	MCF-10A Control	MCF-10A Insulin
Gene description	**Density [INT/mm2]**	**Density [INT/mm2]**	**Density [INT/mm2]**	**Density [INT/mm2]**
Met proto-oncogene (hepatocyte growth factor receptor)	311.24	274.85	422.32	409.60
MHC class I polypeptide-related sequence A	308.72	281.45	420.75	409.64
Matrix metallopeptidase 1 (interstitial collagenase)	1294.53	1147.73	422.21	408.76
Matrix metallopeptidase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)	350.02	292.40	458.08	437.33
Matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)	307.49	256.04	421.52	405.66
Metastasis associated 1			425.15	404.94
Metastasis associated 1 family, member 2	320.31	233.85	421.57	399.91
Metastasis suppressor 1	316.88	228.77	419.26	399.77
V-myc myelocytomatosis viral oncogene homolog (avian)	309.90	289.62	435.68	433.81
Neural cell adhesion molecule 1	296.05	269.35	423.25	410.11
Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)	403.36	361.45	497.67	480.28
Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	574.36	578.76	559.32	479.15
Non-metastatic cells 1, protein (NM23A) expressed in	770.91	747.64	460.65	453.54
Non-metastatic cells 4, protein expressed in	337.00	260.21	423.62	408.25
Platelet-derived growth factor alpha polypeptide	317.50	241.78	417.92	403.57

	MDA-MB-231 Control	MDA-MB-231 Insulin	MCF-10A Control	MCF-10A Insulin
Gene description	**Density [INT/mm2]**	**Density [INT/mm2]**	**Density [INT/mm2]**	**Density [INT/mm2]**
Platelet-derived growth factor beta polypeptide (simian sarcoma viral (v-sis) oncogene homolog)	348.96	266.20	430.90	410.24
Phosphoinositide-3-kinase, catalytic, beta polypeptide	294.91	267.80	426.77	405.53
Phosphoinositide-3-kinase, regulatory subunit 1 (alpha)	302.80	257.51	427.96	415.36
Plasminogen activator, urokinase	771.95	750.53	1213.81	1031.28
Plasminogen activator, urokinase receptor	400.62	352.00	440.47	420.50
Pinin, desmosome associated protein	338.98	291.60	448.04	424.88
Protein kinase, DNA-activated, catalytic polypeptide	294.96	253.91	414.81	405.98
Phosphatase and tensin homolog (mutated in multiple advanced cancers 1)	300.81	251.50	421.86	405.41
V-raf-1 murine leukemia viral oncogene homolog 1	298.78	244.99	423.10	406.34
RAS p21 protein activator (GTPase activating protein) 1	303.15	264.89	418.44	405.85
Retinoblastoma 1 (including osteosarcoma)	338.34	287.97	433.49	424.89
S100 calcium binding protein A4	652.41	571.20	492.71	442.16
Serpin peptidase inhibitor, clade B (ovalbumin), member 2	370.54	342.78	450.62	445.38
Serpin peptidase inhibitor, clade B (ovalbumin), member 5	313.31	279.74	851.05	586.65

	MDA-MB-231 Control	MDA-MB-231 Insulin	MCF-10A Control	MCF-10A Insulin
Gene description	**Density [INT/mm2]**	**Density [INT/mm2]**	**Density [INT/mm2]**	**Density [INT/mm2]**
Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	293.28	266.38	414.20	403.44
Synuclein, gamma (breast cancer-specific protein 1)	308.47	257.75	426.48	411.67
V-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian)	291.93	235.80	417.54	410.51
Spleen tyrosine kinase	311.88	264.86	421.24	404.77
TEK tyrosine kinase, endothelial (venous malformations, multiple cutaneous and mucosal)	319.03	268.12	419.13	404.40
Telomerase reverse transcriptase	314.22	274.48	422.23	404.79
Transforming growth factor, beta 1	302.38	273.09	419.67	402.93
Transforming growth factor, beta receptor I (activin A receptor type II-like kinase, 53kDa)	292.69	267.80	422.41	405.38
Thrombospondin 1	292.64	256.02	419.78	404.25
Thrombospondin 2	304.45	244.96	411.50	405.92
TIMP metallopeptidase inhibitor 1	1589.64	1500.90	1373.70	1213.22
TIMP metallopeptidase inhibitor 3 (Sorsby fundus dystrophy, pseudoinflammatory)	368.39	372.57	742.70	493.35
Tumor necrosis factor (TNF superfamily, member 2)	313.48	286.20	422.81	404.60
Tumor necrosis factor receptor superfamily, member 10b	644.39	703.62	836.77	644.69

	MDA-MB-231 Control	MDA-MB-231 Insulin	MCF-10A Control	MCF-10A Insulin
Gene description	**Density [INT/mm2]**	**Density [INT/mm2]**	**Density [INT/mm2]**	**Density [INT/mm2]**
Tumor necrosis factor receptor superfamily, member 1A	323.89	303.62	454.63	417.27
Tumor necrosis factor receptor superfamily, member 25	304.58	263.74	445.50	433.29
Fas (TNF receptor superfamily, member 6)	299.47	246.72	417.50	402.80
Tumor protein p53	311.93	239.56	412.28	398.89
Twist homolog 1 (acrocephalosyndactyly 3; Saethre-Chotzen syndrome) (Drosophila)	320.06	239.70	413.37	402.60
Ependymin related protein 1 (zebrafish)	298.68	290.26	418.30	407.60
Vascular endothelial growth factor A	302.31	281.18	419.30	406.71

Table 9-2: Densitometry data for controls for all microarrays performed.

	MDA-MB-231 Control	MDA-MB-231 Insulin	MCF-10A Control	MCF-10A Insulin
Gene description	**Density [INT/mm2]**	**Density [INT/mm2]**	**Density [INT/mm2]**	**Density [INT/mm2]**
Controls				
plasmid	308.65	280.09	430.14	405.79
BLANK	311.09	268.65	432.50	407.91
BLANK	327.09	264.25	433.72	413.55
Artificial Sequence	328.23	259.36	428.25	408.70
Artificial Sequence	321.00	251.98	416.10	400.24
Artificial Sequence	319.92	249.29	411.95	402.79
GAPDH	665.38	876.03	663.72	489.39
Beta-2-microglobulin	507.91	628.48	934.89	625.04
Heat shock protein 90kDa	1760.15	2043.07	1507.53	1092.25
Heat shock protein 90kDa	1657.35	1900.26	1446.71	1301.54
beta actin	2343.24	2417.29	1806.32	1449.34
beta actin	2347.66	2405.67	1717.69	1356.20
biotinyated artificial sequence	404.74	349.01	440.19	417.98
biotinyated artificial sequence	1784.54	2046.91	1291.13	1070.58

Publications

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The molecular contribution of TNF-α in the link between obesity and breast cancer

MICHAEL WEICHHAUS, IAIN BROOM and GIOVANNA BERMANO

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Abstract. Obesity is a growing worldwide medical problem, as it pre-disposes the affected hosts to a number of severe diseases, including postmenopausal breast cancer. Obesity development is characterised amongst others by aberrant secretion of adipokines. White fat tissue infiltrating macrophages secrete TNF-α so that its circulating levels correlate positively with BMI. In the study presented here, the effect of TNF-α on cell proliferation, cell signalling pathway activation and cell cycle in two breast cancer cell lines and one breast epithelial cell lines was assessed to determine the contribution of TNF-α on breast cancer progression and aetiology, respectively. TNF-α acted differently on all three cell lines. In MDA-MB-231 breast cancer cells, cell proliferation and PI3-kinase activation were not affected, while MAP-kinase activation and cell cycle progression were decreased, with indications of increased apoptosis. This suggests a growth inhibitory function of TNF-α in these cells. In SK-BR-3 breast cancer cells, cell proliferation and cell signalling pathway activation increased, while cell cycle progression decreased, which contradictorily suggests both growth promoting and growth inhibiting properties of TNF-α on these cells. This makes TNF-α an unlikely candidate for a general contribution to the link between obesity and breast cancer progression, however, individual tumours may be responsive to a proliferative signal of TNF-α. In MCF-10A breast epithelial cells, cell proliferation and MAP-kinase activation increased, while cell cycle progression was unaffected. This suggests a strong proliferative response in these cells, suggesting the possibility that TNF-α may contribute to breast cancer aetiology as a novel link between obesity and increased risk of breast cancer development.

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Introduction

Obesity is a risk factor for the development of postmenopausal breast cancer (1-3). Despite numerous studies, the molecular mechanism by which obesity may contribute to breast cancer aetiology and progression is still poorly understood, suggesting a complex multi-mechanism of interaction. Obesity is characterised, amongst others, by a change in adipocyte secreted adipokines (4-6). Plasma concentrations of several well-known adipokines are positively correlated with BMI, such as leptin (7), interleukin-6 (8) and TNF-α (9). In the study presented here, the contribution of TNF-α in the link between obesity and progression or aetiology of breast cancer cells is assessed.

Tumour necrosis factor-α (TNF-α) is a cytokine involved in the induction of inflammation and initiation of an acute phase immune response. When cells are stimulated to secrete TNF-α, membrane-bound precursor molecules on the surface of secreting cells are cleaved to form 51 kDa circulating TNF-α, consisting of three 17 kDa TNF-α molecules (10). Its name derived from the initial observation that this cytokine was able to induce cell death in the murine fibrosarcoma L-929 cell line (11). Endocrine signalling is accomplished by circulating TNF-α binding to and activating its specific receptor TNF-receptor 1 (TNF-R1) on target cells. The receptor is expressed ubiquitously in all human tissues, including breast epithelial cells (12). Thus TNF-α signalling in breast epithelial cells and breast cancer cells could be affected by obesity associated increases in TNF-α circulatory concentration.

Previous *in vitro* studies observed conflicting results as to the effect of TNF-α on growth promotion in breast cancer cells. It was found that 20 ng/ml TNF-α increased cell proliferation in T47D human breast cancer cells and induced significant activation of PI3-kinase pathway, MAP-kinase pathway and JAK/STAT pathway (13), suggesting a growth promoting effect of TNF-α on these cells. Conversely, treatment with 10 mg/ml TNF-α for 24 h increased G1-phase population, while reducing the cell population in S-phase and G2-phase (14), suggesting a cell cycle inhibiting effect on these cells. Thus TNF-α had different, indeed opposing effects on T47D breast cancer cells. In MCF-7 breast cancer cells 10 ng/ml TNF decreased cell proliferation (15) and an increase in G1-phase cell population was observed after treatment with 10 ng/ml TNF for 36 h in MCF-7 cells (16),

suggesting a growth inhibiting effect in this cell line. In different MCF-7 cell subtypes, however, opposing effects of TNF-α on cell proliferation were observed (17). Thus, the effect of TNF-α on breast cancer cell growth is not elucidated satisfactorily, while the effect of TNF-α on human breast epithelial cells has not been investigated at all.

The study presented here aimed to assess the effect of TNF-α on cell proliferation, PI3-kinase and MAP-kinase cell signalling pathway activation and on changes in the distribution of the cell population across cell cycle stages in an *in vitro* cell culture model. It was of particular interest to determine the possible different impact of TNF-α on breast cancer cells compared to normal breast epithelial cells as a novel way of assessing the molecular mechanisms involved in the link between obesity and breast cancer progression and aetiology. In order to assess the contribution of TNF-α on breast cancer progression, two breast cancer cell lines were selected, MDA-MB-231 and SK-BR-3 breast cancer cells. The contribution of TNF-α on breast cancer aetiology was assessed using normal MCF-10A breast epithelial cells. Previous experiments were performed in oestrogen receptor (ER) positive cells. In the study presented here, ER negative cell lines were used.

Materials and methods

Materials. Human Caucasian breast adenocarcinoma cells MDA-MB-231 (Cat No. 92020424, passage No. 36) cells were purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK). Human Caucasian breast adenocarcinoma cells SK-BR-3 cells (ATCC No. HTB-30, passage No. 28) were purchased from the American Type Culture Collection (ATCC, Manassas, USA). MDA-MB 231 cells and SK-BR-3 cells were routinely cultured in RPMI-1640 Medium (including 25 mM HEPES, 1x Glutamax) [Gibco (Invitrogen), Paisley, UK, Cat No. 72400] supplemented with 10% FCS (Pierce Biosciences, Cramlington, UK, Cat No. CHD0413) and 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco, Cat No. 15140). MDA-MB-231 breast cancer cells have mutations in genes coding for RAS and RAF, increasing their kinase activity, while SK-BR-3 breast cancer cells possess wild-type genes coding for RAS and RAF (18). RAS and RAF are kinase members of the MAP-kinase cell signalling pathway. Human Caucasian breast epithelial cells MCF-10A (ATCC No. CRL-10317, passage No. 102) were purchased from the ATCC and were cultured in DMEM/F-12 Medium (BioWhittaker, UK) (Lonza Biologics, Slough, UK, Cat No. BE12-7199) supplemented with 5% Horse Serum (Sigma-Aldrich, Gillingham, UK, Cat No. H1138), 10 μg/ml human insulin (Sigma, Cat No. I9278), 0.5 μg/ml hydrocortisone (Sigma, Cat No. H0888), 20 μg/ml human epidermal growth factor (Invitrogen, Cat No. 13247-051), 100 ng/ml cholera toxin (Sigma, Cat No. 8052), 50 U/ml penicillin and 50 μg/ml streptomycin (Gibco). Starvation medium was RPMI-1640 (25 mM HEPES, 1x Glutamax) (Gibco) supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco) for MDA-MB 231 cells and SK-BR-3 cells and DMEM/F12 (BioWhittaker) supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin for MCF-10A cells. Human recombinant TNF-α was purchased from Sigma (Cat No. T0157). Cell culture conditions were 37˚C in humidified air supplemented to contain 5% CO_2.

BrdU-incorporation assay. Cell proliferation was detected using a colorimetric Cell Proliferation ELISA Kit (Roche Diagnostics, Penzberg, Germany, Cat No. 11 647 229 001), which assesses DNA replication by measuring bromodeoxyuridine (BrdU) incorporation. Cells ($5x10^3$ cells/well) from each cell line were plated in 96-well plates (Fisher Scientific, Cat No. 167008) with 100 μl/well growth medium and incubated for 24 h at 37˚C. Cells were washed once in 100 μl/well sterile PBS and incubated in starvation medium for 24 h. After starvation, cells were washed as before and treated for 24 or 48 h with 10 ng/ml TNF-α in 100 μl/well starvation medium. During treatment, medium was supplemented with 10 μM BrdU. Incorporated BrdU was detected according to the manufacturer's instructions. In brief, cells were fixed and incubated with supplied peroxidase conjugated anti-BrdU antibody. Bound antibody was detected by colorimetric turn-over. Colour development was quantified on μQuant microplate spectrophotometer (BioTek, UK) by measuring absorption at 450 nm with a reference wavelength of 690 nm. Three experiments were performed for each cell line and each time point. Each experiment consisted of six replicates for each treatment, i.e. six wells for control and six wells for treatment.

Phospho-kinase ELISA. Cell-based ELISA phospho-AKT (S473) immunoassay (Cat No. KCB887) and phospho-ERK1/ERK2 (T202/Y204) immunoassay (Cat No. KCB1018) were purchased from R&D Systems (Abingdon, UK). Cells from all three cell lines were plated into a supplied clear bottom black 96-well plate at a density of $5x10^3$ cells/well with 100 μl/well growth medium and incubated for 24 h at 37˚C. Cells were washed once in 100 μl/well sterile PBS and incubated in starvation medium for 24 h. After starvation, cells were washed as before and treated with 10 ng/ml TNF-α in 100 μl/well starvation medium for times indicated in Fig. 2. Phosphorylation of AKT and ERK1/2 was then assessed following the manufacturer's instructions. In brief, after fixing, the cells were incubated with phospho-AKT or phospho-ERK1/2 specific antibody in conjunction with anti-total AKT or anti-total ERK1/2 specific antibody, respectively. Phospho-specific antibodies were of mouse origin, while antibodies for the total protein were produced in rabbits. Each antibody was identified by mouse or rabbit specific secondary antibodies, which were tagged with HRP or AP, respectively, allowing for simultaneous quantification of phosphorylated and total AKT or ERK1/2 protein. After antibody incubation, excess secondary antibody was removed by several washes in supplied wash buffer and PBS, before 2 fluorescent substrates were added. Fluorescence was measured on Fluoroskan Ascent microplate reader (Labsystem, UK) with excitation at 544 nm and emission at 590 nm (phosphorylated protein), followed by a second read with excitation at 355 nm and emission at 460 nm (total protein). For each cell line two experiments were performed to assess AKT-phosphorylation and two to assess ERK1/2-phosphorylation after TNF-α treatment. Each experiment included two replicates for each treatment time and the control.

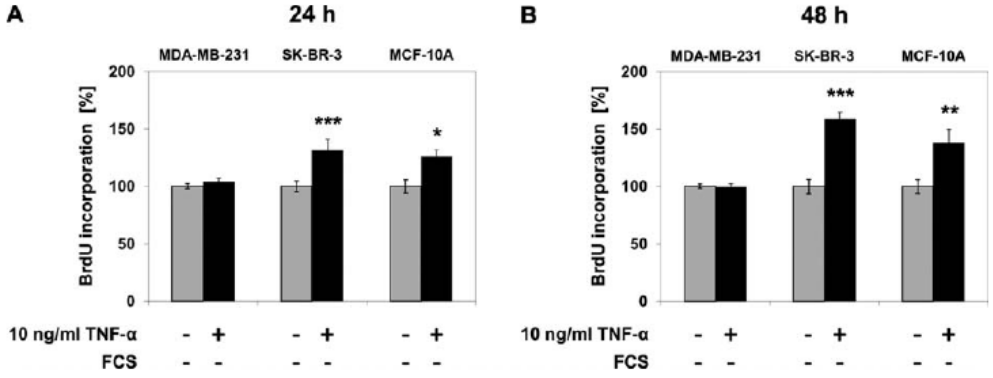

Figure 1. Changes in cell proliferation after treatment with 10 ng/ml TNF-α (A) 24 h or (B) 48 h in MDA-MB-231 breast cancer cells, MCF-10 breast epithelial cells and SK-BR-3 breast cancer cells. Bars represent BrdU-incorporation in relation to the respective control and are expressed as a percentage thereof. Error bars represent ± SEM of three experiments, each consisting of six replicates, i.e. 18 data points for each bar. *Significance value compared to control, obtained using one-way ANOVA analysis. (*0.05>p>0.01; **0.01>p>0.001; ***p<0.001).

Flow cytometry. Changes in the cell distribution across cell cycle stages were assessed by measurement of DNA-content in cells. The DNA specific dye was propidium iodide (PI) (Sigma, Cat No. P4170). Cells were plated at $5\mathbf{x}10^5$ cells/well in 6-well plates with 3 ml growth medium and incubated for 24 h at 37˚C. Cells were starved for 24 h and then treated with 10 ng/ml TNF-α for 24 h. Cells were harvested by trypsin, centrifuged at 500 **x** g for 5 min and each sample was resuspended in 100 μl PBS and 900 μl 70% (v/v) ice-cold ethanol before being incubated at 4˚C for 30 min. Samples were centrifuged at 10,000 **x** g for 5 min, resuspended in 1 ml PBS and again centrifuged at 10,000 **x** g for 5 min. Pellet was resuspended in 500 μl extraction buffer (4 mM citric acid, 0.2 M Na_2HPO_4, pH 7.8) and 500 μl PBS and incubated at room temperature for 5 min. Cells were centrifuged at 10,000 **x** g for 5 min and the pellet was resuspended in 500 μl DNA staining solution (20 μg/ml propidium iodide and 0.2 mg/ml DNAse free RNAse). The cells were incubated at room temperature for 30 min. Flow cytometry of PI stained cells was performed using a Coulter Epics XLMCL flow cytometer (Beckman Coulter, UK). Data were captured and analysed using EXPO 32 Software (Applied Cytometry Systems, Sheffield, UK). Cell cycle distribution was assessed from linear FL-2 area vs. width plots. The percentage of cells in the three cell cycle phases (G1, S and G2) was calculated from histograms of linear FL-2 area plots after assessing 10,000 events. Additionally, percentage of cells with a DNA content <2N (sub-G1) was measured as an indication of apoptosis. Three experiments were performed for each cell line, containing two replicates for control and treatment.

Statistical analysis. Cell proliferation results were assessed using one-way ANOVA. Cell signalling pathway activation and cell cycle results were assessed using Dunnett's post-hoc t-test following univariate analysis of variance. Changes were defined as significantly different at $p<0.05$.

Results

Cell proliferation. Cell proliferation of all cell lines was assessed after 10 ng/ml TNF-α treatment for 24 or 48 h (Fig. 1). Untreated control cells were incubated in starvation medium (Control) at all times. All results are presented as percentage change from this control. In MDA-MB-231 breast cancer cells, cell proliferation did not significantly change after 24- or 48-h treatment with 10 ng/ml TNF-α. In SK-BR-3 breast cancer cells, cell proliferation increased significantly by 31% after 24-h treatment (p=0.016) and by 59% after 48-h treatment (p<0.001) with 10 ng/ml TNF-α. In MCF-10A normal breast epithelial cells, cell proliferation increased significantly by 26% after 24-h treatment (p<0.001) and by 38% after 48-h treatment (p=0.002) with 10 ng/ml TNF-α.

Cell signalling pathway activation. Phosphorylation of AKT or ERK1/2 was measured after treatment with 10 ng/ml TNF-α for between 5-20 min (Fig. 2). Untreated control cells were incubated in starvation medium (Control) at all times. All results are presented as percentage change of this control. In MDA-MB-231 breast cancer cells, AKT-phosphorylation did not change significantly after treatment between 5 and 20 min with 10 ng/ml TNF-α. ERK1/2-phosphorylation was significantly decreased by 22% after 5-min treatment (p=0.044) with 10 ng/ml TNF-α (Fig. 2A). In SK-BR-3 breast cancer cells, AKT-phosphorylation increased significantly by 66, 72 and 91% after 5-min (p=0.04), 10-min (p=0.025) and 20-min treatment (p=0.006)with 10 ng/ml TNF-α, respectively compared to untreated control. ERK1/2-phosphorylation increased significantly by 47% after 5-min treatment (p=0.027) with 10 ng/ml TNF-α (Fig. 2B). In MCF-10A normal breast epithelial cells, AKT-phosphorylation did not change significantly. A non-significant increase of 77% (p=0.169) after 20 min of treatment with 10 ng/ml TNF-α compared to untreated control was, however, observed. ERK1/2-phosphorylation increased

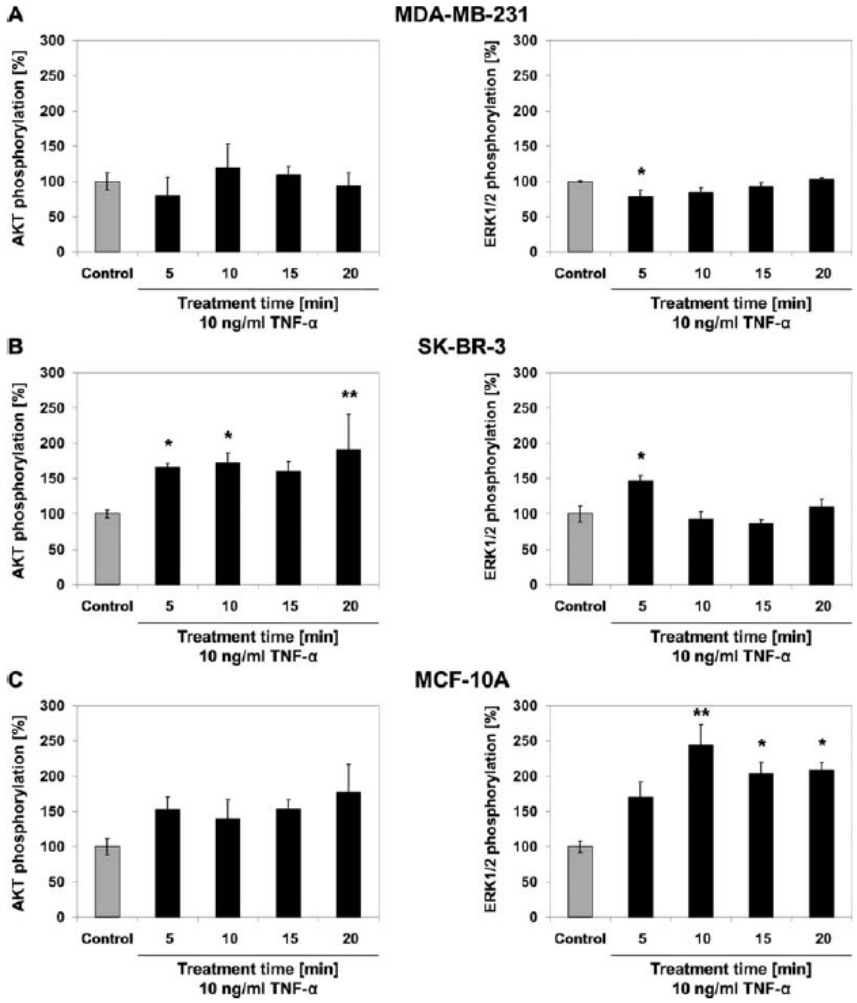

Figure 2. Changes in cell signalling pathway activation [PI3-kinase (left) and MAP-kinase (right))] with 10 ng/ml TNF-α treatment in (A) MDA-MB-231 breast cancer cells (B) SK-BR-3 breast cancer cells and (C) MCF-10A breast epithelial cells. Bars represent AKT-phosphorylation or ERK1/2-phosphorylation in relation to the respective control within each graph and are expressed as a percentage thereof. Error bars represent ± SEM of two experiments, each consisting of two replicates, i.e. four data points for each bar. *Significance value compared to control, obtained using Dunnett's post-hoc t-test following univariate analysis of variance. (*0.05>p>0.01; **0.01>p>0.001).

significantly by 144, 103 and 108% after 10-min (p=0.003), 15-min (p=0.025) and 20-min treatment (p=0.019) with 10 ng/ml TNF-α compared to untreated control (Fig. 2C).

Cell cycle. Cell cycle profiles for all cell lines were established after treatment with 10 ng/ml TNF-α for 24 h (Table I). In MDA-MB-231 breast cancer cells, population of the G1-phase decreased significantly by 3.5 percentage points (a 6% decrease) after 24-h treatment (p=0.006) with 10 ng/ml TNF-α compared to G1-phase population of control cells. S-phase population decreased significantly by 1.8 percentage points (a 15% decrease) after 24-h treatment (p<0.001) with 10 ng/ml TNF-α compared to S-phase population of control cells. No significant difference in G2-phase population after 24-h treatment (p=0.233) with 10 ng/ml TNF-α compared to G2-phase population of control cells was observed. Additionally, the sub-G1 population increased significantly by 4.66 percentage points (a 27% increase) after treatment with 10 ng/ml TNF-α for 24 h (p<0.001). In SK-BR-3 breast cancer cells G1-phase population was not significantly different between 24-h treatment (p=0.811) with 10 ng/ml TNF-α and the control. Population of the S-phase decreased significantly by 1.5 percentage points (a 25% decrease) after 24-h treatment (p=0.011) with 10 ng/ml TNF-α compared to S-phase population of control cells. Population of the G2-phase decreased significantly by 3.8 percentage points (a 24% decrease) after 24-h treatment (p=0.011) with 10 ng/ml TNF-α compared to G2-phase population of control cells. In

Table I. Changes of cell population distribution across cell cycle stages after 24-h treatment with 10 ng/ml TNF-α in MDA-MB-231 cells, SK-BR-3 cells and MCF-10A cells.

	Sub-G1 (%)	G1/G0 (%)	S (%)	G2 (%)
MDA-MB-231				
Control	16.98±1.07	55.12±0.40	7.23±0.65	12.88±0.43
10 ng/ml TNF-α for 24 h	21.64±1.44	51.64±1.14	5.43±0.58	12.38±0.42
UNIANOVA	p<0.001	p=0.006	p<0.001	ns
SK-BR-3				
Control	21.25±1.41	39.90±2.00	6.30±0.45	15.98±0.70
10 ng/ml TNF-α for 24 h	26.62±3.85	37.97±1.14	4.75±0.91	12.19±1.87
UNIANOVA	ns	ns	p=0.011	p=0.011
MCF-10A				
Control	23.17±1.28	65.23±1.50	1.27±0.09	6.60±0.41
10 ng/ml TNF-α for 24 h	27.50±2.33	58.61±2.54	1.54±0.19	7.60 0.76
UNIANOVA	ns	p=0.051	ns	ns

Values represent mean ± standard error of three independent experiments. Each experiment had two replicates, i.e. six data points for control and treatment. ns, not significant.

MCF-10A normal breast epithelial cells no significant difference in any cell cycle phase was observed after 24-h treatment with 10 ng/ml TNF-α compared to control. In the G1-phase population a non-significant decrease of 6.6 percentage points (a 12% decrease) after 24-h treatment (p=0.051) with 10 ng/ml TNF-α compared to G1-phase population of control cells was observed.

Discussion

Adipokines have been hypothesised as molecular explanation of the link between obesity and increased risk of developing postmenopausal breast cancer (19-21). Both leptin (22-26) and adiponectin (22,27-29) have been studied in *in vitro* systems and results have indicated that there is some involvement, even though opposite effects. Their effect on growth promotion in breast cancer cells, however, is small and suggests that additional factors may play a role. The fact that many more adipokines have been discovered recently, the expression of which being affected by obesity, makes the contribution of other adipokines in the molecular link between obesity and postmenopausal breast cancer likely. The effects of TNF-α on a breast cancer cell model were therefore studied to evaluate the contribution of TNF-α in the link between obesity and breast cancer progression. Additionally, a normal breast epithelial cell line was used to examine the potential impact of TNF-α on breast cancer aetiology. The impact of TNF-α on breast epithelial cells has not been assessed previously and represents a novel way of assessing the impact of adipokines on breast cancer aetiology.

In MDA-MB-231 breast cancer cells, cell proliferation did not change after 24- or 48-h treatment with 10 ng/ml TNF-α. Similarly, Mueller and his colleagues observed that 0.25 nM (12.75 ng/ml) TNF-α treatment alone did not change cell proliferation in these cells (30). Thus, there is no indication that TNF-α increases cell proliferation in these cells. In SK-BR-3 breast cancer cells, 10 ng/ml TNF-α treatment increased cell proliferation after 24 and 48 h of treatment. The highest increased was observed after 48-h treatment. Similarly, Rivas and his colleagues observed an increase in SK-BR-3 cell proliferation following 20 ng/ml TNF-α treatment for 48 h (31). There is, therefore, good evidence that TNF-α has a proliferative effect on SK-BR-3 breast cancer cells. Hence, cell proliferation of the two breast cancer cell lines reacted differently to treatment with TNF-α. While both cell lines are ER-negative, there is a difference in HER2 expression with SK-BR-3 cells expressing high levels and MDA-MB-231 cell expressing low levels (32). In addition, MDA-MB-231 cells carry mutations in the genes coding for kinases RAS and RAF, the form part of the MAP-kinase cell signalling pathway (18). These differences may contribute to the different effect of TNF-α on these cells, which may also apply to the other differences observed. In MCF-10A breast epithelial cells, treatment with 10 ng/ml TNF-α increased cell proliferation after 24 and 48 h. In rat breast epithelial cells TNF-α treatment similarly increased proliferation (33,34). Our results indicate that TNF-α treatment has a substantial and sustained effect on promoting cell proliferation in MCF-10A cells.

PI3-kinase signalling is involved in breast cancer development, its activation being linked to increased cell growth (35). In MDA-MB-231 breast cancer cells, TNF-α treatment did not change phosphorylation of AKT, while in SK-BR-3 breast cancer cells, 10 ng/ml TNF-α treatment increased phosphorylation of AKT. These are novel findings as no other studies investigated the phosphorylation of AKT after treatment with TNF-α in these cells. In one study, TNF-α mediated activation of AKT has been linked to activation of

proliferation-stimulating transcription factor NF-κB in T47D breast cancer cells (13). Thus, similarly activation of AKT may be a pathway for TNF-α stimulated cell proliferation in SK-BR-3 breast cancer cells. In MCF-10A breast epithelial cells, 10 ng/ml TNF-α treatment did not change AKT-phosphorylation significantly. A non-significant increase of 77% in AKT-phosphorylation after 20-min treatment was, however, observed.

MAP-kinase signalling is involved in promoting cell proliferation and apoptosis evasion in breast cancer cells (36,37). TNF-α treatment significantly decreased phosphorylation of ERK1/2 in MDA-MB-231 breast cancer cells. In the only comparable study, treatment of MDA-MB-231 breast cancer cells with 10 ng/ml TNF-α for 24 h induced expression of MMP-9, which was reduced after inhibition of MEK1/2 by 10 μM U0126, suggesting that TNF-α activates ERK1/2 to exert this effect (38). This study, however, did not examine ERK1/2-phosphorylation directly. Thus, there is no confirmation to this finding. Additionally, ERK1/2-phosphorylation slowly and non-significantly increases between 5 and 20 min of treatment. Extension of treatment time may be needed to determine, if this trend continues and TNF-α treatment has a time-delay in inducing ERK1/2-phosphorylation after increased treatment time. A reduction in ERK1/2-phosphorylation is explained by TNF-α's interference with the, by mutation of RAS and RAF, constitutively activated MAP-kinase pathway. It also indicates, however, a growth inhibitory effect of TNF-α on these cells, which in the previous results had not been observed. In SK-BR-3 breast cancer cells, 10 ng/ml TNF-α treatment significantly increased phosphorylation of ERK1/2. This is a novel finding and further supports the growth promoting effect of TNF-α in this breast cancer cell line. With both PI3-kinase and MAP-kinase pathway activated in response to TNF-α treatment, it can not be ascertained which pathway may promote the observed increase in cell proliferation. In MCF-10A breast epithelial cells, 10 ng/ml TNF-α increased phosphorylation of ERK1/2 significantly after between 10- and 20-min treatment. The highest increase was observed after 10-min treatment. This indicates a sustained activation of at least 10 min. The non-significant increase in AKT-phosphorylation may suggest that the MAP-kinase may be majorly responsible for mediating TNF-α induced cell proliferation in this cell line.

In MDA-MB-231 breast cancer cells, 10 ng/ml TNF-α treatment for 24 h significantly decreased the G1- and S-phase population of the cell cycle profile. In addition, cells with a DNA-content below that expected of cells in G1-phase were measured. These cells were indicative of apoptotic cells. In MDA-MB-231 breast cancer cells, this 'subG1' population increased significantly after TNF-α treatment. This suggests that TNF-α may promote apoptosis in these cells. This is a novel finding and in addition to the decrease in ERK1/2 phosphorylation after TNF-α treatment suggests a growth inhibiting and even apoptosis inducing effect of TNF-α on MDA-MB-231 breast cancer cells. In SK-BR-3 breast cancer cells, 10 ng/ml TNF-α treatment significantly decreased S-phase and G2-phase population after 24-h treatment. There was, however, no indication of increased apoptosis. A similar result was observed for T47D breast cancer cells, where S-phase and G2-phase decreased after treatment with 10 ng/ml TNF-α for 24 h (14). These authors concluded that TNF-α induced cell cycle arrest at the G1/S cell cycle checkpoint (14), which may similarly apply to SK-BR-3 breast cancer cells. Cell cycle arrest is typically associated with growth inhibition, thus, the cell cycle results oppose the previous findings, all of which could be interpreted as TNF-α promoting cell growth in SK-BR-3 breast cancer cells. There is no conclusive explanation for this observation. One may be confronted with a similar situation as observed for different subtypes of the MCF-7 breast cancer cell line, which showed opposing effects to TNF-α treatment (17). In MCF-10A breast epithelial cells, 10 ng/ml TNF-α did not change the distribution of the cell population across cell cycle phases. G1-phase population decreased non-significantly after TNF-α treatment (p=0.051). The probability value is just above the cut-off point. A decrease in G1-phase population could be interpreted as an increase in cell cycle progression, thus complementing findings from the cell proliferation results and cell signalling pathways. This suggests that all experiments point to an increase in cell growth in MCF-10A cells, following TNF-α treatment.

In conclusion, TNF-α affects breast cancer and the metabolism of breast epithelial cells. The effect on breast cancer cells is, however, not as comprehensive as the effect on breast epithelial cells. In MDA-MB-231 breast cancer cells the results generally suggest no effect on cell growth, with some indications of growth inhibition and even increased apoptosis after TNF-α treatment. Conversely, results for SK-BR-3 cells generally indicate a growth promoting effect of TNF-α, while the cell cycle results are contradicting these findings. TNF-α exerts different and even opposing effects between just two breast cancer cell lines, suggesting there is no general mode of action of TNF-α on breast cancer cells, but that different cells and thus, tumours may react differently to increased TNF-α concentrations. If one assumes cell mosaicism in tumours, one may also speculate that cells of the same tumour could react differently to TNF-α. As the majority of our results indicate growth promotion in SK-BR-3 breast cancer cells, TNF-α can not be excluded as a potential mediator in the link between obesity and breast cancer progression. In MCF-10A breast epithelial cells, TNF-α increases cell proliferation, with no indication of cell cycle arrest or onset of apoptosis. This significant finding high-lights a potential and novel way of impact of TNF-α on breast cancer aetiology and may provide a new understanding in the link between obesity and increased risk of breast cancer development.

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References

1. Calle EE, Rodriguez C, Walker-Thurmond K and Thun MJ: Overweight, obesity, and mortality from cancer in a prospectively studied cohort of U.S. adults. N Engl J Med 348: 1625-1638, 2003.

2. Lahmann PH, Hoffmann K, Allen N, van Gils CH, Khaw KT, Tehard B, Berrino F, Tjonneland A, Bigaard J, Olsen A, Overvad K, Clavel-Chapelon F, Nagel G, Boeing H, Trichopoulos D, Economou G, Bellos G, Palli D, Tumino R, Panico S, Sacerdote C, Krogh V, Peeters PH, Bueno-de-Mesquita HB, Lund E, Ardanaz E, Amiano P, Pera G, Quiros JR, Martinez C, Tormo MJ, Wirfalt E, Berglund G, Hallmans G, Key TJ, Reeves G, Bingham S, Norat T, Biessy C, Kaaks R and Riboli E: Body size and breast cancer risk: findings from the European prospective investigation into cancer and nutrition (EPIC). Int J Cancer 111: 762-771, 2004.
3. Abrahamson PE, Gammon MD, Lund MJ, Flagg EW, Porter PL, Stevens J, Swanson CA, Brinton LA, Eley JW and Coates RJ: General and abdominal obesity and survival among young women with breast cancer. Cancer Epidemiol Biomarkers Prev 15: 1871-1877, 2006.
4. Fain JN, Madan AK, Hiler ML, Cheema P and Bahouth SW: Comparison of the release of adipokines by adipose tissue, adipose tissue matrix, and adipocytes from visceral and subcutaneous abdominal adipose tissues of obese humans. Endocrinology 145: 2273-2282, 2004.
5. Trayhurn P and Wood IS: Adipokines: inflammation and the pleiotropic role of white adipose tissue. Br J Nutr 92: 347-355, 2004.
6. Samaras K, Botelho NK, Chisholm DJ and Lord RV: Subcutaneous and visceral adipose tissue gene expression of serum adipokines that predict type 2 diabetes. Obesity (Silver Spring) 18: 884-889, 2010.
7. Considine RV, Sinha MK, Heiman ML, Kriauciunas A, Stephens TW, Nyce MR, Ohannesian JP, Marco CC, McKee LJ, Bauer TL and Caro JF: Serum immunoreactive-leptin concentrations in normal-weight and obese humans. N Engl J Med 334: 292-295, 1996.
8. Park HS, Park JY and Yu R: Relationship of obesity and visceral adiposity with serum concentrations of CRP, TNF-alpha and IL-6. Diabetes Res Clin Pract 69: 29-35, 2005.
9. Olszanecka-Glinianowicz M, Zahorska-Markiewicz B, Janowska J and Zurakowski A: Serum concentrations of nitric oxide, tumor necrosis factor (TNF)-alpha and TNF soluble receptors in women with overweight and obesity. Metabolism 53: 1268-1273, 2004.
10. Black RA, Rauch CT, Kozlosky CJ, Peschon JJ, Slack JL, Wolfson MF, Castner BJ, Stocking KL, Reddy P, Srinivasan S, Nelson N, Boiani N, Schooley KA, Gerhart M, Davis R, Fitzner JN, Johnson RS, Paxton RJ, March CJ and Cerretti DP: A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells. Nature 385: 729-733, 1997.
11. Carswell EA, Old LJ, Kassel RL, Green S, Fiore N and Williamson B: An endotoxin-induced serum factor that causes necrosis of tumors. Proc Natl Acad Sci USA 72: 3666-3670, 1975.
12. Krajewska M, Krajewski S, Zapata JM, Van Arsdale T, Gascoyne RD, Berern K, McFadden D, Shabaik A, Hugh J, Reynolds A, Clevenger CV and Reed JC: TRAF-4 expression in epithelial progenitor cells: analysis in normal adult, fetal, and tumor tissues. Am J Pathol 152: 1549-1561, 1998.
13. Rivas MA, Carnevale RP, Proietti CJ, Rosemblit C, Beguelin W, Salatino M, Charreau EH, Frahm I, Sapia S, Brouckaert P, Elizalde PV and Schillaci R: TNF alpha acting on TNFR1 promotes breast cancer growth via p42/P44 MAPK, JNK, akt and NF-kappa B-dependent pathways. Exp Cell Res 314: 509-529, 2008.
14. Pusztai L, Lewis CE and McGee JO: Growth arrest of the breast cancer cell line, T47D, by TNF alpha; cell cycle specificity and signal transduction. Br J Cancer 67: 290-296, 1993.
15. Lee SH and Nam HS: TNF alpha-induced down-regulation of estrogen receptor alpha in MCF-7 breast cancer cells. Mol Cells 26: 285-290, 2008.
16. Bogin L, Papa MZ, Polak-Charcon S and Degani H: TNF-induced modulations of phospholipid metabolism in human breast cancer cells. Biochim Biophys Acta 1392: 217-232, 1998.
17. Burow ME, Weldon CB, Tang Y, Navar GL, Krajewski S, Reed JC, Hammond TG, Clejan S and Beckman BS: Differences in susceptibility to tumor necrosis factor alpha-induced apoptosis among MCF-7 breast cancer cell variants. Cancer Res 58: 4940-4946, 1998.
18. Hollestelle A, Elstrodt F, Nagel JH, Kallemeijn WW and Schutte M: Phosphatidylinositol-3-OH kinase or RAS pathway mutations in human breast cancer cell lines. Mol Cancer Res 5: 195-201, 2007.
19. Lorincz AM and Sukumar S: Molecular links between obesity and breast cancer. Endocr Relat Cancer 13: 279-292, 2006.
20. Housa D, Housova J, Vernerova Z and Haluzik M: Adipocytokines and cancer. Physiol Res 55: 233-244, 2006.
21. Hou WK, Xu YX, Yu T, Zhang L, Zhang WW, Fu CL, Sun Y, Wu Q and Chen L: Adipocytokines and breast cancer risk. Chin Med J (Engl) 120: 1592-1596, 2007.
22. Nkhata KJ, Ray A, Schuster TF, Grossmann ME and Cleary MP: Effects of adiponectin and leptin co-treatment on human breast cancer cell growth. Oncol Rep 21: 1611-1619, 2009.
23. Frankenberry KA, Skinner H, Somasundar P, McFadden DW and Vona-Davis LC: Leptin receptor expression and cell signaling in breast cancer. Int J Oncol 28: 985-993, 2006.
24. Garofalo C and Surmacz E: Leptin and cancer. J Cell Physiol 207: 12-22, 2006.
25. Dieudonne MN, Machinal-Quelin F, Serazin-Leroy V, Leneveu MC, Pecquery R and Giudicelli Y: Leptin mediates a proliferative response in human MCF7 breast cancer cells. Biochem Biophys Res Commun 293: 622-628, 2002.
26. O'brien SN, Welter BH and Price TM: Presence of leptin in breast cell lines and breast tumors. Biochem Biophys Res Commun 259: 695-698, 1999.
27. Dos Santos E, Benaitreau D, Dieudonne MN, Leneveu MC, Serazin V, Giudicelli Y and Pecquery R: Adiponectin mediates an antiproliferative response in human MDA-MB 231 breast cancer cells. Oncol Rep 20: 971-977, 2008.
28. Korner A, Pazaitou-Panayiotou K, Kelesidis T, Kelesidis I, Williams CJ, Kaprara A, Bullen J, Neuwirth A, Tseleni S, Mitsiades N, Kiess W and Mantzoros CS: Total and high-molecular-weight adiponectin in breast cancer: in vitro and in vivo studies. J Clin Endocrinol Metab 92: 1041-1048, 2007.
29. Dieudonne MN, Bussiere M, Dos Santos E, Leneveu MC, Giudicelli Y and Pecquery R: Adiponectin mediates antiproliferative and apoptotic responses in human MCF7 breast cancer cells. Biochem Biophys Res Commun 345: 271-279, 2006.
30. Mueller H, Flury N, Liu R, Scheidegger S and Eppenberger U: Tumour necrosis factor and interferon are selectively cytostatic in vitro for hormone-dependent and hormone-independent human breast cancer cells. Eur J Cancer 32A: 2312-2318, 1996.
31. Rivas MA, Tkach M, Beguelin W, Proietti CJ, Rosemblit C, Charreau EH, Elizalde PV and Schillaci R: Transactivation of ErbB-2 induced by tumor necrosis factor alpha promotes NF-kappaB activation and breast cancer cell proliferation. Breast Cancer Res Treat 122: 111-124, 2010.
32. Kurebayashi J: Biological and clinical significance of HER2 overexpression in breast cancer. Breast Cancer 8: 45-51, 2001.
33. Ip MM, Shoemaker SF and Darcy KM: Regulation of rat mammary epithelial cell proliferation and differentiation by tumor necrosis factor-alpha. Endocrinology 130: 2833-2844, 1992.
34. Varela LM, Stangle-Castor NC, Shoemaker SF, Shea-Eaton WK and Ip MM: TNFalpha induces NFkappaB/p50 in association with the growth and morphogenesis of normal and transformed rat mammary epithelial cells. J Cell Physiol 188: 120-131, 2001.
35. Scheid MP and Woodgett JR: Phosphatidylinositol 3′ kinase signaling in mammary tumorigenesis. J Mammary Gland Biol Neoplasia 6: 83-99, 2001.
36. Reddy KB, Nabha SM and Atanaskova N: Role of MAP kinase in tumor progression and invasion. Cancer Metastasis Rev 22: 395-403, 2003.
37. McCubrey JA, Steelman LS, Chappell WH, Abrams SL, Wong EW, Chang F, Lehmann B, Terrian DM, Milella M, Tafuri A, Stivala F, Libra M, Basecke J, Evangelisti C, Martelli AM and Franklin R: Roles of the Raf/MEK/ERK pathway in cell growth, malignant transformation and drug resistance. Biochim Biophys Acta 1773: 1263-1284, 2007.
38. Kim S, Choi JH, Kim JB, Nam SJ, Yang JH, Kim JH and Lee JE: Berberine suppresses TNF-alpha-induced MMP-9 and cell invasion through inhibition of AP-1 activity in MDA-MB-231 human breast cancer cells. Molecules 13: 2975-2985, 2008.

Effect of hyperinsulinaemia and impaired adipokines secretion on proliferation of human breast cancer and normal human breast epithelial cells

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Recent findings suggest a connection between obesity and breast cancer. Obesity is associated with impaired secretion of adipokines and, through the metabolic syndrome, promotes hyperinsulinaemia.
This project investigated the effect of hyperinsulinaemia and impaired adipokine secretion on proliferation of two Estrogen-Receptor (ER) negative breast cancer cell lines (MDA-MB-231, SK-BR-3) and one ER-negative normal breast epithelial cell line (MCF-10a).
Cells were incubated with insulin (100 nM), leptin (100 nM), TNF-alpha (10 ng/ml), IL-6 (10 ng/ml) and adiponectin (500 ng/ml) for 24 or 48h and cell proliferation was assessed by BrdU incorporation. Mean of control was compared to treatment by two-tailed t-test. *p<0.05; **p<0.01; ***p<0.001.

Results:

Cell line	**BrdU-incorporation** [% of control]									
	Insulin		**Leptin**		**TNF-alpha**		**IL-6**		**Adiponectin**	
	24 h	**48h**	**24 h**	**48h**	**24 h**	**48h**	**24 h**	**48h**	**24 h**	**48h**
MDA-MB-231	99	82***	121**	101	104	99	104	96	102	97
MCF-10a	284***	137**	97	114*	126**	138**	109	138**	91	91

MDA-MB-231 cancer cells are least likely to respond to insulin and adipokines stimulation. SK-BR-3 cancer cell proliferation is increased by TNF-alpha treatment, whereas 24h IL-6 and adiponectin treatments reduced cell proliferation. Normal breast epithelial cell (MCF-10a) proliferation is highly increased with insulin treatment, and stimulated with 48h leptin, TNF-alpha and IL-6 treatments. These preliminary results suggest that hyperinsulinaemia and impaired adipokines secretion may affect carcinogenesis of breast epithelial cells rather than increasing cancer progression.

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Insulin-induced gene expression changes in breast cancer cells and normal breast epithelial cells

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Obesity increases breast cancer incidence rates in postmenopausal women. Chronic high levels of insulin, present in the majority of obese and insulin resistant patients, may provide the growth promoting stimulus to explain this connection. In this work, the cancer progression and cancer initiating properties of high insulin levels were examined in breast cancer cells (MDA-MB-231) and breast epithelial cells (MCF-10a), respectively.
High insulin levels (100 nM) induced differential changes in cell proliferation in the two cell lines used. Human Cancer PathwayFinder DNA Microarrays (SABiosciences) were used to examine gene expression changes after insulin treatment. High insulin levels increased expression of genes involved in cell cycle control (e.g. cyclin D1) and DNA damage repair (e.g. ATM) in MDA-MB 231 cells and in MCF-10a cells (e.g. cyclin E1, CDC25a). Expression of genes responsible for mediating apoptosis and cell senescence (e.g. APAF, BAD, bcl-X) was decreased after insulin treatment in MDA-MB 231 cells but the expression of the same group of genes did not change in MCF-10a cells. High insulin levels increased expression of genes encoding for signal transduction molecules (e.g. AKT1) and transcription factors (e.g. FOS, JUN, MYC), and of genes responsible for invasion and metastasis (e.g. MMP2) in MCF-10a cells whereas gene expression of the same groups of genes did not change or was decreased in MDA-MB 231 cells. These results suggest a role for insulin resistance in breast cancer initiation and progression, aggravating the potential of breast cancer to evade apoptosis, to metastasise and may promote carcinogenesis of healthy epithelial cells.

Conflict of interest:
None

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Insulin-induced gene expression changes in breast cancer cells and normal breast epithelial cells

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CENTRE FOR OBESITY RESEARCH AND EPIDEMIOLOGY
THE ROBERT GORDON UNIVERSITY, ABERDEEN

Introduction

Breast cancer is prevailing as the most diagnosed cancer in women. Obesity and its co-morbidities, including type-II diabetes, are increasing to epidemic proportions.

A pathological link between obesity, breast cancer risk and mortality has been established recently.

Insulin resistance has been closely associated with obesity. It is considered a pre-stage of type 2 diabetes and is characterised by chronic high circulating levels of insulin.

Previously we have demonstrated the ability of high insulin levels to differentially activate insulin receptor, PI3- kinase and MAP-kinase cell signalling pathways in MDA-MB 231 human breast cancer cells and in MCF-10a human normal breast epithelial cells in addition to increase cell proliferation in MCF-10a cells.

We here demonstrate changes in gene expression profiles after treatment of both cell lines with 100 nM insulin for 1 h.

Objective

To examine the effects of high insulin levels (100 nM) on gene expression in MDA-MB 231 cells and MCF-10a cells.

Method

Oligo GEArray® Human Cancer PathwayFinder™ Microarray from SABiosciences was used to detect gene expression changes.

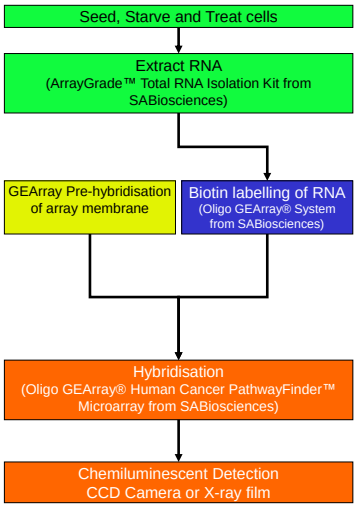
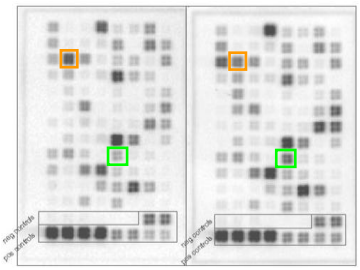


Figure 1: Flowchart of Microarray-analysis

Results



Control 100 nM Insulin

Gene expression increased after treatment

Gene expression decreased after treatment

Figure 2: Representative image of microarray result

Summary and Conclusion

High insulin levels increased expression of genes involved in cell cycle control (e.g. cyclin E1) and DNA damage repair (e.g. ATM) in MDA-MB 231 cells and in MCF-10a cells (e.g. CDC25a).

Expression of genes responsible for mediating apoptosis and cell senescence (e.g. APAF, BAD, bcl-X) was decreased after insulin treatment in MDA-MB 231 cells but the expression of the same group of genes did not change in MCF-10a cells.

High insulin levels increased expression of genes encoding for signal transduction molecules (e.g. AKT1) and transcription factors (e.g. FOS, JUN, MYC), and of genes responsible for invasion and metastasis (e.g. MMP2) in MCF-10a cells whereas gene expression of the same groups of genes did not change or was decreased in MDA-MB 231 cells.

These results suggest a role for insulin resistance in breast cancer initiation and progression, aggravating the potential of breast cancer cells to evade apoptosis, to metastasise and may promote carcinogenesis of healthy epithelial cells.

Detailed Results

Gene description	MDA-MB-231	MCF-10a
Ribosomal protein S27a	1.1	8.3
V-akt murine thymoma viral oncogene homolog 1	0.6	UP
Angiopoietin 1	0.2	ND
Angiopoietin 2	0.9	ND
Apoptotic peptidase activating factor 1	0.3	ND
Ataxia telangiectasia mutated	0.8	ND
BCL2-antagonist of cell death	0.7	ND
Brain-specific angiogenesis inhibitor 1	0.7	ND
BCL2-associated X protein	1.2	UP
B-cell CLL/lymphoma 2	6.1	UP
BCL2-like 1	2.5	ND
Baculoviral IAP repeat-containing 5 (survivin)	1.3	2.9
Breast cancer 1, early onset	2.8	ND
Breast cancer 2, early onset	0.6	UP
Caspase 8, apoptosis-related cysteine peptidase	0.7	ND
Caspase 9, apoptosis-related cysteine peptidase	0.9	UP
Cyclin D1	UP	ND
Cyclin E1	4.0	UP
CD44 molecule (Indian blood group)	3.7	UP
Cell division cycle 25 homolog A (S. pombe)	1.3	4.1
Cadherin 1, type 1, E-cadherin (epithelial)	DOWN	ND
Cyclin-dependent kinase 2	0.5	UP
Cyclin-dependent kinase 4	1.1	2.4
Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	0.9	1.4
Cyclin-dependent kinase inhibitor 1B (p27, Kip1)	UP	ND
Cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)	ND	ND
CASP8 and FADD-like apoptosis regulator	ND	ND
CHK2 checkpoint homolog (S. pombe)	ND	ND
Collagen, type XVIII, alpha 1	1.7	ND
Catenin (cadherin-associated protein), beta 1, 88kDa	DOWN	ND
E2F transcription factor 1	0.9	ND
Epidermal growth factor (beta-urogastrone)	0.3	ND
Epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian)	2.6	4.6
V-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)	1.2	2.5
V-Ets erythroblastosis virus E26 oncogene homolog 2 (avian)	1.7	ND
Fibroblast growth factor 2 (basic)	ND	ND

Gene description	MDA-MB-231	MCF-10a
Jun oncogene	1.5	3.8
CD82 molecule	ND	ND
KiSS-1 metastasis-suppressor	ND	UP
Mitogen-activated protein kinase kinase 1	0.7	1.2
Mitogen-activated protein kinase 14	DOWN	ND
Melanoma cell adhesion molecule	0.4	ND
Mdm2, transformed 3T3 cell double minute 2, p53 binding protein (mouse)	DOWN	ND
Met proto-oncogene (hepatocyte growth factor receptor)	UP	ND
MHC class I polypeptide-related sequence A	UP	ND
Matrix metallopeptidase 1 (interstitial collagenase)	0.8	ND
Matrix metallopeptidase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)	0.4	1.5
Matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)	DOWN	ND
Metastasis associated 1	DOWN	ND
Metastasis associated 1 family, member 2	DOWN	ND
Metastasis suppressor 1	ND	ND
V-myc myelocytomatosis viral oncogene homolog (avian)	ND	UP
Neural cell adhesion molecule 1	ND	ND
Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)	0.8	1.4
Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	1.1	1.0
Non-metastatic cells 1, protein (NM23A) expressed in	1.0	1.9
Non-metastatic cells 4, protein expressed in	0.4	0.7
Platelet-derived growth factor alpha polypeptide	DOWN	ND
Platelet-derived growth factor beta polypeptide (simian sarcoma viral (v-sis) oncogene homolog)	0.3	ND
Phosphoinositide-3-kinase, catalytic, beta polypeptide	UP	UP
Phosphoinositide-3-kinase, regulatory subunit 1 (alpha)	ND	1.8
Plasminogen activator, urokinase	1.0	1.2
Plasminogen activator, urokinase receptor	1.1	1.7
Pinin, desmosome associated protein	1.5	1.2
Protein kinase, DNA-activated, catalytic polypeptide	ND	ND
Phosphatase and tensin homolog (mutated in multiple advanced cancers 1)	DOWN	ND
V-raf-1 murine leukemia viral oncogene homolog 1	DOWN	ND
RAS p21 protein activator (GTPase activating protein) 1	ND	ND
Retinoblastoma 1 (including osteosarcoma)	2.4	2.6
S100 calcium binding protein A4	0.9	0.9
Serpin peptidase inhibitor, clade B (ovalbumin), member 2	1.2	2.0
Serpin peptidase inhibitor, clade B (ovalbumin), member 5	0.9	1.0

Table 1: Changes in gene expression expressed as fold of expression in control cells, i.e. expression in control cells is 1.0

≥1.5 gene expression increased with treatment (green)
≤0.7 gene expression decreased with treatment (orange)

"UP": expression only detected in treated cells
"DOWN": expression only detected in control cells
"ND": No expression detected in either cells

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breast cancer CAMPAIGN researching the cure

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Molecular aspects linking insulin resistance to breast cancer by activation of cell signalling pathways

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Recent findings suggest a connection between obesity and breast cancer. Obesity is linked with higher incidences of insulin resistance as part of the metabolic syndrome, resulting in chronically elevated insulin plasma levels. We examined the effect of high insulin concentrations (100 nM) on estrogen-receptor (ER) negative breast cancer cells (MDA-MB-231) and normal breast epithelial cells (MCF-10a).
Treatment with high insulin concentrations increased insulin receptor (IR) phosphorylation significantly in both cell lines. Phosphorylation of protein kinase B (Akt), representative of PI3-kinase cell signalling pathway activation was increased by 101% ($p=0.0112$) in MDA-MB-231 cells and by 81% ($p=0.0031$) in MCF-10a cells after 10 min insulin treatment. Phosphorylation of extracellular regulated kinase 1/2 (ERK1/2), representative of MAP-kinase cell signalling pathway activation did not change in both cell lines after 10 min of insulin treatment. Cell proliferation did not change in MDA-MB-231 cells and increased by 75% ($p=0.0067$) in MCF-10a cells after 24 h insulin treatment. Cell proliferation was decreased in MDA-MB-231 cells by 15% ($p=0.0083$) after 1 h treatment with PD98059, a MAP-kinase inhibitor. In MCF-10a cells cell proliferation was decreased by 51% ($p<0.0001$) after 1 h treatment with wortmannin, a PI3-kinase inhibitor and by 56% ($p=0.236$) after 1 h treatment with PD98059.
These preliminary findings suggest that insulin resistance may increase carcinogenesis of breast epithelial cells but may not increase cancer progression in ER-negative breast tumours. Both the PI3-kinase and MAP-kinase pathway may be responsible for mediating insulin induced cell proliferation. Further studies are needed to verify the involvement of the MAP-kinase pathway in cell proliferation in MDA-MB-231 cells.

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Molecular aspects linking insulin resistance to breast cancer by activation of cell signalling pathways

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Abstract

Recent findings suggest a connection between obesity and breast cancer. Obesity is linked with higher incidences of insulin resistance as part of the metabolic syndrome, resulting in chronically elevated insulin plasma levels. We examined the effect of high insulin concentrations (100 nM) on estrogen-receptor (ER) negative breast cancer cells (MDA-MB-231) and normal breast epithelial cells (MCF-10a).

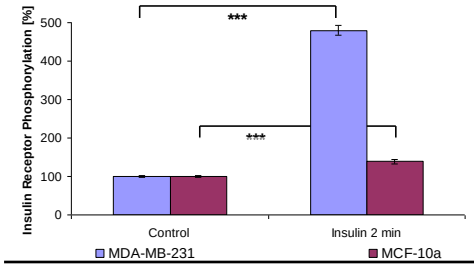
Treatment with high insulin concentrations increased insulin receptor (IR) phosphorylation significantly in both cell lines. Phosphorylation of protein kinase B (Akt), representative of PI3-kinase cell signalling pathway activation was increased by 101% (p=0.0112) in MDA-MB-231 cells and by 81% (p=0.0031) in MCF-10a cells after 10 min insulin treatment. Phosphorylation of extracellular regulated kinase 1/2 (ERK1/2), representative of MAP-kinase cell signalling pathway activation did not change in both cell lines after 10 min of insulin treatment. Cell proliferation did not change in MDA-MB-231 cells and increased by 75% (p=0.0067) in MCF-10a cells after 24 h insulin treatment. Cell proliferation was decreased in MDA-MB-231 cells by 15% (p=0.0083) after 24 h treatment with PD98059, a MAP-kinase inhibitor. In MCF-10a cells cell proliferation was decreased by 51% (p<0.0001) after 24 h treatment with wortmannin, a PI3-kinase inhibitor and by 56% (p=0.236) after 24 h treatment with PD98059.

These preliminary findings suggest that insulin resistance may increase carcinogenesis of breast epithelial cells but may not increase cancer progression in ER-negative breast tumours. Both the PI3-kinase and MAP-kinase pathway may be responsible for mediating insulin induced cell proliferation. Further studies are needed to verify the involvement of the MAP-kinase pathway in cell proliferation in MDA-MB-231 cells.

Results

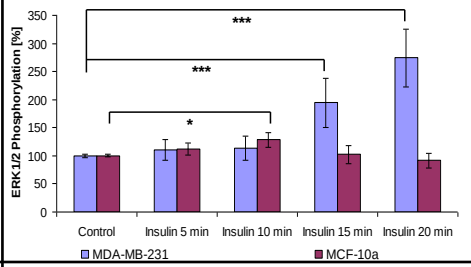
High insulin levels and Insulin Receptor phosphorylation

ELISA kits were used to measure IR phosphorylation after 2 min treatment with 100 nM Insulin



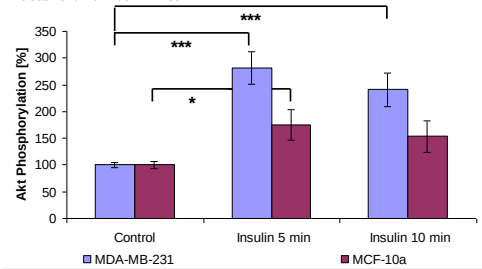
High insulin levels and MAP-kinase cell signalling pathway activation

ELISA kits were used to measure ERK1/2 phosphorylation after 5-20 min treatment with 100 nM Insulin



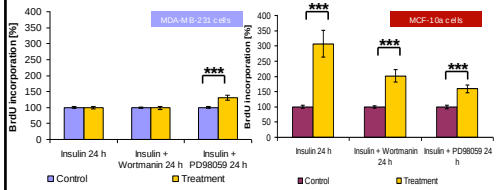
High insulin levels and PI3-kinase cell signalling pathway activation

ELISA kits were used to measure protein kinase B (Akt) phosphorylation after 5-10 min treatment with 100 nM Insulin



High insulin levels and cell proliferation

BrdU-incorporation was used to measure cell proliferation after treatment for 24 h with 100 nM Insulin alone or in combination with either 100 nM wortmannin or 50 µM PD98059.



All experiments are expressed as mean ± sem of three experiments with two replicates (n≥6), except BrdU (n≥18). T-test has been used to compare control to treatment.
* p<0.05; *** p<0.001

Objectives

1. **To examine the effects of high insulin levels (100 nM) on phosphorylation of Insulin Receptor in cancerous MDA-MB-231 and normal breast epithelial MCF-10a cells.**
2. **To examine activation of PI3-kinase and MAP-kinase cell signalling pathways after insulin treatment.**
3. **To examine cell proliferation in cancerous MDA-MB-231 and normal breast epithelial MCF-10a cells after insulin treatment.**
4. **To examine the role of PI3-kinase and MAP-kinase cell signalling pathways in cell proliferation.**

Conclusion

1. **High insulin levels affect both cancerous MDA-MB-231 cells and normal breast epithelial MCF-10a cells.**
2. **Activation of Insulin Receptor, PI3-kinase and MAP-kinase cell signalling pathways is higher in cancerous MDA-MB-231 cells than in normal breast epithelial MCF-10a cells.**
3. **But only normal breast epithelial MCF-10a cells respond to high insulin stimulation with increased cell proliferation, pointing to a role of insulin resistance in breast cancer initiation.**
4. **High insulin levels stimulate cancerous MDA-MB-231 cells, but the physiological effects need to be investigated.**

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- **Breast Cancer Campaign**
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Molecular aspects of insulin resistance, cell signalling pathways and breast cancer in relation to obesity

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A growing number of clinical studies validate a relation of insulin resistance and breast cancer in obese patients. We hypothesised that high plasma insulin levels cause aberrant insulin signalling in breast epithelial cells which may be responsible for an increase in cell proliferation, indicative of potential carcinogenesis and increased cancer progression. It was of particular interest to determine any differences of high insulin concentrations in activating the phosphoinositide-3 kinase (PI-3 kinase) pathway or the mitogen-activated protein kinase (MAP kinase) pathway, the latter being linked to increased cell proliferation.
We used two cell line models to investigate the carcinogenic (MCF-10A, immortalised breast epithelial cells) and cancer progression (MDA-MB-231, ER-negative breast cancer cells) potential of insulin. Insulin treatment (100 nM, 24 h) increased cell proliferation in MCF-10A cells, but had no cell proliferative effect on MDA-MB-231 cells. Additionally expression of PCNA as marker of proliferation was tested. The use of PI-3 kinase and MAP kinase specific inhibitors (Wortmannin and PD98059, respectively) demonstrated both pathways being responsible for the observed increase in cell proliferation (MCF-10A). Simultaneous treatment with both inhibitors eliminated insulin induced cell proliferation entirely. Phosphorylation of ERK1/2 was examined as specific activity measurement of MAP kinase pathway. Insulin induced higher phosphorylation levels in MCF-10A cells than in MDA-MB-231.
These preliminary results suggest that insulin may initiate carcinogenesis of breast epithelial cells by increasing cell proliferation rather than increasing cancer progression of existing tumours. These effects may be mediated by insulin activating both the PI-3 kinase and the MAP kinase signalling pathways.

Conflict of interest:
None disclosed

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Molecular aspects of insulin resistance, cell signaling pathways and breast cancer in relation to obesity

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Introduction

Breast cancer is prevailing as the most diagnosed cancer in women. Obesity and its co-morbidities, including type-II diabetes, are increasing to epidemic proportions.

A pathological link between obesity, breast cancer risk and mortality has been established recently[1].

Insulin resistance is a pre-malignant indicator of type-II diabetes and closely linked with obesity. Its molecular implications result in chronically elevated circulating insulin plasma levels.

Here we examine the effect of high insulin levels on cell proliferation, activation of PI3-kinase and MAP-kinase cell signalling pathways in estrogen-receptor negative (ER-) breast cancer cells (MDA-MB-231) and on normal breast epithelial cells (MCF-10a).

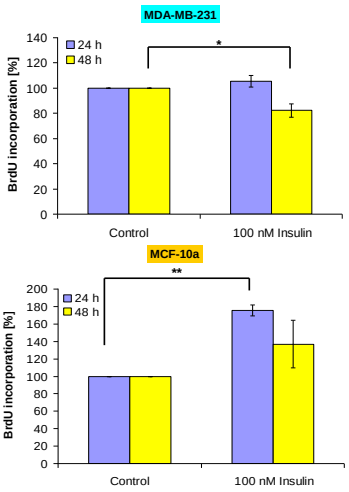
Objectives

1. **To examine the effects of high insulin levels (100 nM) on cell proliferation in MDA-MB-231 and MCF-10a cells.**
2. **To examine activation of PI3-kinase and MAP-kinase cell signalling pathways after insulin treatment.**
3. **To examine the role of PI3-kinase and MAP-kinase cell signalling pathways in insulin-mediated cell proliferation.**

Results

Effects of high insulin levels on cell proliferation

BrdU-incorporation was used to measure DNA-synthesis after 24 and 48 h treatment with 100 nM insulin

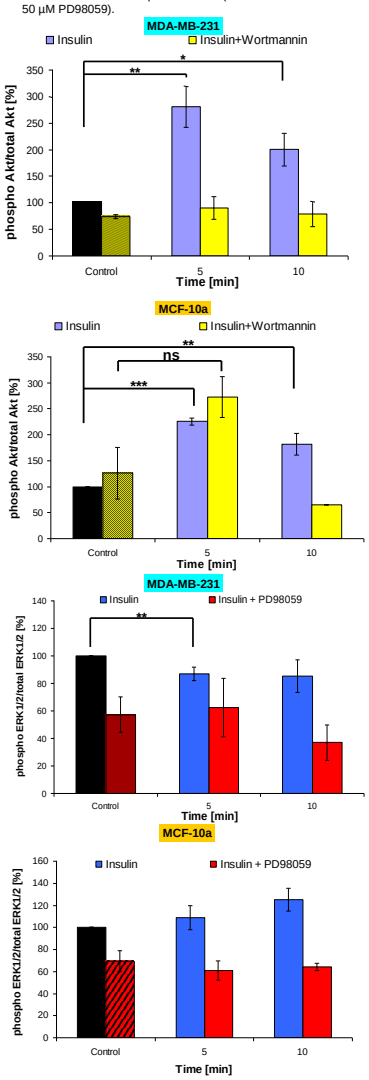


High insulin concentrations increased cell proliferation in normal breast epithelial cells, but not in ER- breast cancer cells.

Results

Effect of high insulin levels on activation of PI3-kinase and MAP-kinase cell signalling pathways

ELISA kits were used to measure Akt and ERK1/2 phosphorylation after 5 and 10 min treatment with 100 nM insulin and with/without pre-treatment (100 nM wortmannin or 50 µM PD98059).

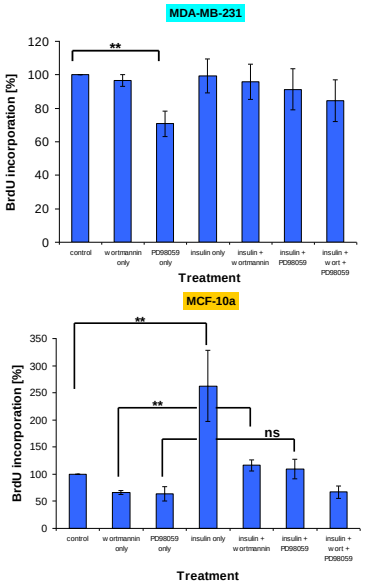


High insulin concentrations activated the PI3-kinase, but not the MAP-kinase cell signalling pathway in ER- breast cancer cells and in normal breast epithelial cells.

Results

Inhibition of cell signalling pathways and effects on cell proliferation after insulin stimulation

DNA-synthesis was measured during 24 h treatment with 100 nM insulin following cell signalling pathway inhibition by 1 h pre-treatment with 100 nM wortmannin and/or 50 µM PD98059



Inhibition of the MAP-kinase pathway reduced cell proliferation in ER- breast cancer cells. In normal breast epithelial cells, inhibition of MAP-kinase pathway reduced insulin-induced cell proliferation.

Conclusions

1. Further preliminary results indicate an activation of MAP-kinase cell signalling pathway after 15 min of insulin treatment in MCF-10a cells (data not shown).
2. Insulin may initiate carcinogenesis of breast epithelial cells by increasing cell proliferation (MCF-10a), rather than increasing cancer progression of existing tumours (MDA-MB-231).
3. These effects may be mediated by insulin activating the MAP-kinase pathway.
4. The MAP-kinase pathway may be a future target for breast cancer prevention in obese women.

References

1. Calle, E.E., Rodriguez, C., Walker-Thurmond, K. and Thun, M.J. (2003) Overweight, obesity and mortality from cancer in a prospectively studied cohort of U.S. adults. N. Engl. J. Med., 348, 1625-1638.

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References

Abate, N., Garg, A., Coleman, R., Grundy, S. M. and Peshock, R. M. (1997) Prediction of total subcutaneous abdominal, intraperitoneal, and retroperitoneal adipose tissue masses in men by a single axial magnetic resonance imaging slice. *Am. J. Clin. Nutr.*, **65**(2), 403-408.

Abel, E.D., Peroni, O., Kim, J. K., Kim, Y. B., Boss, O., Hadro, E., Minnemann, T., Shulman, G. I. and Kahn, B. B. (2001) Adipose-selective targeting of the GLUT4 gene impairs insulin action in muscle and liver. *Nature*, **409**(6821), 729-733.

Abrahamson, P.E., Gammon, M. D., Lund, M. J., Flagg, E. W., Porter, P. L., Stevens, J., Swanson, C. A., Brinton, L. A., Eley, J. W. and Coates, R. J. (2006) General and abdominal obesity and survival among young women with breast cancer. *Cancer Epidemiol. Biomarkers Prev.*, **15**(10), 1871-1877.

Alberti, K.G. and Zimmet, P. Z. (1998) Definition, diagnosis and classification of diabetes mellitus and its complications. part 1: Diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. *Diabet. Med.*, **15**(7), 539-553.

Alblas, J., Slager-Davidov, R., Steenbergh, P. H., Sussenbach, J. S. and van der Burg, B. (1998) The role of MAP kinase in TPA-mediated cell cycle arrest of human breast cancer cells. *Oncogene*, **16**(1), 131-139.

Alimova, I.N., Liu, B., Fan, Z., Edgerton, S. M., Dillon, T., Lind, S. E. and Thor, A. D. (2009) Metformin inhibits breast cancer cell growth, colony formation and induces cell cycle arrest in vitro. *Cell. Cycle*, **8**(6), 909-915.

Allen, N.E., Appleby, P. N., Kaaks, R., Rinaldi, S., Davey, G. K. and Key, T. J. (2003) Lifestyle determinants of serum insulin-like growth-factor-I (IGF-I), C-peptide and hormone binding protein levels in british women. *Cancer Causes Control*, **14**(1), 65-74.

Allison, D.B., Fontaine, K. R., Manson, J. E., Stevens, J. and VanItallie, T. B. (1999) Annual deaths attributable to obesity in the united states. *JAMA*, **282**(16), 1530-1538.

Alokail, M.S., Al-Daghri, N. M., Al-Attas, O. S. and Hussain, T. (2009) Combined effects of obesity and type 2 diabetes contribute to increased breast cancer risk in premenopausal women. *Cardiovasc. Diabetol.*, **8**, 33.

Ambrosini, G., Nath, A. K., Sierra-Honigmann, M. R. and Flores-Riveros, J. (2002) Transcriptional activation of the human leptin gene in response to hypoxia. involvement of hypoxia-inducible factor 1. *J. Biol. Chem.*, **277**(37), 34601-34609.

Amling, C.L. (2004) The association between obesity and the progression of prostate and renal cell carcinoma. *Urol. Oncol.*, **22**(6), 478-484.

Anderson, A.S. and Caswell, S. (2009) Obesity management--an opportunity for cancer prevention. *Surgeon*, **7**(5), 282-285.

Ando, S., Panno, M. L., Salerno, M., Sisci, D., Mauro, L., Lanzino, M. and Surmacz, E. (1998) Role of IRS-1 signaling in insulin-induced modulation of estrogen receptors in breast cancer cells. *Biochem. Biophys. Res. Commun.*, **253**(2), 315-319.

Antuna-Puente, B., Feve, B., Fellahi, S. and Bastard, J. P. (2008) Adipokines: The missing link between insulin resistance and obesity. *Diabetes Metab.*, **34**(1), 2-11.

Applanat, M.P., Buteau-Lozano, H., Herve, M. A. and Corpet, A. (2008) Vascular endothelial growth factor is a target gene for estrogen receptor and contributes to breast cancer progression. *Adv. Exp. Med. Biol.*, **617**, 437-444.

Arditi, J.D., Venihaki, M., Karalis, K. P. and Chrousos, G. P. (2007) Antiproliferative effect of adiponectin on MCF7 breast cancer cells: A potential hormonal link between obesity and cancer. *Horm. Metab. Res.*, **39**(1), 9-13.

Ardizzoia, A., Lissoni, P., Brivio, F., Tisi, E., Perego, M. S., Grassi, M. G., Pittalis, S., Crispino, S., Barni, S. and Tancini, G. (1992) Tumor necrosis factor in solid tumors: Increased blood levels in the metastatic disease. *J. Biol. Regul. Homeost. Agents*, **6**(3), 103-107.

Arita, Y., Kihara, S., Ouchi, N., Takahashi, M., Maeda, K., Miyagawa, J., Hotta, K., Shimomura, I., Nakamura, T., Miyaoka, K., Kuriyama, H., Nishida, M., Yamashita, S., Okubo, K., Matsubara, K., Muraguchi, M., Ohmoto, Y., Funahashi, T. and Matsuzawa, Y. (1999) Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. *Biochem. Biophys. Res. Commun.*, **257**(1), 79-83.

Asano, T., Fujishiro, M., Kushiyama, A., Nakatsu, Y., Yoneda, M., Kamata, H. and Sakoda, H. (2007) Role of phosphatidylinositol 3-kinase activation on insulin action and its alteration in diabetic conditions. *Biol. Pharm. Bull.*, **30**(9), 1610-1616.

Asgeirsson, K.S., Olafsdottir, K., Jonasson, J. G. and Ogmundsdottir, H. M. (1998) The effects of IL-6 on cell adhesion and e-cadherin expression in breast cancer. *Cytokine*, **10**(9), 720-728.

Auwerx, J. and Staels, B. (1998) Leptin. *Lancet*, **351**(9104), 737-742.

Bachelot, T., Ray-Coquard, I., Menetrier-Caux, C., Rastkha, M., Duc, A. and Blay, J. Y. (2003) Prognostic value of serum levels of interleukin 6 and of serum and

plasma levels of vascular endothelial growth factor in hormone-refractory metastatic breast cancer patients. *Br. J. Cancer*, **88**(11), 1721-1726.

Backer, J.M., Myers, M. G.,Jr, Shoelson, S. E., Chin, D. J., Sun, X. J., Miralpeix, M., Hu, P., Margolis, B., Skolnik, E. Y., Schlessinger, J. and White, M. F. (1992) Phosphatidylinositol 3'-kinase is activated by association with IRS-1 during insulin stimulation. *EMBO J.*, **11**(9), 3469-3479.

Bacon, B.R., Farahvash, M. J., Janney, C. G. and Neuschwander-Tetri, B. A. (1994) Nonalcoholic steatohepatitis: An expanded clinical entity. *Gastroenterology*, **107**(4), 1103-1109.

Badache, A. and Hynes, N. E. (2001) Interleukin 6 inhibits proliferation and, in cooperation with an epidermal growth factor receptor autocrine loop, increases migration of T47D breast cancer cells. *Cancer Res.*, **61**(1), 383-391.

Bado, A., Levasseur, S., Attoub, S., Kermorgant, S., Laigneau, J. P., Bortoluzzi, M. N., Moizo, L., Lehy, T., Guerre-Millo, M., Le Marchand-Brustel, Y. and Lewin, M. J. (1998) The stomach is a source of leptin. *Nature*, **394**(6695), 790-793.

Baglietto, L., English, D. R., Hopper, J. L., Morris, H. A., Tilley, W. D. and Giles, G. G. (2007) Circulating insulin-like growth factor-I and binding protein-3 and the risk of breast cancer. *Cancer Epidemiol. Biomarkers Prev.*, **16**(4), 763-768.

Bahceci, M., Gokalp, D., Bahceci, S., Tuzcu, A., Atmaca, S. and Arikan, S. (2007) The correlation between adiposity and adiponectin, tumor necrosis factor alpha, interleukin-6 and high sensitivity C-reactive protein levels. is adipocyte size associated with inflammation in adults? *J. Endocrinol. Invest.*, **30**(3), 210-214.

Balkau, B., Shipley, M., Jarrett, R. J., Pyorala, K., Pyorala, M., Forhan, A. and Eschwege, E. (1998) High blood glucose concentration is a risk factor for mortality in middle-aged nondiabetic men. 20-year follow-up in the whitehall study, the paris prospective study, and the helsinki policemen study. *Diabetes Care*, **21**(3), 360-367.

Balkau, B., Deanfield, J. E., Despres, J. P., Bassand, J. P., Fox, K. A., Smith, S. C.,Jr, Barter, P., Tan, C. E., Van Gaal, L., Wittchen, H. U., Massien, C. and Haffner, S. M. (2007) International day for the evaluation of abdominal obesity (IDEA): A study of waist circumference, cardiovascular disease, and diabetes mellitus in 168,000 primary care patients in 63 countries. *Circulation*, **116**(17), 1942-1951.

Balkwill, F.R., Lee, A., Aldam, G., Moodie, E., Thomas, J. A., Tavernier, J. and Fiers, W. (1986) Human tumor xenografts treated with recombinant human tumor necrosis factor alone or in combination with interferons. *Cancer Res.*, **46**(8), 3990-3993.

Banegas, J.R., Lopez-Garcia, E., Gutierrez-Fisac, J. L., Guallar-Castillon, P. and Rodriguez-Artalejo, F. (2003) A simple estimate of mortality attributable to excess weight in the european union. *Eur. J. Clin. Nutr.,* **57**(2), 201-208.

Bardon, S. and Razanamahefa, L. (1998) Retinoic acid suppresses insulin-induced cell growth and cyclin D1 gene expression in human breast cancer cells. *Int. J. Oncol.,* **12**(2), 355-359.

Barsh, G.S., Farooqi, I. S. and O'Rahilly, S. (2000) Genetics of body-weight regulation. *Nature,* **404**(6778), 644-651.

Bartella, V., Cascio, S., Fiorio, E., Auriemma, A., Russo, A. and Surmacz, E. (2008) Insulin-dependent leptin expression in breast cancer cells. *Cancer Res.,* **68**(12), 4919-4927.

Bartucci, M., Morelli, C., Mauro, L., Ando, S. and Surmacz, E. (2001) Differential insulin-like growth factor I receptor signaling and function in estrogen receptor (ER)-positive MCF-7 and ER-negative MDA-MB-231 breast cancer cells. *Cancer Res.,* **61**(18), 6747-6754.

Basolo, F., Calvo, S., Fiore, L., Conaldi, P. G., Falcone, V. and Toniolo, A. (1993) Growth-stimulating activity of interleukin 6 on human mammary epithelial cells transfected with the int-2 gene. *Cancer Res.,* **53**(13), 2957-2960.

Basolo, F., Fiore, L., Fontanini, G., Conaldi, P. G., Calvo, S., Falcone, V. and Toniolo, A. (1996) Expression of and response to interleukin 6 (IL6) in human mammary tumors. *Cancer Res.,* **56**(13), 3118-3122.

Bastard, J.P., Pieroni, L. and Hainque, B. (2000) Relationship between plasma plasminogen activator inhibitor 1 and insulin resistance. *Diabetes Metab. Res. Rev.,* **16**(3), 192-201.

Bastard, J.P., Maachi, M., Lagathu, C., Kim, M. J., Caron, M., Vidal, H., Capeau, J. and Feve, B. (2006) Recent advances in the relationship between obesity, inflammation, and insulin resistance. *Eur. Cytokine Netw.,* **17**(1), 4-12.

Bastard, J.P., Maachi, M., Van Nhieu, J. T., Jardel, C., Bruckert, E., Grimaldi, A., Robert, J. J., Capeau, J. and Hainque, B. (2002) Adipose tissue IL-6 content correlates with resistance to insulin activation of glucose uptake both in vivo and in vitro. *J. Clin. Endocrinol. Metab.,* **87**(5), 2084-2089.

Batterham, R.L., Cohen, M. A., Ellis, S. M., Le Roux, C. W., Withers, D. J., Frost, G. S., Ghatei, M. A. and Bloom, S. R. (2003) Inhibition of food intake in obese subjects by peptide YY3-36. *N. Engl. J. Med.,* **349**(10), 941-948.

Batterham, R.L., Cowley, M. A., Small, C. J., Herzog, H., Cohen, M. A., Dakin, C. L., Wren, A. M., Brynes, A. E., Low, M. J., Ghatei, M. A., Cone, R. D. and

Bloom, S. R. (2002) Gut hormone PYY(3-36) physiologically inhibits food intake. *Nature,* **418**(6898), 650-654.

Baumann, C.A., Ribon, V., Kanzaki, M., Thurmond, D. C., Mora, S., Shigematsu, S., Bickel, P. E., Pessin, J. E. and Saltiel, A. R. (2000) CAP defines a second signalling pathway required for insulin-stimulated glucose transport. *Nature,* **407**(6801), 202-207.

Becker, S., Dossus, L. and Kaaks, R. (2009) Obesity related hyperinsulinaemia and hyperglycaemia and cancer development. *Arch. Physiol. Biochem.,* **115**(2), 86-96.

Belfiore, A. and Frasca, F. (2008) IGF and insulin receptor signaling in breast cancer. *J. Mammary Gland Biol. Neoplasia,* **13**(4), 381-406.

Belfiore, A., Frasca, F., Pandini, G., Sciacca, L. and Vigneri, R. (2009) Insulin receptor isoforms and insulin receptor/insulin-like growth factor receptor hybrids in physiology and disease. *Endocr. Rev.,* **30**(6), 586-623.

Belfiore, A., Frittitta, L., Costantino, A., Frasca, F., Pandini, G., Sciacca, L., Goldfine, I. D. and Vigneri, R. (1996a) Insulin receptors in breast cancer. *Ann. N. Y. Acad. Sci.,* **784**, 173-188.

Belfiore, A., Costantino, A., Frasca, F., Pandini, G., Mineo, R., Vigneri, P., Maddux, B., Goldfine, I. D. and Vigneri, R. (1996b) Overexpression of membrane glycoprotein PC-1 in MDA-MB231 breast cancer cells is associated with inhibition of insulin receptor tyrosine kinase activity. *Mol. Endocrinol.,* **10**(11), 1318-1326.

Bellacosa, A., de Feo, D., Godwin, A. K., Bell, D. W., Cheng, J. Q., Altomare, D. A., Wan, M., Dubeau, L., Scambia, G., Masciullo, V., Ferrandina, G., Benedetti Panici, P., Mancuso, S., Neri, G. and Testa, J. R. (1995) Molecular alterations of the AKT2 oncogene in ovarian and breast carcinomas. *Int. J. Cancer,* **64**(4), 280-285.

Belobrajdic, D., Frystyk, J., Jeyaratnaganthan, N., Espelund, U., Flyvbjerg, A., Clifton, P. and Noakes, M. (2010) Moderate energy restriction induced weight loss affects circulating IGF levels independent of dietary composition. *Eur. J. Endocrinol.,* .

Beltowski, J. (2006) Apelin and visfatin: Unique "beneficial" adipokines upregulated in obesity? *Med. Sci. Monit.,* **12**(6), RA112-9.

Benoy, I., Salgado, R., Colpaert, C., Weytjens, R., Vermeulen, P. B. and Dirix, L. Y. (2002) Serum interleukin 6, plasma VEGF, serum VEGF, and VEGF platelet load in breast cancer patients. *Clin. Breast Cancer.,* **2**(4), 311-315.

Berberoglu, U., Yildirim, E. and Celen, O. (2004) Serum levels of tumor necrosis factor alpha correlate with response to neoadjuvant chemotherapy in locally advanced breast cancer. *Int. J. Biol. Markers,* **19**(2), 130-134.

Berclaz, G., Li, S., Price, K. N., Coates, A. S., Castiglione-Gertsch, M., Rudenstam, C. M., Holmberg, S. B., Lindtner, J., Erien, D., Collins, J., Snyder, R., Thurlimann, B., Fey, M. F., Mendiola, C., Werner, I. D., Simoncini, E., Crivellari, D., Gelber, R. D., Goldhirsch, A. and International Breast Cancer Study Group (2004) Body mass index as a prognostic feature in operable breast cancer: The international breast cancer study group experience. *Ann. Oncol.,* **15**(6), 875-884.

Berrington de Gonzalez, A., Sweetland, S. and Spencer, E. (2003) A meta-analysis of obesity and the risk of pancreatic cancer. *Br. J. Cancer,* **89**(3), 519-523.

Berrino, F., Muti, P., Micheli, A., Bolelli, G., Krogh, V., Sciajno, R., Pisani, P., Panico, S. and Secreto, G. (1996) Serum sex hormone levels after menopause and subsequent breast cancer. *J. Natl. Cancer Inst.,* **88**(5), 291-296.

Bertacca, A., Ciccarone, A., Cecchetti, P., Vianello, B., Laurenza, I., Maffei, M., Chiellini, C., Del Prato, S. and Benzi, L. (2005) Continually high insulin levels impair akt phosphorylation and glucose transport in human myoblasts. *Metabolism,* **54**(12), 1687-1693.

Bhat-Nakshatri, P., Campbell, R. A., Patel, N. M., Newton, T. R., King, A. J., Marshall, M. S., Ali, S. and Nakshatri, H. (2004) Tumour necrosis factor and PI3-kinase control oestrogen receptor alpha protein level and its transrepression function. *Br. J. Cancer,* **90**(4), 853-859.

Binai, N.A., Damert, A., Carra, G., Steckelbroeck, S., Lower, J., Lower, R. and Wessler, S. (2009) Expression of estrogen receptor alpha increases leptin-induced STAT3 activity in breast cancer cells. *Int. J. Cancer,* .

Binkley, J.K., Eales, J. and Jekanowski, M. (2000) The relation between dietary change and rising US obesity. *Int. J. Obes. Relat. Metab. Disord.,* **24**(8), 1032-1039.

Bjørbæk, C., El-Haschimi, K., Frantz, J. D. and Flier, J. S. (1999) The role of SOCS-3 in leptin signaling and leptin resistance. *J. Biol. Chem.,* **274**(42), 30059-30065.

Bjørbæk, C., Elmquist, J. K., Frantz, J. D., Shoelson, S. E. and Flier, J. S. (1998) Identification of SOCS-3 as a potential mediator of central leptin resistance. *Mol. Cell,* **1**(4), 619-625.

Black, R.A., Rauch, C. T., Kozlosky, C. J., Peschon, J. J., Slack, J. L., Wolfson, M. F., Castner, B. J., Stocking, K. L., Reddy, P., Srinivasan, S., Nelson, N., Boiani, N., Schooley, K. A., Gerhart, M., Davis, R., Fitzner, J. N., Johnson, R. S.,

Paxton, R. J., March, C. J. and Cerretti, D. P. (1997) A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells. *Nature,* **385**(6618), 729-733.

Blackwood, M.A. and Weber, B. L. (1998) BRCA1 and BRCA2: From molecular genetics to clinical medicine. *J. Clin. Oncol.,* **16**(5), 1969-1977.

Blake, A.D., Hayes, N. S., Slater, E. E. and Strader, C. D. (1987) Insulin receptor desensitization correlates with attenuation of tyrosine kinase activity, but not of receptor endocytosis. *Biochem. J.,* **245**(2), 357-364.

Blundell, T.L. and Humbel, R. E. (1980) Hormone families: Pancreatic hormones and homologous growth factors. *Nature,* **287**(5785), 781-787.

Bodmer, M., Meier, C., Krahenbuhl, S., Jick, S. S., Meier, C. R. and Meier, C. R. (2010) Long-term metformin use is associated with decreased risk of breast cancer. *Diabetes Care,* .

Bogin, L., Papa, M. Z., Polak-Charcon, S. and Degani, H. (1998) TNF-induced modulations of phospholipid metabolism in human breast cancer cells. *Biochim. Biophys. Acta,* **1392**(2-3), 217-232.

Boni-Schnetzler, M., Schmid, C., Meier, P. J. and Froesch, E. R. (1991) Insulin regulates insulin-like growth factor I mRNA in rat hepatocytes. *Am. J. Physiol.,* **260**(6 Pt 1), E846-51.

Bornstein, S.R., Abu-Asab, M., Glasow, A., Path, G., Hauner, H., Tsokos, M., Chrousos, G. P. and Scherbaum, W. A. (2000) Immunohistochemical and ultrastructural localization of leptin and leptin receptor in human white adipose tissue and differentiating human adipose cells in primary culture. *Diabetes,* **49**(4), 532-538.

Bourn, J. (2001) Tackling obesity in England. **Report HC 220 of the National Audit Office**.

Boyd, D.B. (2003) Insulin and cancer. *Integr. Cancer. Ther.,* **2**(4), 315-329.

Boyd, N.F., Rommens, J. M., Vogt, K., Lee, V., Hopper, J. L., Yaffe, M. J. and Paterson, A. D. (2005) Mammographic breast density as an intermediate phenotype for breast cancer. *Lancet Oncol.,* **6**(10), 798-808.

Boyd, N.F., Martin, L. J., Rommens, J. M., Paterson, A. D., Minkin, S., Yaffe, M. J., Stone, J. and Hopper, J. L. (2009) Mammographic density: A heritable risk factor for breast cancer. *Methods Mol. Biol.,* **472**, 343-360.

Bozcuk, H., Uslu, G., Samur, M., Yildiz, M., Ozben, T., Ozdogan, M., Artac, M., Altunbas, H., Akan, I. and Savas, B. (2004) Tumour necrosis factor-alpha, interleukin-6, and fasting serum insulin correlate with clinical outcome in

metastatic breast cancer patients treated with chemotherapy. *Cytokine,* **27**(2-3), 58-65.

Brader, S. and Eccles, S. A. (2004) Phosphoinositide 3-kinase signalling pathways in tumor progression, invasion and angiogenesis. *Tumori,* **90**(1), 2-8.

Brahimi-Horn, M.C. and Pouyssegur, J. (2007) Oxygen, a source of life and stress. *FEBS Lett.,* **581**(19), 3582-3591.

Brakenhielm, E., Veitonmaki, N., Cao, R., Kihara, S., Matsuzawa, Y., Zhivotovsky, B., Funahashi, T. and Cao, Y. (2004) Adiponectin-induced antiangiogenesis and antitumor activity involve caspase-mediated endothelial cell apoptosis. *Proc. Natl. Acad. Sci. U. S. A.,* **101**(8), 2476-2481.

Brodie, A., Lu, Q. and Nakamura, J. (1997) Aromatase in the normal breast and breast cancer. *J. Steroid Biochem. Mol. Biol.,* **61**(3-6), 281-286.

Brodin, A. (2007) Theoretical models of adaptive energy management in small wintering birds. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.,* **362**(1486), 1857-1871.

Brown, R.A., Domin, J., Arcaro, A., Waterfield, M. D. and Shepherd, P. R. (1999) Insulin activates the alpha isoform of class II phosphoinositide 3-kinase. *J. Biol. Chem.,* **274**(21), 14529-14532.

Bulun, S.E., Mahendroo, M. S. and Simpson, E. R. (1994) Aromatase gene expression in adipose tissue: Relationship to breast cancer. *J. Steroid Biochem. Mol. Biol.,* **49**(4-6), 319-326.

Burow, M.E., Weldon, C. B., Melnik, L. I., Duong, B. N., Collins-Burow, B. M., Beckman, B. S. and McLachlan, J. A. (2000) PI3-K/AKT regulation of NF-kappaB signaling events in suppression of TNF-induced apoptosis. *Biochem. Biophys. Res. Commun.,* **271**(2), 342-345.

Burow, M.E., Weldon, C. B., Tang, Y., Navar, G. L., Krajewski, S., Reed, J. C., Hammond, T. G., Clejan, S. and Beckman, B. S. (1998) Differences in susceptibility to tumor necrosis factor alpha-induced apoptosis among MCF-7 breast cancer cell variants. *Cancer Res.,* **58**(21), 4940-4946.

Butland, B., Jebb, S., Kopleman, P., McPherson, K., Thomas., S., Mardell., J. and Parry, V. (2007) Foresight Tackling Obesities: Future Choices – Project report. Available on http://www.foresight.gov.uk/Obesity/17.pdf. Accessed on 12th August 2010.

Cai, Z., Capoulade, C., Moyret-Lalle, C., Amor-Gueret, M., Feunteun, J., Larsen, A. K., Paillerets, B. B. and Chouaib, S. (1997) Resistance of MCF7 human breast carcinoma cells to TNF-induced cell death is associated with loss of p53 function. *Oncogene,* **15**(23), 2817-2826.

Cailleau, R., Olive, M. and Cruciger, Q. V. (1978) Long-term human breast carcinoma cell lines of metastatic origin: Preliminary characterization. *In. Vitro.,* **14**(11), 911-915.

Calle, E.E., Rodriguez, C., Walker-Thurmond, K. and Thun, M. J. (2003) Overweight, obesity, and mortality from cancer in a prospectively studied cohort of U.S. adults. *N. Engl. J. Med.,* **348**(17), 1625-1638.

Calvin, M. (1975) Carcinogenesis. *Naturwissenschaften,* **62**(9), 405-413.

Cameron, A.J., Zimmet, P. Z., Shaw, J. E. and Alberti, K. G. (2009) The metabolic syndrome: In need of a global mission statement. *Diabet. Med.,* **26**(3), 306-309.

Camirand, A., Lu, Y. and Pollak, M. (2002) Co-targeting HER2/ErbB2 and insulin-like growth factor-1 receptors causes synergistic inhibition of growth in HER2-overexpressing breast cancer cells. *Med. Sci. Monit.,* **8**(12), BR521-6.

Cancer Research UK (2006) The ten most common cancers, females, UK, 2006, http://info.cancerresearchuk.org/prod_consump/groups/cr_common/@nre/@sta/documents/generalcontent/crukmig_1000ast-2740.xls, accessed on 3rd December 2009.

Cancer Research UK (2007) The ten most common causes of cancer deaths, female, UK, 2007, http://info.cancerresearchuk.org/prod_consump/groups/cr_common/@nre/@sta/documents/generalcontent/crukmig_1000ast-2754.xls, accessed on 3rd December 2009.

Cancer Research UK (2009a) Types of breast cancer, http://www.cancerhelp.org.uk/type/breast-cancer/about/types/index.htm, accessed on 4th December 2009.

Cancer Research UK (2009b) Definite breast cancer risks, http://www.cancerhelp.org.uk/type/breast-cancer/about/risks/definite-breast-cancer-risks, accessed on 4th December 2009.

Carboni, J.M., Lee, A. V., Hadsell, D. L., Rowley, B. R., Lee, F. Y., Bol, D. K., Camuso, A. E., Gottardis, M., Greer, A. F., Ho, C. P., Hurlburt, W., Li, A., Saulnier, M., Velaparthi, U., Wang, C., Wen, M. L., Westhouse, R. A., Wittman, M., Zimmermann, K., Rupnow, B. A. and Wong, T. W. (2005) Tumor development by transgenic expression of a constitutively active insulin-like growth factor I receptor. *Cancer Res.,* **65**(9), 3781-3787.

Cardone, M.H., Roy, N., Stennicke, H. R., Salvesen, G. S., Franke, T. F., Stanbridge, E., Frisch, S. and Reed, J. C. (1998) Regulation of cell death protease caspase-9 by phosphorylation. *Science,* **282**(5392), 1318-1321.

Carmichael, A.R. (2006) Obesity and prognosis of breast cancer. *Obes. Rev.,* **7**(4), 333-340.

Carswell, E.A., Old, L. J., Kassel, R. L., Green, S., Fiore, N. and Williamson, B. (1975) An endotoxin-induced serum factor that causes necrosis of tumors. *Proc. Natl. Acad. Sci. U. S. A.,* **72**(9), 3666-3670.

Casabiell, X., Pineiro, V., Tome, M. A., Peino, R., Dieguez, C. and Casanueva, F. F. (1997) Presence of leptin in colostrum and/or breast milk from lactating mothers: A potential role in the regulation of neonatal food intake. *J. Clin. Endocrinol. Metab.,* **82**(12), 4270-4273.

Cascio, S., Bartella, V., Auriemma, A., Johannes, G. J., Russo, A., Giordano, A. and Surmacz, E. (2008) Mechanism of leptin expression in breast cancer cells: Role of hypoxia-inducible factor-1alpha. *Oncogene,* **27**(4), 540-547.

Castelli, W.P. (1996) Lipids, risk factors and ischaemic heart disease. *Atherosclerosis,* **124 Suppl**, S1-9.

Castrellon, A.B. and Gluck, S. (2008) Chemoprevention of breast cancer. *Expert Rev. Anticancer Ther.,* **8**(3), 443-452.

Catalano, S., Marsico, S., Giordano, C., Mauro, L., Rizza, P., Panno, M. L. and Ando, S. (2003) Leptin enhances, via AP-1, expression of aromatase in the MCF-7 cell line. *J. Biol. Chem.,* **278**(31), 28668-28676.

Catalano, S., Mauro, L., Marsico, S., Giordano, C., Rizza, P., Rago, V., Montanaro, D., Maggiolini, M., Panno, M. L. and Ando, S. (2004) Leptin induces, via ERK1/ERK2 signal, functional activation of estrogen receptor alpha in MCF-7 cells. *J. Biol. Chem.,* **279**(19), 19908-19915.

Caterson, I.D. and Gill, T. P. (2002) Obesity: Epidemiology and possible prevention. *Best Pract. Res. Clin. Endocrinol. Metab.,* **16**(4), 595-610.

Cavanagh, D., Marsden, D. E. and Ruffolo, E. H. (1984) Carcinoma of the endometrium. *Obstet. Gynecol. Annu.,* **13**, 211-260.

Chakravarthy, M.V. and Booth, F. W. (2004) Eating, exercise, and "thrifty" genotypes: Connecting the dots toward an evolutionary understanding of modern chronic diseases. *J. Appl. Physiol.,* **96**(1), 3-10.

Chang, P.Y., Le Marchand-Brustel, Y., Cheatham, L. A. and Moller, D. E. (1995) Insulin stimulation of mitogen-activated protein kinase, p90rsk, and p70 S6 kinase in skeletal muscle of normal and insulin-resistant mice. implications for the regulation of glycogen synthase. *J. Biol. Chem.,* **270**(50), 29928-29935.

Chen, C., Chang, Y. C., Liu, C. L., Chang, K. J. and Guo, I. C. (2006a) Leptin-induced growth of human ZR-75-1 breast cancer cells is associated with up-regulation of cyclin D1 and c-myc and down-regulation of tumor suppressor p53 and p21WAF1/CIP1. *Breast Cancer Res. Treat.,* **98**(2), 121-132.

Chen, H.J., Bai, C. H., Yeh, W. T., Chiu, H. C. and Pan, W. H. (2006b) Influence of metabolic syndrome and general obesity on the risk of ischemic stroke. *Stroke,* **37**(4), 1060-1064.

Chen, S., Itoh, T., Wu, K., Zhou, D. and Yang, C. (2002) Transcriptional regulation of aromatase expression in human breast tissue. *J. Steroid Biochem. Mol. Biol.,* **83**(1-5), 93-99.

Cheng, A.Y. and Leiter, L. A. (2009) Diabetes and cardiovascular disease: The role of glycemic control. *Curr. Diab Rep.,* **9**(1), 65-72.

Cheng, J.Q., Ruggeri, B., Klein, W. M., Sonoda, G., Altomare, D. A., Watson, D. K. and Testa, J. R. (1996) Amplification of AKT2 in human pancreatic cells and inhibition of AKT2 expression and tumorigenicity by antisense RNA. *Proc. Natl. Acad. Sci. U. S. A.,* **93**(8), 3636-3641.

Cherel, M., Campion, L., Bezieau, S., Campone, M., Charrier, J., Gaschet, J., Ricolleau, G., Gouraud, W., Charbonnel, C. and Jezequel, P. (2009) Molecular screening of interleukin-6 gene promoter and influence of -174G/C polymorphism on breast cancer. *Cytokine,* **47**(3), 214-223.

Chiang, S.H., Hwang, J., Legendre, M., Zhang, M., Kimura, A. and Saltiel, A. R. (2003) TCGAP, a multidomain rho GTPase-activating protein involved in insulin-stimulated glucose transport. *EMBO J.,* **22**(11), 2679-2691.

Chiang, S.H., Baumann, C. A., Kanzaki, M., Thurmond, D. C., Watson, R. T., Neudauer, C. L., Macara, I. G., Pessin, J. E. and Saltiel, A. R. (2001) Insulin-stimulated GLUT4 translocation requires the CAP-dependent activation of TC10. *Nature,* **410**(6831), 944-948.

Chisholm, D.J., Samaras, K., Markovic, T., Carey, D., Lapsys, N. and Campbell, L. V. (1998) Obesity: Genes, glands or gluttony? *Reprod. Fertil. Dev.,* **10**(1), 49-53.

Chiu, J.J., Sgagias, M. K. and Cowan, K. H. (1996) Interleukin 6 acts as a paracrine growth factor in human mammary carcinoma cell lines. *Clin. Cancer Res.,* **2**(1), 215-221.

Cho Mar, K., Eimoto, T., Nagaya, S. and Tateyama, H. (2006) Cell proliferation marker MCM2, but not Ki67, is helpful for distinguishing between minimally invasive follicular carcinoma and follicular adenoma of the thyroid. *Histopathology,* **48**(7), 801-807.

Cho, H., Aronica, S. M. and Katzenellenbogen, B. S. (1994) Regulation of progesterone receptor gene expression in MCF-7 breast cancer cells: A comparison of the effects of cyclic adenosine 3',5'-monophosphate, estradiol, insulin-like growth factor-I, and serum factors. *Endocrinology,* **134**(2), 658-664.

Cianfrocca, M. and Gradishar, W. (2009) New molecular classifications of breast cancer. *CA Cancer. J. Clin.,* **59**(5), 303-313.

Cinti, S. (2005) The adipose organ. *Prostaglandins Leukot. Essent. Fatty Acids,* **73**(1), 9-15.

Cirillo, D., Rachiglio, A. M., la Montagna, R., Giordano, A. and Normanno, N. (2008) Leptin signaling in breast cancer: An overview. *J. Cell. Biochem.,* **105**(4), 956-964.

Cleary, M.P., Juneja, S. C., Phillips, F. C., Hu, X., Grande, J. P. and Maihle, N. J. (2004) Leptin receptor-deficient MMTV-TGF-alpha/Lepr(db)lepr(db) female mice do not develop oncogene-induced mammary tumors. *Exp. Biol. Med. (Maywood),* **229**(2), 182-193.

Cleary, M.P., Phillips, F. C., Getzin, S. C., Jacobson, T. L., Jacobson, M. K., Christensen, T. A., Juneja, S. C., Grande, J. P. and Maihle, N. J. (2003) Genetically obese MMTV-TGF-alpha/Lep(ob)lep(ob) female mice do not develop mammary tumors. *Breast Cancer Res. Treat.,* **77**(3), 205-215.

Cleland, W.H., Mendelson, C. R. and Simpson, E. R. (1985) Effects of aging and obesity on aromatase activity of human adipose cells. *J. Clin. Endocrinol. Metab.,* **60**(1), 174-177.

Clement, K., Vaisse, C., Lahlou, N., Cabrol, S., Pelloux, V., Cassuto, D., Gourmelen, M., Dina, C., Chambaz, J., Lacorte, J. M., Basdevant, A., Bougneres, P., Lebouc, Y., Froguel, P. and Guy-Grand, B. (1998) A mutation in the human leptin receptor gene causes obesity and pituitary dysfunction. *Nature,* **392**(6674), 398-401.

Coleman, D.L. (1978) Obese and diabetes: Two mutant genes causing diabetes-obesity syndromes in mice. *Diabetologia,* **14**(3), 141-148.

Cong, L., Gasser, J., Zhao, J., Yang, B., Li, F. and Zhao, A. Z. (2007) Human adiponectin inhibits cell growth and induces apoptosis in human endometrial carcinoma cells, HEC-1-A and RL95 2. *Endocr. Relat. Cancer,* **14**(3), 713-720.

Considine, R.V., Sinha, M. K., Heiman, M. L., Kriauciunas, A., Stephens, T. W., Nyce, M. R., Ohannesian, J. P., Marco, C. C., McKee, L. J., Bauer, T. L. and Caro, J. F. (1996) Serum immunoreactive-leptin concentrations in normal-weight and obese humans. *N. Engl. J. Med.,* **334**(5), 292-295.

Conze, D., Weiss, L., Regen, P. S., Bhushan, A., Weaver, D., Johnson, P. and Rincon, M. (2001) Autocrine production of interleukin 6 causes multidrug resistance in breast cancer cells. *Cancer Res.,* **61**(24), 8851-8858.

Cook, M.B., Greenwood, D. C., Hardie, L. J., Wild, C. P. and Forman, D. (2008) A systematic review and meta-analysis of the risk of increasing adiposity on barrett's esophagus. *Am. J. Gastroenterol.*, **103**(2), 292-300.

Costantino, A., Milazzo, G., Giorgino, F., Russo, P., Goldfine, I. D., Vigneri, R. and Belfiore, A. (1993) Insulin-resistant MDA-MB231 human breast cancer cells contain a tyrosine kinase inhibiting activity. *Mol. Endocrinol.*, **7**(12), 1667-1676.

Cross, D.A., Alessi, D. R., Cohen, P., Andjelkovich, M. and Hemmings, B. A. (1995) Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature*, **378**(6559), 785-789.

Curb, J.D. and Marcus, E. B. (1991) Body fat and obesity in japanese americans. *Am. J. Clin. Nutr.*, **53**(6 Suppl), 1552S-1555S.

Cusi, K., Maezono, K., Osman, A., Pendergrass, M., Patti, M. E., Pratipanawatr, T., DeFronzo, R. A., Kahn, C. R. and Mandarino, L. J. (2000) Insulin resistance differentially affects the PI 3-kinase- and MAP kinase-mediated signaling in human muscle. *J. Clin. Invest.*, **105**(3), 311-320.

Cust, A.E., Stocks, T., Lukanova, A., Lundin, E., Hallmans, G., Kaaks, R., Jonsson, H. and Stattin, P. (2009) The influence of overweight and insulin resistance on breast cancer risk and tumour stage at diagnosis: A prospective study. *Breast Cancer Res. Treat.*, **113**(3), 567-576.

Cymbaluk, A., Chudecka-Glaz, A. and Rzepka-Gorska, I. (2008) Leptin levels in serum depending on body mass index in patients with endometrial hyperplasia and cancer. *Eur. J. Obstet. Gynecol. Reprod. Biol.*, **136**(1), 74-77.

Dahlman-Wright, K., Cavailles, V., Fuqua, S. A., Jordan, V. C., Katzenellenbogen, J. A., Korach, K. S., Maggi, A., Muramatsu, M., Parker, M. G. and Gustafsson, J. A. (2006) International union of pharmacology. LXIV. estrogen receptors. *Pharmacol. Rev.*, **58**(4), 773-781.

Dai, Z., Xu, Y. C. and Niu, L. (2007) Obesity and colorectal cancer risk: A meta-analysis of cohort studies. *World J. Gastroenterol.*, **13**(31), 4199-4206.

Daling, J.R., Malone, K. E., Doody, D. R., Johnson, L. G., Gralow, J. R. and Porter, P. L. (2001) Relation of body mass index to tumor markers and survival among young women with invasive ductal breast carcinoma. *Cancer*, **92**(4), 720-729.

Daughaday, W.H. and Rotwein, P. (1989) Insulin-like growth factors I and II. peptide, messenger ribonucleic acid and gene structures, serum, and tissue concentrations. *Endocr. Rev.*, **10**(1), 68-91.

Davies, H., Bignell, G. R., Cox, C., Stephens, P., Edkins, S., Clegg, S., Teague, J., Woffendin, H., Garnett, M. J., Bottomley, W., Davis, N., Dicks, E., Ewing, R.,

Floyd, Y., Gray, K., Hall, S., Hawes, R., Hughes, J., Kosmidou, V., Menzies, A., Mould, C., Parker, A., Stevens, C., Watt, S., Hooper, S., Wilson, R., Jayatilake, H., Gusterson, B. A., Cooper, C., Shipley, J., Hargrave, D., Pritchard-Jones, K., Maitland, N., Chenevix-Trench, G., Riggins, G. J., Bigner, D. D., Palmieri, G., Cossu, A., Flanagan, A., Nicholson, A., Ho, J. W., Leung, S. Y., Yuen, S. T., Weber, B. L., Seigler, H. F., Darrow, T. L., Paterson, H., Marais, R., Marshall, C. J., Wooster, R., Stratton, M. R. and Futreal, P. A. (2002) Mutations of the BRAF gene in human cancer. *Nature,* **417**(6892), 949-954.

de la Maza, M.P., Estevez, A., Bunout, D., Klenner, C., Oyonarte, M. and Hirsch, S. (1994) Ventricular mass in hypertensive and normotensive obese subjects. *Int. J. Obes. Relat. Metab. Disord.,* **18**(4), 193-197.

de Souza Batista, C.M., Yang, R. Z., Lee, M. J., Glynn, N. M., Yu, D. Z., Pray, J., Ndubuizu, K., Patil, S., Schwartz, A., Kligman, M., Fried, S. K., Gong, D. W., Shuldiner, A. R., Pollin, T. I. and McLenithan, J. C. (2007) Omentin plasma levels and gene expression are decreased in obesity. *Diabetes,* **56**(6), 1655-1661.

Deurenberg, P., Weststrate, J. A. and Seidell, J. C. (1991) Body mass index as a measure of body fatness: Age- and sex-specific prediction formulas. *Br. J. Nutr.,* **65**(2), 105-114.**de Waard, F.** (1979) Premenopausal and postmenopausal breast cancer: One disease or two? *J. Natl. Cancer Inst.,* **63**(3), 549-552.

Debnath, J., Muthuswamy, S. K. and Brugge, J. S. (2003) Morphogenesis and oncogenesis of MCF-10A mammary epithelial acini grown in three-dimensional basement membrane cultures. *Methods,* **30**(3), 256-268.

DeFronzo, R.A. (1997) Insulin resistance: A multifaceted syndrome responsible for NIDDM, obesity, hypertension, dyslipidaemia and atherosclerosis. *Neth. J. Med.,* **50**(5), 191-197.

Dejean, L.M., Martinez-Caballero, S., Manon, S. and Kinnally, K. W. (2006) Regulation of the mitochondrial apoptosis-induced channel, MAC, by BCL-2 family proteins. *Biochim. Biophys. Acta,* **1762**(2), 191-201.

Del Giudice, M.E., Fantus, I. G., Ezzat, S., McKeown-Eyssen, G., Page, D. and Goodwin, P. J. (1998) Insulin and related factors in premenopausal breast cancer risk. *Breast Cancer Res. Treat.,* **47**(2), 111-120.

Dent, P., Reardon, D. B., Park, J. S., Bowers, G., Logsdon, C., Valerie, K. and Schmidt-Ullrich, R. (1999) Radiation-induced release of transforming growth factor alpha activates the epidermal growth factor receptor and mitogen-activated protein kinase pathway in carcinoma cells, leading to increased proliferation and protection from radiation-induced cell death. *Mol. Biol. Cell,* **10**(8), 2493-2506.

Deutsch, C., Portik-Dobos, V., Smith, A. D., Ergul, A. and Dorrance, A. M. (2009) Diet-induced obesity causes cerebral vessel remodeling and increases the damage caused by ischemic stroke. *Microvasc. Res.,* **78**(1), 100-106.

Dick, G.M. and Sturek, M. (1996) Effects of a physiological insulin concentration on the endothelin-sensitive Ca2+ store in porcine coronary artery smooth muscle. *Diabetes,* **45**(7), 876-880.

Dickson, R.B. and Stancel, G. M. (2000) Estrogen receptor-mediated processes in normal and cancer cells. *J. Natl. Cancer. Inst. Monogr.,* **(27)**(27), 135-145.

Dickson, R.B. and Lippman, M. E. (1986) Hormonal control of human breast cancer cell lines. *Cancer Surv.,* **5**(3), 617-624.

Dieudonne, M.N., Bussiere, M., Dos Santos, E., Leneveu, M. C., Giudicelli, Y. and Pecquery, R. (2006) Adiponectin mediates antiproliferative and apoptotic responses in human MCF7 breast cancer cells. *Biochem. Biophys. Res. Commun.,* **345**(1), 271-279.

Dieudonne, M.N., Machinal-Quelin, F., Serazin-Leroy, V., Leneveu, M. C., Pecquery, R. and Giudicelli, Y. (2002) Leptin mediates a proliferative response in human MCF7 breast cancer cells. *Biochem. Biophys. Res. Commun.,* **293**(1), 622-628.

Diorio, C., Pollak, M., Byrne, C., Masse, B., Hebert-Croteau, N., Yaffe, M., Cote, G., Berube, S., Morin, C. and Brisson, J. (2005) Insulin-like growth factor-I, IGF-binding protein-3, and mammographic breast density. *Cancer Epidemiol. Biomarkers Prev.,* **14**(5), 1065-1073.

Doecke, J.D., Zhao, Z. Z., Stark, M. S., Green, A. C., Hayward, N. K., Montgomery, G. W., Webb, P. M., Whiteman, D. C. and Australian Cancer Study (2008) Single nucleotide polymorphisms in obesity-related genes and the risk of esophageal cancers. *Cancer Epidemiol. Biomarkers Prev.,* **17**(4), 1007-1012.

Dos Santos, E., Benaitreau, D., Dieudonne, M. N., Leneveu, M. C., Serazin, V., Giudicelli, Y. and Pecquery, R. (2008) Adiponectin mediates an antiproliferative response in human MDA-MB 231 breast cancer cells. *Oncol. Rep.,* **20**(4), 971-977.

Dossus, L., McKay, J. D., Canzian, F., Wilkening, S., Rinaldi, S., Biessy, C., Olsen, A., Tjonneland, A., Jakobsen, M. U., Overvad, K., Clavel-Chapelon, F., Boutron-Ruault, M. C., Fournier, A., Linseisen, J., Lukanova, A., Boeing, H., Fisher, E., Trichopoulou, A., Georgila, C., Trichopoulos, D., Palli, D., Krogh, V., Tumino, R., Vineis, P., Quiros, J. R., Sala, N., Martinez-Garcia, C., Dorronsoro, M., Chirlaque, M. D., Barricarte, A., van Duijnhoven, F. J., Bueno-de-Mesquita, H. B., van Gils, C. H., Peeters, P. H., Hallmans, G., Lenner, P., Bingham, S., Khaw, K. T., Key, T. J., Travis, R. C., Ferrari, P., Jenab, M., Riboli, E. and Kaaks, R. (2008) Polymorphisms of genes coding for ghrelin and its receptor in relation to anthropometry, circulating levels of IGF-I and IGFBP-3, and breast cancer risk: A case-control study nested within the european prospective investigation into cancer and nutrition (EPIC). *Carcinogenesis,* **29**(7), 1360-1366.

Dubois, S.G., Heilbronn, L. K., Smith, S. R., Albu, J. B., Kelley, D. E., Ravussin, E. and Look AHEAD Adipose Research Group (2006) Decreased expression of adipogenic genes in obese subjects with type 2 diabetes. *Obesity (Silver Spring),* **14**(9), 1543-1552.

Dubska, L., Andera, L. and Sheard, M. A. (2005) HER2 signaling downregulation by trastuzumab and suppression of the PI3K/Akt pathway: An unexpected effect on TRAIL-induced apoptosis. *FEBS Lett.*, **579**(19), 4149-4158.

Dufourny, B., Alblas, J., van Teeffelen, H. A., van Schaik, F. M., van der Burg, B., Steenbergh, P. H. and Sussenbach, J. S. (1997) Mitogenic signaling of insulin-like growth factor I in MCF-7 human breast cancer cells requires phosphatidylinositol 3-kinase and is independent of mitogen-activated protein kinase. *J. Biol. Chem.*, **272**(49), 31163-31171.

Dunn, K.L., Espino, P. S., Drobic, B., He, S. and Davie, J. R. (2005) The ras-MAPK signal transduction pathway, cancer and chromatin remodeling. *Biochem. Cell Biol.*, **83**(1), 1-14.

Dupont, J. and Le Roith, D. (2001) Insulin-like growth factor 1 and oestradiol promote cell proliferation of MCF-7 breast cancer cells: New insights into their synergistic effects. *Mol. Pathol.*, **54**(3), 149-154.

Eaton, S.B., Eaton, S. B.,3rd, Konner, M. J. and Shostak, M. (1996) An evolutionary perspective enhances understanding of human nutritional requirements. *J. Nutr.*, **126**(6), 1732-1740.

Eisen, G.M., Sandler, R. S., Murray, S. and Gottfried, M. (1997) The relationship between gastroesophageal reflux disease and its complications with barrett's esophagus. *Am. J. Gastroenterol.*, **92**(1), 27-31.

Eliassen, A.H., Tworoger, S. S., Mantzoros, C. S., Pollak, M. N. and Hankinson, S. E. (2007) Circulating insulin and c-peptide levels and risk of breast cancer among predominately premenopausal women. *Cancer Epidemiol. Biomarkers Prev.*, **16**(1), 161-164.

Ellisen, L.W. and Haber, D. A. (1998) Hereditary breast cancer. *Annu. Rev. Med.*, **49**, 425-436.

El-Serag, H. (2008) Role of obesity in GORD-related disorders. *Gut*, **57**(3), 281-284.

Esparis-Ogando, A., Ocana, A., Rodriguez-Barrueco, R., Ferreira, L., Borges, J. and Pandiella, A. (2008) Synergic antitumoral effect of an IGF-IR inhibitor and trastuzumab on HER2-overexpressing breast cancer cells. *Ann. Oncol.*, **19**(11), 1860-1869.

Esposito, K., Giugliano, F., De Sio, M., Carleo, D., Di Palo, C., D'Armiento, M. and Giugliano, D. (2006) Dietary factors in erectile dysfunction. *Int. J. Impot. Res.*, **18**(4), 370-374.

Facchini, F.S., Hua, N., Abbasi, F. and Reaven, G. M. (2001) Insulin resistance as a predictor of age-related diseases. *J. Clin. Endocrinol. Metab.*, **86**(8), 3574-3578.

Fader, A.N., Arriba, L. N., Frasure, H. E. and von Gruenigen, V. E. (2009) Endometrial cancer and obesity: Epidemiology, biomarkers, prevention and survivorship. *Gynecol. Oncol.*, **114**(1), 121-127.

Fagan, D.H. and Yee, D. (2008) Crosstalk between IGF1R and estrogen receptor signaling in breast cancer. *J. Mammary Gland Biol. Neoplasia*, **13**(4), 423-429.

Fain, J.N., Madan, A. K., Hiler, M. L., Cheema, P. and Bahouth, S. W. (2004) Comparison of the release of adipokines by adipose tissue, adipose tissue matrix, and adipocytes from visceral and subcutaneous abdominal adipose tissues of obese humans. *Endocrinology*, **145**(5), 2273-2282.

Fajardo, L.F., Kwan, H. H., Kowalski, J., Prionas, S. D. and Allison, A. C. (1992) Dual role of tumor necrosis factor-alpha in angiogenesis. *Am. J. Pathol.*, **140**(3), 539-544.

Faridi, J., Fawcett, J., Wang, L. and Roth, R. A. (2003) Akt promotes increased mammalian cell size by stimulating protein synthesis and inhibiting protein degradation. *Am. J. Physiol. Endocrinol. Metab.*, **285**(5), E964-72.

Farooqi, I.S., Yeo, G. S., Keogh, J. M., Aminian, S., Jebb, S. A., Butler, G., Cheetham, T. and O'Rahilly, S. (2000) Dominant and recessive inheritance of morbid obesity associated with melanocortin 4 receptor deficiency. *J. Clin. Invest.*, **106**(2), 271-279.

Farooqi, I.S., Jebb, S. A., Langmack, G., Lawrence, E., Cheetham, C. H., Prentice, A. M., Hughes, I. A., McCamish, M. A. and O'Rahilly, S. (1999) Effects of recombinant leptin therapy in a child with congenital leptin deficiency. *N. Engl. J. Med.*, **341**(12), 879-884.

Feigelson, H.S. and Henderson, B. E. (1996) Estrogens and breast cancer. *Carcinogenesis*, **17**(11), 2279-2284.

Ferlay, J., Parkin, D. M. and Steliarova-Foucher, E. (2010) Estimates of cancer incidence and mortality in europe in 2008. *Eur. J. Cancer*, **46**(4), 765-781.

Ferlay, J., Autier, P., Boniol, M., Heanue, M., Colombet, M. and Boyle, P. (2007) Estimates of the cancer incidence and mortality in europe in 2006. *Ann. Oncol.*, **18**(3), 581-592.

Fields, D.A., Goran, M. I. and McCrory, M. A. (2002) Body-composition assessment via air-displacement plethysmography in adults and children: A review. *Am. J. Clin. Nutr.,* **75**(3), 453-467.

Fiorio, E., Mercanti, A., Terrasi, M., Micciolo, R., Remo, A., Auriemma, A., Molino, A., Parolin, V., Di Stefano, B., Bonetti, F., Giordano, A., Cetto, G. L. and Surmacz, E. (2008) Leptin/HER2 crosstalk in breast cancer: In vitro study and preliminary in vivo analysis. *BMC Cancer,* **8**, 305.

Flegal, K.M., Carroll, M. D., Kuczmarski, R. J. and Johnson, C. L. (1998) Overweight and obesity in the united states: Prevalence and trends, 1960-1994. *Int. J. Obes. Relat. Metab. Disord.,* **22**(1), 39-47.

Fleischmann, E., Kurz, A., Niedermayr, M., Schebesta, K., Kimberger, O., Sessler, D. I., Kabon, B. and Prager, G. (2005) Tissue oxygenation in obese and non-obese patients during laparoscopy. *Obes. Surg.,* **15**(6), 813-819.

Fletcher, O., Gibson, L., Johnson, N., Altmann, D. R., Holly, J. M., Ashworth, A., Peto, J. and Silva Idos, S. (2005) Polymorphisms and circulating levels in the insulin-like growth factor system and risk of breast cancer: A systematic review. *Cancer Epidemiol. Biomarkers Prev.,* **14**(1), 2-19.

Flotho, C., Valcamonica, S., Mach-Pascual, S., Schmahl, G., Corral, L., Ritterbach, J., Hasle, H., Arico, M., Biondi, A. and Niemeyer, C. M. (1999) RAS mutations and clonality analysis in children with juvenile myelomonocytic leukemia (JMML). *Leukemia,* **13**(1), 32-37.

Forbes, G.B. (1987) Human Body Composition: growth, aging, nutrition and activity, New York: Springer Verlag.

Frankenberry, K.A., Skinner, H., Somasundar, P., McFadden, D. W. and Vona-Davis, L. C. (2006) Leptin receptor expression and cell signaling in breast cancer. *Int. J. Oncol.,* **28**(4), 985-993.

Frayling, T.M., Timpson, N. J., Weedon, M. N., Zeggini, E., Freathy, R. M., Lindgren, C. M., Perry, J. R., Elliott, K. S., Lango, H., Rayner, N. W., Shields, B., Harries, L. W., Barrett, J. C., Ellard, S., Groves, C. J., Knight, B., Patch, A. M., Ness, A. R., Ebrahim, S., Lawlor, D. A., Ring, S. M., Ben-Shlomo, Y., Jarvelin, M. R., Sovio, U., Bennett, A. J., Melzer, D., Ferrucci, L., Loos, R. J., Barroso, I., Wareham, N. J., Karpe, F., Owen, K. R., Cardon, L. R., Walker, M., Hitman, G. A., Palmer, C. N., Doney, A. S., Morris, A. D., Smith, G. D., Hattersley, A. T. and McCarthy, M. I. (2007) A common variant in the FTO gene is associated with body mass index and predisposes to childhood and adult obesity. *Science,* **316**(5826), 889-894.

Freiberg, M.S., Pencina, M. J., D'Agostino, R. B., Lanier, K., Wilson, P. W. and Vasan, R. S. (2008) BMI vs. waist circumference for identifying vascular risk. *Obesity (Silver Spring),* **16**(2), 463-469.

Fresno Vara, J.A., Casado, E., de Castro, J., Cejas, P., Belda-Iniesta, C. and Gonzalez-Baron, M. (2004) PI3K/Akt signalling pathway and cancer. *Cancer Treat. Rev.*, **30**(2), 193-204.

Fried, S.K., Bunkin, D. A. and Greenberg, A. S. (1998) Omental and subcutaneous adipose tissues of obese subjects release interleukin-6: Depot difference and regulation by glucocorticoid. *J. Clin. Endocrinol. Metab.*, **83**(3), 847-850.

Friedman, J.M. (2000) Obesity in the new millennium. *Nature*, **404**(6778), 632-634.

Friedman, J.M. and Halaas, J. L. (1998) Leptin and the regulation of body weight in mammals. *Nature*, **395**(6704), 763-770.

Fruhbeck, G. (2006) Intracellular signalling pathways activated by leptin. *Biochem. J.*, **393**(Pt 1), 7-20.

Frystyk, J., Brick, D. J., Gerweck, A. V., Utz, A. L. and Miller, K. K. (2009) Bioactive insulin-like growth factor-I in obesity. *J. Clin. Endocrinol. Metab.*, **94**(8), 3093-3097.

Frystyk, J., Skjaerbaek, C., Vestbo, E., Fisker, S. and Orskov, H. (1999) Circulating levels of free insulin-like growth factors in obese subjects: The impact of type 2 diabetes. *Diabetes Metab. Res. Rev.*, **15**(5), 314-322.

Frystyk, J., Vestbo, E., Skjaerbaek, C., Mogensen, C. E. and Orskov, H. (1995) Free insulin-like growth factors in human obesity. *Metabolism*, **44**(10 Suppl 4), 37-44.

Fukuhara, A., Matsuda, M., Nishizawa, M., Segawa, K., Tanaka, M., Kishimoto, K., Matsuki, Y., Murakami, M., Ichisaka, T., Murakami, H., Watanabe, E., Takagi, T., Akiyoshi, M., Ohtsubo, T., Kihara, S., Yamashita, S., Makishima, M., Funahashi, T., Yamanaka, S., Hiramatsu, R., Matsuzawa, Y. and Shimomura, I. (2005) Visfatin: A protein secreted by visceral fat that mimics the effects of insulin. *Science*, **307**(5708), 426-430.

Gao, Z., Hwang, D., Bataille, F., Lefevre, M., York, D., Quon, M. J. and Ye, J. (2002) Serine phosphorylation of insulin receptor substrate 1 by inhibitor kappa B kinase complex. *J. Biol. Chem.*, **277**(50), 48115-48121.

Garcia, J.M., Silva, J. M., Dominguez, G., Gonzalez, R., Navarro, A., Carretero, L., Provencio, M., Espana, P. and Bonilla, F. (1999) Allelic loss of the PTEN region (10q23) in breast carcinomas of poor pathophenotype. *Breast Cancer Res. Treat.*, **57**(3), 237-243.

Garmendia, M.L., Pereira, A., Alvarado, M. E. and Atalah, E. (2007) Relation between insulin resistance and breast cancer among chilean women. *Ann. Epidemiol.*, **17**(6), 403-409.

Garofalo, C. and Surmacz, E. (2006) Leptin and cancer. *J. Cell. Physiol.,* **207**(1), 12-22.

Garofalo, C., Sisci, D. and Surmacz, E. (2004) Leptin interferes with the effects of the antiestrogen ICI 182,780 in MCF-7 breast cancer cells. *Clin. Cancer Res.,* **10**(19), 6466-6475.

Garofalo, C., Koda, M., Cascio, S., Sulkowska, M., Kanczuga-Koda, L., Golaszewska, J., Russo, A., Sulkowski, S. and Surmacz, E. (2006) Increased expression of leptin and the leptin receptor as a marker of breast cancer progression: Possible role of obesity-related stimuli. *Clin. Cancer Res.,* **12**(5), 1447-1453.

Garrido, M.C., Cordell, J. L., Becker, M. H., Key, G., Gerdes, J., Jones, M., Gatter, K. C. and Mason, D. Y. (1992) Monoclonal antibody JC1: New reagent for studying cell proliferation. *J. Clin. Pathol.,* **45**(10), 860-865.

Gaur, U. and Aggarwal, B. B. (2003) Regulation of proliferation, survival and apoptosis by members of the TNF superfamily. *Biochem. Pharmacol.,* **66**(8), 1403-1408.

Geier, A., Beery, R., Haimshon, M., Hemi, R. and Lunenfeld, B. (1992) Serum and insulin inhibit cell death induced by cycloheximide in the human breast cancer cell line MCF-7. *In Vitro Cell. Dev. Biol.,* **28A**(6), 415-418.

Geisler, J., Haynes, B., Ekse, D., Dowsett, M. and Lonning, P. E. (2007) Total body aromatization in postmenopausal breast cancer patients is strongly correlated to plasma leptin levels. *J. Steroid Biochem. Mol. Biol.,* **104**(1-2), 27-34.

Gerhardt, C.C., Romero, I. A., Cancello, R., Camoin, L. and Strosberg, A. D. (2001) Chemokines control fat accumulation and leptin secretion by cultured human adipocytes. *Mol. Cell. Endocrinol.,* **175**(1-2), 81-92.

Gesta, S., Tseng, Y. H. and Kahn, C. R. (2007) Developmental origin of fat: Tracking obesity to its source. *Cell,* **131**(2), 242-256.

Gibbs, J., Young, R. C. and Smith, G. P. (1973) Cholecystokinin decreases food intake in rats. *J. Comp. Physiol. Psychol.,* **84**(3), 488-495.

Gliozzo, B., Sung, C. K., Scalia, P., Papa, V., Frasca, F., Sciacca, L., Giorgino, F., Milazzo, G., Goldfine, I. D., Vigneri, R. and Pezzino, V. (1998) Insulin-stimulated cell growth in insulin receptor substrate-1-deficient ZR-75-1 cells is mediated by a phosphatidylinositol-3-kinase-independent pathway. *J. Cell. Biochem.,* **70**(2), 268-280.

Gnacinska, M., Malgorzewicz, S., Lysiak-Szydlowska, W. and Sworczak, K. (2010) The serum profile of adipokines in overweight patients with metabolic syndrome. *Endokrynol. Pol.,* **61**(1), 36-41.

Godden, J., Leake, R. and Kerr, D. J. (1992) The response of breast cancer cells to steroid and peptide growth factors. *Anticancer Res.,* **12**(5), 1683-1688.

Godsland, I.F. (2009) Insulin resistance and hyperinsulinaemia in the development and progression of cancer. *Clin. Sci. (Lond),* **118**(5), 315-332.

Gonullu, G., Basturk, B., Evrensel, T., Oral, B., Gozkaman, A. and Manavoglu, O. (2007) Association of breast cancer and cytokine gene polymorphism in turkish women. *Saudi Med. J.,* **28**(11), 1728-1733.

Gonullu, G., Ersoy, C., Ersoy, A., Evrensel, T., Basturk, B., Kurt, E., Oral, B., Gokgoz, S. and Manavoglu, O. (2005) Relation between insulin resistance and serum concentrations of IL-6 and TNF-alpha in overweight or obese women with early stage breast cancer. *Cytokine,* **31**(4), 264-269.

Gonzalez-Angulo, A.M. and Meric-Bernstam, F. (2010) Metformin: A therapeutic opportunity in breast cancer. *Clin. Cancer Res.,* **16**(6), 1695-1700.

Goodwin, P.J., Ennis, M., Pritchard, K. I., Trudeau, M. E., Koo, J., Madarnas, Y., Hartwick, W., Hoffman, B. and Hood, N. (2002) Fasting insulin and outcome in early-stage breast cancer: Results of a prospective cohort study. *J. Clin. Oncol.,* **20**(1), 42-51.

Gordon-Larsen, P., Zemel, B. S. and Johnston, F. E. (1997) Secular changes in stature, weight, fatness, overweight, and obesity in urban african american adolescents from the mid-1950's to the mid-1990's. *Am. J. Hum. Biol.,* **9**(6), 675-688.

Graham, T.E., Yang, Q., Bluher, M., Hammarstedt, A., Ciaraldi, T. P., Henry, R. R., Wason, C. J., Oberbach, A., Jansson, P. A., Smith, U. and Kahn, B. B. (2006) Retinol-binding protein 4 and insulin resistance in lean, obese, and diabetic subjects. *N. Engl. J. Med.,* **354**(24), 2552-2563.

Green, B.B., Weiss, N. S. and Daling, J. R. (1988) Risk of ovulatory infertility in relation to body weight. *Fertil. Steril.,* **50**(5), 721-726.

Greer, J.B. and Whitcomb, D. C. (2009) Inflammation and pancreatic cancer: An evidence-based review. *Curr. Opin. Pharmacol.,* **9**(4), 411-418.

Griffin, M.E., Marcucci, M. J., Cline, G. W., Bell, K., Barucci, N., Lee, D., Goodyear, L. J., Kraegen, E. W., White, M. F. and Shulman, G. I. (1999) Free fatty acid-induced insulin resistance is associated with activation of protein kinase C theta and alterations in the insulin signaling cascade. *Diabetes,* **48**(6), 1270-1274.

Grodin, J.M., Siiteri, P. K. and MacDonald, P. C. (1973) Source of estrogen production in postmenopausal women. *J. Clin. Endocrinol. Metab.,* **36**(2), 207-214.

Grosfeld, A., Andre, J., Hauguel-De Mouzon, S., Berra, E., Pouyssegur, J. and Guerre-Millo, M. (2002) Hypoxia-inducible factor 1 transactivates the human leptin gene promoter. *J. Biol. Chem.,* **277**(45), 42953-42957.

Gross, G.E., Boldt, D. H. and Osborne, C. K. (1984) Perturbation by insulin of human breast cancer cell cycle kinetics. *Cancer Res.,* **44**(8), 3570-3575.

Grossmann, M.E., Nkhata, K. J., Mizuno, N. K., Ray, A. and Cleary, M. P. (2008) Effects of adiponectin on breast cancer cell growth and signaling. *Br. J. Cancer,* **98**(2), 370-379.

Gruen, M.L., Hao, M., Piston, D. W. and Hasty, A. H. (2007) Leptin requires canonical migratory signaling pathways for induction of monocyte and macrophage chemotaxis. *Am. J. Physiol. Cell. Physiol.,* **293**(5), C1481-8.

Gu, W., Tu, Z., Kleyn, P. W., Kissebah, A., Duprat, L., Lee, J., Chin, W., Maruti, S., Deng, N., Fisher, S. L., Franco, L. S., Burn, P., Yagaloff, K. A., Nathan, J., Heymsfield, S., Albu, J., Pi-Sunyer, F. X. and Allison, D. B. (1999) Identification and functional analysis of novel human melanocortin-4 receptor variants. *Diabetes,* **48**(3), 635-639.

Gumbs, A.A. (2008) Obesity, pancreatitis, and pancreatic cancer. *Obes. Surg.,* **18**(9), 1183-1187.

Gunter, M.J., Hoover, D. R., Yu, H., Wassertheil-Smoller, S., Rohan, T. E., Manson, J. E., Li, J., Ho, G. Y., Xue, X., Anderson, G. L., Kaplan, R. C., Harris, T. G., Howard, B. V., Wylie-Rosett, J., Burk, R. D. and Strickler, H. D. (2009) Insulin, insulin-like growth factor-I, and risk of breast cancer in postmenopausal women. *J. Natl. Cancer Inst.,* **101**(1), 48-60.

Gupta, K., Krishnaswamy, G., Karnad, A. and Peiris, A. N. (2002) Insulin: A novel factor in carcinogenesis. *Am. J. Med. Sci.,* **323**(3), 140-145.

Habel, L.A. and Stanford, J. L. (1993) Hormone receptors and breast cancer. *Epidemiol. Rev.,* **15**(1), 209-219.

Hahn, T.M., Breininger, J. F., Baskin, D. G. and Schwartz, M. W. (1998) Coexpression of agrp and NPY in fasting-activated hypothalamic neurons. *Nat. Neurosci.,* **1**(4), 271-272.

Halford, J.C. and Blundell, J. E. (2000) Separate systems for serotonin and leptin in appetite control. *Ann. Med.,* **32**(3), 222-232.

Hankinson, S.E., Willett, W. C., Colditz, G. A., Hunter, D. J., Michaud, D. S., Deroo, B., Rosner, B., Speizer, F. E. and Pollak, M. (1998) Circulating concentrations of insulin-like growth factor-I and risk of breast cancer. *Lancet,* **351**(9113), 1393-1396.

Hardie, D.G. and Carling, D. (1997) The AMP-activated protein kinase--fuel gauge of the mammalian cell? *Eur. J. Biochem.,* **246**(2), 259-273.

Harris, C.C. and Hollstein, M. (1993) Clinical implications of the p53 tumor-suppressor gene. *N. Engl. J. Med.,* **329**(18), 1318-1327.

Harvey, J.A. and Bovbjerg, V. E. (2004) Quantitative assessment of mammographic breast density: Relationship with breast cancer risk. *Radiology,* **230**(1), 29-41.

Haslam, D.W. and James, W. P. (2005) Obesity. *Lancet,* **366**(9492), 1197-1209.

Hausman, G.J. and Richardson, R. L. (2004) Adipose tissue angiogenesis. *J. Anim. Sci.,* **82**(3), 925-934.

Haverty, A.A., Harmey, J. H., Redmond, H. P. and Bouchier-Hayes, D. J. (1997) Interleukin-6 upregulates GP96 expression in breast cancer. *J. Surg. Res.,* **69**(1), 145-149.

Hefler, L.A., Grimm, C., Lantzsch, T., Lampe, D., Leodolter, S., Koelbl, H., Heinze, G., Reinthaller, A., Tong-Cacsire, D., Tempfer, C. and Zeillinger, R. (2005) Interleukin-1 and interleukin-6 gene polymorphisms and the risk of breast cancer in caucasian women. *Clin. Cancer Res.,* **11**(16), 5718-5721.

Heliovaara, M.K., Strandberg, T. E., Karonen, S. L. and Ebeling, P. (2006) Association of serum adiponectin concentration to lipid and glucose metabolism in healthy humans. *Horm. Metab. Res.,* **38**(5), 336-340.

Hida, K., Wada, J., Eguchi, J., Zhang, H., Baba, M., Seida, A., Hashimoto, I., Okada, T., Yasuhara, A., Nakatsuka, A., Shikata, K., Hourai, S., Futami, J., Watanabe, E., Matsuki, Y., Hiramatsu, R., Akagi, S., Makino, H. and Kanwar, Y. S. (2005) Visceral adipose tissue-derived serine protease inhibitor: A unique insulin-sensitizing adipocytokine in obesity. *Proc. Natl. Acad. Sci. U. S. A.,* **102**(30), 10610-10615.

Hill, D.J. and Milner, R. D. (1985) Insulin as a growth factor. *Pediatr. Res.,* **19**(9), 879-886.

Hinney, A., Schmidt, A., Nottebom, K., Heibult, O., Becker, I., Ziegler, A., Gerber, G., Sina, M., Gorg, T., Mayer, H., Siegfried, W., Fichter, M., Remschmidt, H. and Hebebrand, J. (1999) Several mutations in the melanocortin-4 receptor gene including a nonsense and a frameshift mutation associated with dominantly inherited obesity in humans. *J. Clin. Endocrinol. Metab.,* **84**(4), 1483-1486.

Hirosumi, J., Tuncman, G., Chang, L., Gorgun, C. Z., Uysal, K. T., Maeda, K., Karin, M. and Hotamisligil, G. S. (2002) A central role for JNK in obesity and insulin resistance. *Nature,* **420**(6913), 333-336.

Hoda, M.R. and Popken, G. (2008) Mitogenic and anti-apoptotic actions of adipocyte-derived hormone leptin in prostate cancer cells. *BJU Int.,* **102**(3), 383-388.

Hoda, M.R., Keely, S. J., Bertelsen, L. S., Junger, W. G., Dharmasena, D. and Barrett, K. E. (2007) Leptin acts as a mitogenic and antiapoptotic factor for colonic cancer cells. *Br. J. Surg.,* **94**(3), 346-354.

Hollestelle, A., Elstrodt, F., Nagel, J. H., Kallemeijn, W. W. and Schutte, M. (2007) Phosphatidylinositol-3-OH kinase or RAS pathway mutations in human breast cancer cell lines. *Mol. Cancer. Res.,* **5**(2), 195-201.

Honma, S., Shimodaira, K., Shimizu, Y., Tsuchiya, N., Saito, H., Yanaihara, T. and Okai, T. (2002) The influence of inflammatory cytokines on estrogen production and cell proliferation in human breast cancer cells. *Endocr. J.,* **49**(3), 371-377.

Horvath, T.L., Diano, S., Sotonyi, P., Heiman, M. and Tschop, M. (2001) Minireview: Ghrelin and the regulation of energy balance--a hypothalamic perspective. *Endocrinology,* **142**(10), 4163-4169.

Hotamisligil, G.S. (2003) Inflammatory pathways and insulin action. *Int. J. Obes. Relat. Metab. Disord.,* **27 Suppl 3**, S53-5.

Hotamisligil, G.S., Shargill, N. S. and Spiegelman, B. M. (1993) Adipose expression of tumor necrosis factor-alpha: Direct role in obesity-linked insulin resistance. *Science,* **259**(5091), 87-91.

Housa, D., Housova, J., Vernerova, Z. and Haluzik, M. (2006) Adipocytokines and cancer. *Physiol. Res.,* **55**(3), 233-244.

Houseknecht, K.L., McGuire, M. K., Portocarrero, C. P., McGuire, M. A. and Beerman, K. (1997) Leptin is present in human milk and is related to maternal plasma leptin concentration and adiposity. *Biochem. Biophys. Res. Commun.,* **240**(3), 742-747.

Hryniuk, W.M., Banerjee, M., Du, W., Heppler, G., Severson, R. and Song, E.Y. (2001) Adjuvant tamoxifen may be relatively ineffective in woman with diabetes and breast cancer [abstract 141]. *Proc Am Soc Clin Oncol*., **30a**.

Hu, X., Juneja, S. C., Maihle, N. J. and Cleary, M. P. (2002) Leptin--a growth factor in normal and malignant breast cells and for normal mammary gland development. *J. Natl. Cancer Inst.,* **94**(22), 1704-1711.

Huang, Z., Willett, W. C., Colditz, G. A., Hunter, D. J., Manson, J. E., Rosner, B., Speizer, F. E. and Hankinson, S. E. (1999) Waist circumference, waist:Hip ratio, and risk of breast cancer in the nurses' health study. *Am. J. Epidemiol.,* **150**(12), 1316-1324.

Hudziak, R.M., Shepard, H. M., Ullrich, A. and Fendly, B. M. (1997) Monoclonal antibodies directed to the HER2 receptor. **286303**(5677171).

Huguet, E.L., McMahon, J. A., McMahon, A. P., Bicknell, R. and Harris, A. L. (1994) Differential expression of human wnt genes 2, 3, 4, and 7B in human breast cell lines and normal and disease states of human breast tissue. *Cancer Res.,* **54**(10), 2615-2621.

Hussein, M.Z., Al Fikky, A., Abdel Bar, I. and Attia, O. (2004) Serum IL-6 and IL-12 levels in breast cancer patients. *Egypt. J. Immunol.,* **11**(2), 165-170.

Huxley, R.R., Ansary-Moghaddam, A., Clifton, P., Czernichow, S., Parr, C. L. and Woodward, M. (2009) The impact of dietary and lifestyle risk factors on risk of colorectal cancer: A quantitative overview of the epidemiological evidence. *Int. J. Cancer,* **125**(1), 171-180.

Ihle, J.N., Witthuhn, B. A., Quelle, F. W., Yamamoto, K. and Silvennoinen, O. (1995) Signaling through the hematopoietic cytokine receptors. *Annu. Rev. Immunol.,* **13**, 369-398.

Ildaphonse, G., George, P. S. and Mathew, A. (2009) Obesity and kidney cancer risk in men: A meta-analysis (1992-2008). *Asian Pac. J. Cancer. Prev.,* **10**(2), 279-286.

Ingalls, A.M., Dickie, M. M. and Snell, G. D. (1950) Obese, a new mutation in the house mouse. *J. Hered.,* **41**(12), 317-318.

Inoue, T., Kotooka, N., Morooka, T., Komoda, H., Uchida, T., Aso, Y., Inukai, T., Okuno, T. and Node, K. (2007) High molecular weight adiponectin as a predictor of long-term clinical outcome in patients with coronary artery disease. *Am. J. Cardiol.,* **100**(4), 569-574.

International Diabetes Federation (2006) The IDF consensus worldwide definition of the metabolic syndrome, http://www.idf.org/webdata/docs/IDF_Meta_def_final.pdf, accessed on 1st October 2009

Ip, M.M., Shoemaker, S. F. and Darcy, K. M. (1992) Regulation of rat mammary epithelial cell proliferation and differentiation by tumor necrosis factor-alpha. *Endocrinology,* **130**(5), 2833-2844.

Ip, M.S., Lam, B., Ng, M. M., Lam, W. K., Tsang, K. W. and Lam, K. S. (2002) Obstructive sleep apnea is independently associated with insulin resistance. *Am. J. Respir. Crit. Care Med.,* **165**(5), 670-676.

Irvin, W.J.,Jr and Carey, L. A. (2008) What is triple-negative breast cancer? *Eur. J. Cancer,* **44**(18), 2799-2805.

Ishikawa, M., Kitayama, J. and Nagawa, H. (2004) Enhanced expression of leptin and leptin receptor (OB-R) in human breast cancer. *Clin. Cancer Res.,* **10**(13), 4325-4331.

Ito, K., Utsunomiya, H., Yaegashi, N. and Sasano, H. (2007) Biological roles of estrogen and progesterone in human endometrial carcinoma--new developments in potential endocrine therapy for endometrial cancer. *Endocr. J.,* **54**(5), 667-679.

Jablonska, E. (1998) Serum levels of tumor necrosis factor alpha and production of this cytokine by polymorphonuclear cells in breast cancer patients. *Arch. Immunol. Ther. Exp. (Warsz),* **46**(2), 93-96.

Jackson, J.G., White, M. F. and Yee, D. (1998) Insulin receptor substrate-1 is the predominant signaling molecule activated by insulin-like growth factor-I, insulin, and interleukin-4 in estrogen receptor-positive human breast cancer cells. *J. Biol. Chem.,* **273**(16), 9994-10003.

Jarow, J.P., Kirkland, J., Koritnik, D. R. and Cefalu, W. T. (1993) Effect of obesity and fertility status on sex steroid levels in men. *Urology,* **42**(2), 171-174.

Jeng, M.H., Yue, W., Eischeid, A., Wang, J. P. and Santen, R. J. (2000) Role of MAP kinase in the enhanced cell proliferation of long term estrogen deprived human breast cancer cells. *Breast Cancer Res. Treat.,* **62**(3), 167-175.

Jeoung, D.I., Tang, B. and Sonenberg, M. (1995) Effects of tumor necrosis factor-alpha on antimitogenicity and cell cycle-related proteins in MCF-7 cells. *J. Biol. Chem.,* **270**(31), 18367-18373.

Jiang, H., Yu, J., Guo, H., Song, H. and Chen, S. (2008) Upregulation of survivin by leptin/STAT3 signaling in MCF-7 cells. *Biochem. Biophys. Res. Commun.,* **368**(1), 1-5.

Jiang, X.C., Moulin, P., Quinet, E., Goldberg, I. J., Yacoub, L. K., Agellon, L. B., Compton, D., Schnitzer-Polokoff, R. and Tall, A. R. (1991) Mammalian adipose tissue and muscle are major sources of lipid transfer protein mRNA. *J. Biol. Chem.,* **266**(7), 4631-4639.

Joel, P.B., Smith, J., Sturgill, T. W., Fisher, T. L., Blenis, J. and Lannigan, D. A. (1998) pp90rsk1 regulates estrogen receptor-mediated transcription through phosphorylation of ser-167. *Mol. Cell. Biol.,* **18**(4), 1978-1984.

Johnson, A.L., Cornoni, J. C., Cassel, J. C., Tyroler, H. A., Heyden, S. and Hames, C. G. (1975) Influence of race, sex and weight on blood pressure behavior in young adults. *Am. J. Cardiol.,* **35**(4), 523-530.

Jokela, M., Elovainio, M. and Kivimaki, M. (2008) Lower fertility associated with obesity and underweight: The US national longitudinal survey of youth. *Am. J. Clin. Nutr.,* **88**(4), 886-893.

Junttila, T.T., Akita, R. W., Parsons, K., Fields, C., Lewis Phillips, G. D., Friedman, L. S., Sampath, D. and Sliwkowski, M. X. (2009) Ligand-independent HER2/HER3/PI3K complex is disrupted by trastuzumab and is effectively inhibited by the PI3K inhibitor GDC-0941. *Cancer. Cell.,* **15**(5), 429-440.

Kaaks, R. and Lukanova, A. (2001) Energy balance and cancer: The role of insulin and insulin-like growth factor-I. *Proc. Nutr. Soc.,* **60**(1), 91-106.

Kahn, B.B. (1998) Type 2 diabetes: When insulin secretion fails to compensate for insulin resistance. *Cell,* **92**(5), 593-596.

Kahn, B.B. and Flier, J. S. (2000) Obesity and insulin resistance. *J. Clin. Invest.,* **106**(4), 473-481.

Kahn, S.E., Hull, R. L. and Utzschneider, K. M. (2006) Mechanisms linking obesity to insulin resistance and type 2 diabetes. *Nature,* **444**(7121), 840-846.

Kaklamani, V.G., Sadim, M., Hsi, A., Offit, K., Oddoux, C., Ostrer, H., Ahsan, H., Pasche, B. and Mantzoros, C. (2008) Variants of the adiponectin and adiponectin receptor 1 genes and breast cancer risk. *Cancer Res.,* **68**(9), 3178-3184.

Kang, J.H., Lee, Y. Y., Yu, B. Y., Yang, B. S., Cho, K. H., Yoon, D. K. and Roh, Y. K. (2005) Adiponectin induces growth arrest and apoptosis of MDA-MB-231 breast cancer cell. *Arch. Pharm. Res.,* **28**(11), 1263-1269.

Kannel, W.B., Brand, N., Skinner, J. J.,Jr, Dawber, T. R. and McNamara, P. M. (1967) The relation of adiposity to blood pressure and development of hypertension. the framingham study. *Ann. Intern. Med.,* **67**(1), 48-59.

Karaduman, M., Bilici, A., Ozet, A., Sengul, A., Musabak, U. and Alomeroglu, M. (2007) Tissue levels of adiponectin in breast cancer patients. *Med. Oncol.,* **24**(4), 361-366.

Karagiannides, I., Tchkonia, T., Dobson, D. E., Steppan, C. M., Cummins, P., Chan, G., Salvatori, K., Hadzopoulou-Cladaras, M. and Kirkland, J. L. (2001) Altered expression of C/EBP family members results in decreased adipogenesis with aging. *Am. J. Physiol. Regul. Integr. Comp. Physiol.,* **280**(6), R1772-80.

Karey, K.P. and Sirbasku, D. A. (1988) Differential responsiveness of human breast cancer cell lines MCF-7 and T47D to growth factors and 17 beta-estradiol. *Cancer Res.,* **48**(14), 4083-4092.

Karin, M. (2009) NF-kappaB as a critical link between inflammation and cancer. *Cold Spring Harbor Perspect. Biol.,* **1**(5), a000141.

Kato, S., Endoh, H., Masuhiro, Y., Kitamoto, T., Uchiyama, S., Sasaki, H., Masushige, S., Gotoh, Y., Nishida, E., Kawashima, H., Metzger, D. and

Chambon, P. (1995) Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. *Science,* **270**(5241), 1491-1494.

Kazer, R.R. (1995) Insulin resistance, insulin-like growth factor I and breast cancer: A hypothesis. *Int. J. Cancer,* **62**(4), 403-406.

Kelley, D.E. and Mandarino, L. J. (2000) Fuel selection in human skeletal muscle in insulin resistance: A reexamination. *Diabetes,* **49**(5), 677-683.

Kennedy, G.C. (1953) The role of depot fat in the hypothalamic control of food intake in the rat. *Proc. R. Soc. Lond. B. Biol. Sci.,* **140**(901), 578-596.

Kennedy, R.T., Kauri, L. M., Dahlgren, G. M. and Jung, S. K. (2002) Metabolic oscillations in beta-cells. *Diabetes,* **51 Suppl 1**, S152-61.

Key, T.J. and Pike, M. C. (1988) The dose-effect relationship between 'unopposed' oestrogens and endometrial mitotic rate: Its central role in explaining and predicting endometrial cancer risk. *Br. J. Cancer,* **57**(2), 205-212.

Kiess, W., Reich, A., Meyer, K., Glasow, A., Deutscher, J., Klammt, J., Yang, Y., Muller, G. and Kratzsch, J. (1999) A role for leptin in sexual maturation and puberty? *Horm. Res.,* **51 Suppl 3**, 55-63.

Kim, J.H., Bachmann, R. A. and Chen, J. (2009) Interleukin-6 and insulin resistance. *Vitam. Horm.,* **80**, 613-633.

Kim, S., Choi, J. H., Kim, J. B., Nam, S. J., Yang, J. H., Kim, J. H. and Lee, J. E. (2008) Berberine suppresses TNF-alpha-induced MMP-9 and cell invasion through inhibition of AP-1 activity in MDA-MB-231 human breast cancer cells. *Molecules,* **13**(12), 2975-2985.

Klein, S., Allison, D. B., Heymsfield, S. B., Kelley, D. E., Leibel, R. L., Nonas, C. and Kahn, R. (2007) Waist circumference and cardiometabolic risk: A consensus statement from shaping america's health: Association for weight management and obesity prevention; NAASO, the obesity society; the american society for nutrition; and the american diabetes association. *Obesity (Silver Spring),* **15**(5), 1061-1067.

Klinge, C.M. (2000) Estrogen receptor interaction with co-activators and co-repressors. *Steroids,* **65**(5), 227-251.

Klok, M.D., Jakobsdottir, S. and Drent, M. L. (2007) The role of leptin and ghrelin in the regulation of food intake and body weight in humans: A review. *Obes. Rev.,* **8**(1), 21-34.

Knowlden, J.M., Hutcheson, I. R., Barrow, D., Gee, J. M. and Nicholson, R. I. (2005) Insulin-like growth factor-I receptor signaling in tamoxifen-resistant breast cancer: A supporting role to the epidermal growth factor receptor. *Endocrinology,* **146**(11), 4609-4618.

Knupfer, H. and Preiss, R. (2007) Significance of interleukin-6 (IL-6) in breast cancer (review). *Breast Cancer Res. Treat.,* **102**(2), 129-135.

Koerner, A., Kratzsch, J. and Kiess, W. (2005) Adipocytokines: Leptin--the classical, resistin--the controversical, adiponectin--the promising, and more to come. *Best Pract. Res. Clin. Endocrinol. Metab.,* **19**(4), 525-546.

Kohn, A.D., Summers, S. A., Birnbaum, M. J. and Roth, R. A. (1996) Expression of a constitutively active akt Ser/Thr kinase in 3T3-L1 adipocytes stimulates glucose uptake and glucose transporter 4 translocation. *J. Biol. Chem.,* **271**(49), 31372-31378.

Kojima, M. and Kangawa, K. (2005) Ghrelin: Structure and function. *Physiol. Rev.,* **85**(2), 495-522.

Kojima, M., Hosoda, H., Date, Y., Nakazato, M., Matsuo, H. and Kangawa, K. (1999) Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature,* **402**(6762), 656-660.

Kolch, W. (2000) Meaningful relationships: The regulation of the Ras/Raf/MEK/ERK pathway by protein interactions. *Biochem. J.,* **351 Pt 2**, 289-305.

Kopelman, P. (2007) Health risks associated with overweight and obesity. *Obes. Rev.,* **8 Suppl 1**, 13-17.

Kopelman, P.G. (2000) Obesity as a medical problem. *Nature,* **404**(6778), 635-643.

Kopelman, P.G., Apps, M. C., Cope, T. and Empey, D. W. (1985) The influence of menstrual status, body weight and hypothalamic function on nocturnal respiration in women. *J. R. Coll. Physicians Lond.,* **19**(4), 243-247.

Kopelman, P.G., Apps, M. C., Cope, T., Ingram, D. A., Empey, D. W. and Evans, S. J. (1986) Nocturnal hypoxia and sleep apnoea in asymptomatic obese men. *Int. J. Obes.,* **10**(3), 211-217.

Korner, A., Wabitsch, M., Seidel, B., Fischer-Posovszky, P., Berthold, A., Stumvoll, M., Bluher, M., Kratzsch, J. and Kiess, W. (2005) Adiponectin expression in humans is dependent on differentiation of adipocytes and down-regulated by humoral serum components of high molecular weight. *Biochem. Biophys. Res. Commun.,* **337**(2), 540-550.

Korner, A., Pazaitou-Panayiotou, K., Kelesidis, T., Kelesidis, I., Williams, C. J., Kaprara, A., Bullen, J., Neuwirth, A., Tseleni, S., Mitsiades, N., Kiess, W. and Mantzoros, C. S. (2007) Total and high-molecular-weight adiponectin in breast cancer: In vitro and in vivo studies. *J. Clin. Endocrinol. Metab.,* **92**(3), 1041-1048.

Kozlowski, L., Zakrzewska, I., Tokajuk, P. and Wojtukiewicz, M. Z. (2003) Concentration of interleukin-6 (IL-6), interleukin-8 (IL-8) and interleukin-10 (IL-10) in blood serum of breast cancer patients. *Rocz. Akad. Med. Bialymst.,* **48**, 82-84.

Krajcik, R.A., Massardo, S. and Orentreich, N. (2003) No association between serum levels of tumor necrosis factor-alpha (TNF-alpha) or the soluble receptors sTNFR1 and sTNFR2 and breast cancer risk. *Cancer Epidemiol. Biomarkers Prev.,* **12**(9), 945-946.

Krajewska, M., Krajewski, S., Zapata, J. M., Van Arsdale, T., Gascoyne, R. D., Berern, K., McFadden, D., Shabaik, A., Hugh, J., Reynolds, A., Clevenger, C. V. and Reed, J. C. (1998) TRAF-4 expression in epithelial progenitor cells. analysis in normal adult, fetal, and tumor tissues. *Am. J. Pathol.,* **152**(6), 1549-1561.

Kumar, R. and Mendelsohn, J. (1994) Reduced expression of c-erbB2 gene product in human mammary carcinoma SK-BR-3 cells treated with interferon-gamma and tumor necrosis factor-alpha. *Anticancer Res.,* **14**(3A), 1001-1008.

Kunisue, H., Kurebayashi, J., Otsuki, T., Tang, C. K., Kurosumi, M., Yamamoto, S., Tanaka, K., Doihara, H., Shimizu, N. and Sonoo, H. (2000) Anti-HER2 antibody enhances the growth inhibitory effect of anti-oestrogen on breast cancer cells expressing both oestrogen receptors and HER2. *Br. J. Cancer,* **82**(1), 46-51.

Kurbel, S. (2005) Selective reduction of estrogen receptor (ER) positive breast cancer occurrence by estrogen receptor modulators supports etiological distinction between ER positive and ER negative breast cancers. *Med. Hypotheses,* **64**(6), 1182-1187.

Kurebayashi, J. (2001) Biological and clinical significance of HER2 overexpression in breast cancer. *Breast Cancer,* **8**(1), 45-51.

Lacey, J.V.,Jr, Ioffe, O. B., Ronnett, B. M., Rush, B. B., Richesson, D. A., Chatterjee, N., Langholz, B., Glass, A. G. and Sherman, M. E. (2008) Endometrial carcinoma risk among women diagnosed with endometrial hyperplasia: The 34-year experience in a large health plan. *Br. J. Cancer,* **98**(1), 45-53.

Lahmann, P.H., Hoffmann, K., Allen, N., van Gils, C. H., Khaw, K. T., Tehard, B., Berrino, F., Tjonneland, A., Bigaard, J., Olsen, A., Overvad, K., Clavel-Chapelon, F., Nagel, G., Boeing, H., Trichopoulos, D., Economou, G., Bellos, G., Palli, D., Tumino, R., Panico, S., Sacerdote, C., Krogh, V., Peeters, P. H., Bueno-de-Mesquita, H. B., Lund, E., Ardanaz, E., Amiano, P., Pera, G., Quiros, J. R., Martinez, C., Tormo, M. J., Wirfalt, E., Berglund, G., Hallmans, G., Key, T. J., Reeves, G., Bingham, S., Norat, T., Biessy, C., Kaaks, R. and Riboli, E. (2004) Body size and breast cancer risk: Findings from the european prospective investigation into cancer and nutrition (EPIC). *Int. J. Cancer,* **111**(5), 762-771.

Lalande, S. and Johnson, B. D. (2008) Diastolic dysfunction: A link between hypertension and heart failure. *Drugs Today (Barc)*, **44**(7), 503-513.

Lam, J.C. and Ip., M.S. (2009) Obstructive sleep apnea and the metabolic syndrome. *Expert Rev. Respir. Med*. **3**(2), 177-186.

Lam, Q.L. and Lu, L. (2007) Role of leptin in immunity. *Cell. Mol. Immunol.*, **4**(1), 1-13.

Lanzino, M., Morelli, C., Garofalo, C., Panno, M. L., Mauro, L., Ando, S. and Sisci, D. (2008) Interaction between estrogen receptor alpha and insulin/IGF signaling in breast cancer. *Curr. Cancer. Drug Targets*, **8**(7), 597-610.

Larsson, S.C. and Wolk, A. (2007) Obesity and colon and rectal cancer risk: A meta-analysis of prospective studies. *Am. J. Clin. Nutr.*, **86**(3), 556-565.

Larsson, S.C., Mantzoros, C. S. and Wolk, A. (2007) Diabetes mellitus and risk of breast cancer: A meta-analysis. *Int. J. Cancer*, **121**(4), 856-862.

Laud, K., Gourdou, I., Pessemesse, L., Peyrat, J. P. and Djiane, J. (2002) Identification of leptin receptors in human breast cancer: Functional activity in the T47-D breast cancer cell line. *Mol. Cell. Endocrinol.*, **188**(1-2), 219-226.

Laven, J.S., Imani, B., Eijkemans, M. J. and Fauser, B. C. (2002) New approach to polycystic ovary syndrome and other forms of anovulatory infertility. *Obstet. Gynecol. Surv.*, **57**(11), 755-767.

Lee, A.V., Weng, C. N., Jackson, J. G. and Yee, D. (1997) Activation of estrogen receptor-mediated gene transcription by IGF-I in human breast cancer cells. *J. Endocrinol.*, **152**(1), 39-47.

Lee, A.V., Jackson, J. G., Gooch, J. L., Hilsenbeck, S. G., Coronado-Heinsohn, E., Osborne, C. K. and Yee, D. (1999) Enhancement of insulin-like growth factor signaling in human breast cancer: Estrogen regulation of insulin receptor substrate-1 expression in vitro and in vivo. *Mol. Endocrinol.*, **13**(5), 787-796.

Lee, S., Bacha, F., Gungor, N. and Arslanian, S. A. (2006) Waist circumference is an independent predictor of insulin resistance in black and white youths. *J. Pediatr.*, **148**(2), 188-194.

Lee, S.H. and Nam, H. S. (2008) TNF alpha-induced down-regulation of estrogen receptor alpha in MCF-7 breast cancer cells. *Mol. Cells*, **26**(3), 285-290.

Lefebvre, P.J., Paolisso, G., Scheen, A. J. and Henquin, J. C. (1987) Pulsatility of insulin and glucagon release: Physiological significance and pharmacological implications. *Diabetologia*, **30**(7), 443-452.

Leinonen, E., Hurt-Camejo, E., Wiklund, O., Hulten, L. M., Hiukka, A. and Taskinen, M. R. (2003) Insulin resistance and adiposity correlate with acute-phase reaction and soluble cell adhesion molecules in type 2 diabetes. *Atherosclerosis,* **166**(2), 387-394.

Lenormand, P., McMahon, M. and Pouyssegur, J. (1996) Oncogenic raf-1 activates p70 S6 kinase via a mitogen-activated protein kinase-independent pathway. *J. Biol. Chem.,* **271**(26), 15762-15768.

Lettner, A. and Roden, M. (2008) Ectopic fat and insulin resistance. *Curr. Diab Rep.,* **8**(3), 185-191.

Levin, B.E. (2002) Metabolic sensors: Viewing glucosensing neurons from a broader perspective. *Physiol. Behav.,* **76**(3), 397-401.

Li, N., Batzer, A., Daly, R., Yajnik, V., Skolnik, E., Chardin, P., Bar-Sagi, D., Margolis, B. and Schlessinger, J. (1993) Guanine-nucleotide-releasing factor hSos1 binds to Grb2 and links receptor tyrosine kinases to ras signalling. *Nature,* **363**(6424), 85-88.

Lieblein, J.C., Ball, S., Hutzen, B., Sasser, A. K., Lin, H. J., Huang, T. H., Hall, B. M. and Lin, J. (2008) STAT3 can be activated through paracrine signaling in breast epithelial cells. *BMC Cancer,* **8**, 302.

Lijnen, H.R., Christiaens, V., Scroyen, I., Voros, G., Tjwa, M., Carmeliet, P. and Collen, D. (2006) Impaired adipose tissue development in mice with inactivation of placental growth factor function. *Diabetes,* **55**(10), 2698-2704.

Lindgren, C.M., Heid, I. M., Randall, J. C., Lamina, C., Steinthorsdottir, V., Qi, L., Speliotes, E. K., Thorleifsson, G., Willer, C. J., Herrera, B. M., Jackson, A. U., Lim, N., Scheet, P., Soranzo, N., Amin, N., Aulchenko, Y. S., Chambers, J. C., Drong, A., Luan, J., Lyon, H. N., Rivadeneira, F., Sanna, S., Timpson, N. J., Zillikens, M. C., Zhao, J. H., Almgren, P., Bandinelli, S., Bennett, A. J., Bergman, R. N., Bonnycastle, L. L., Bumpstead, S. J., Chanock, S. J., Cherkas, L., Chines, P., Coin, L., Cooper, C., Crawford, G., Doering, A., Dominiczak, A., Doney, A. S., Ebrahim, S., Elliott, P., Erdos, M. R., Estrada, K., Ferrucci, L., Fischer, G., Forouhi, N. G., Gieger, C., Grallert, H., Groves, C. J., Grundy, S., Guiducci, C., Hadley, D., Hamsten, A., Havulinna, A. S., Hofman, A., Holle, R., Holloway, J. W., Illig, T., Isomaa, B., Jacobs, L. C., Jameson, K., Jousilahti, P., Karpe, F., Kuusisto, J., Laitinen, J., Lathrop, G. M., Lawlor, D. A., Mangino, M., McArdle, W. L., Meitinger, T., Morken, M. A., Morris, A. P., Munroe, P., Narisu, N., Nordstrom, A., Nordstrom, P., Oostra, B. A., Palmer, C. N., Payne, F., Peden, J. F., Prokopenko, I., Renstrom, F., Ruokonen, A., Salomaa, V., Sandhu, M. S., Scott, L. J., Scuteri, A., Silander, K., Song, K., Yuan, X., Stringham, H. M., Swift, A. J., Tuomi, T., Uda, M., Vollenweider, P., Waeber, G., Wallace, C., Walters, G. B., Weedon, M. N., Wellcome Trust Case Control Consortium, Witteman, J. C., Zhang, C., Zhang, W., Caulfield, M. J., Collins, F. S., Davey Smith, G., Day, I. N., Franks, P. W., Hattersley, A. T., Hu,

F. B., Jarvelin, M. R., Kong, A., Kooner, J. S., Laakso, M., Lakatta, E., Mooser, V., Morris, A. D., Peltonen, L., Samani, N. J., Spector, T. D., Strachan, D. P., Tanaka, T., Tuomilehto, J., Uitterlinden, A. G., van Duijn, C. M., Wareham, N. J., Hugh, W., Procardis Consortia, Waterworth, D. M., Boehnke, M., Deloukas, P., Groop, L., Hunter, D. J., Thorsteinsdottir, U., Schlessinger, D., Wichmann, H. E., Frayling, T. M., Abecasis, G. R., Hirschhorn, J. N., Loos, R. J., Stefansson, K., Mohlke, K. L., Barroso, I., McCarthy, M. I. and Giant Consortium (2009) Genome-wide association scan meta-analysis identifies three loci influencing adiposity and fat distribution. *PLoS Genet.*, **5**(6), e1000508.

Lippman, M.E., Krueger, K. A., Eckert, S., Sashegyi, A., Walls, E. L., Jamal, S., Cauley, J. A. and Cummings, S. R. (2001) Indicators of lifetime estrogen exposure: Effect on breast cancer incidence and interaction with raloxifene therapy in the multiple outcomes of raloxifene evaluation study participants. *J. Clin. Oncol.*, **19**(12), 3111-3116.

Litovkin, K.V., Domenyuk, V. P., Bubnov, V. V. and Zaporozhan, V. N. (2007) Interleukin-6 -174G/C polymorphism in breast cancer and uterine leiomyoma patients: A population-based case control study. *Exp. Oncol.*, **29**(4), 295-298.

Liu, B., Fan, Z., Edgerton, S. M., Deng, X. S., Alimova, I. N., Lind, S. E. and Thor, A. D. (2009) Metformin induces unique biological and molecular responses in triple negative breast cancer cells. *Cell. Cycle*, **8**(13), 2031-2040.

Liu, C.L., Chang, Y. C., Cheng, S. P., Chern, S. R., Yang, T. L., Lee, J. J., Guo, I. C. and Chen, C. P. (2007) The roles of serum leptin concentration and polymorphism in leptin receptor gene at codon 109 in breast cancer. *Oncology*, **72**(1-2), 75-81.

Lobenhofer, E.K., Huper, G., Iglehart, J. D. and Marks, J. R. (2000) Inhibition of mitogen-activated protein kinase and phosphatidylinositol 3-kinase activity in MCF-7 cells prevents estrogen-induced mitogenesis. *Cell Growth Differ.*, **11**(2), 99-110.

Lockner, D.W., Heyward, V. H., Baumgartner, R. N. and Jenkins, K. A. (2000) Comparison of air-displacement plethysmography, hydrodensitometry, and dual X-ray absorptiometry for assessing body composition of children 10 to 18 years of age. *Ann. N. Y. Acad. Sci.*, **904**, 72-78.

Longcope, C., Baker, R. and Johnston, C. C.,Jr (1986) Androgen and estrogen metabolism: Relationship to obesity. *Metabolism*, **35**(3), 235-237.

Loos, R.J., Lindgren, C. M., Li, S., Wheeler, E., Zhao, J. H., Prokopenko, I., Inouye, M., Freathy, R. M., Attwood, A. P., Beckmann, J. S., Berndt, S. I., Prostate, Lung, Colorectal, and Ovarian (PLCO) Cancer Screening Trial, Jacobs, K. B., Chanock, S. J., Hayes, R. B., Bergmann, S., Bennett, A. J., Bingham, S. A., Bochud, M., Brown, M., Cauchi, S., Connell, J. M., Cooper,

C., Smith, G. D., Day, I., Dina, C., De, S., Dermitzakis, E. T., Doney, A. S., Elliott, K. S., Elliott, P., Evans, D. M., Sadaf Farooqi, I., Froguel, P., Ghori, J., Groves, C. J., Gwilliam, R., Hadley, D., Hall, A. S., Hattersley, A. T., Hebebrand, J., Heid, I. M., KORA, Lamina, C., Gieger, C., Illig, T., Meitinger, T., Wichmann, H. E., Herrera, B., Hinney, A., Hunt, S. E., Jarvelin, M. R., Johnson, T., Jolley, J. D., Karpe, F., Keniry, A., Khaw, K. T., Luben, R. N., Mangino, M., Marchini, J., McArdle, W. L., McGinnis, R., Meyre, D., Munroe, P. B., Morris, A. D., Ness, A. R., Neville, M. J., Nica, A. C., Ong, K. K., O'Rahilly, S., Owen, K. R., Palmer, C. N., Papadakis, K., Potter, S., Pouta, A., Qi, L., Nurses' Health Study, Randall, J. C., Rayner, N. W., Ring, S. M., Sandhu, M. S., Scherag, A., Sims, M. A., Song, K., Soranzo, N., Speliotes, E. K., Diabetes Genetics Initiative, Syddall, H. E., Teichmann, S. A., Timpson, N. J., Tobias, J. H., Uda, M., SardiNIA Study, Vogel, C. I., Wallace, C., Waterworth, D. M., Weedon, M. N., Wellcome Trust Case Control Consortium, Willer, C. J., FUSION, Wraight, Yuan, X., Zeggini, E., Hirschhorn, J. N., Strachan, D. P., Ouwehand, W. H., Caulfield, M. J., Samani, N. J., Frayling, T. M., Vollenweider, P., Waeber, G., Mooser, V., Deloukas, P., McCarthy, M. I., Wareham, N. J., Barroso, I., Jacobs, K. B., Chanock, S. J., Hayes, R. B., Lamina, C., Gieger, C., Illig, T., Meitinger, T., Wichmann, H. E., Kraft, P., Hankinson, S. E., Hunter, D. J., Hu, F. B., Lyon, H. N., Voight, B. F., Ridderstrale, M., Groop, L., Scheet, P., Sanna, S., Abecasis, G. R., Albai, G., Nagaraja, R., Schlessinger, D., Jackson, A. U., Tuomilehto, J., Collins, F. S., Boehnke, M. and Mohlke, K. L. (2008) Common variants near MC4R are associated with fat mass, weight and risk of obesity. *Nat. Genet.*, **40**(6), 768-775.

Lord, G.M., Matarese, G., Howard, J. K., Baker, R. J., Bloom, S. R. and Lechler, R. I. (1998) Leptin modulates the T-cell immune response and reverses starvation-induced immunosuppression. *Nature,* **394**(6696), 897-901.

Lorincz, A.M. and Sukumar, S. (2006) Molecular links between obesity and breast cancer. *Endocr. Relat. Cancer,* **13**(2), 279-292.

Lowry, O.H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) Protein measurement with the folin phenol reagent. *J. Biol. Chem.,* **193**(1), 265-275.

Lu, Y., Zi, X. and Pollak, M. (2004) Molecular mechanisms underlying IGF-I-induced attenuation of the growth-inhibitory activity of trastuzumab (herceptin) on SKBR3 breast cancer cells. *Int. J. Cancer,* **108**(3), 334-341.

Lu, Y., Zi, X., Zhao, Y., Mascarenhas, D. and Pollak, M. (2001) Insulin-like growth factor-I receptor signaling and resistance to trastuzumab (herceptin). *J. Natl. Cancer Inst.,* **93**(24), 1852-1857.

Lu, Y., Lin, Y. Z., LaPushin, R., Cuevas, B., Fang, X., Yu, S. X., Davies, M. A., Khan, H., Furui, T., Mao, M., Zinner, R., Hung, M. C., Steck, P., Siminovitch, K. and Mills, G. B. (1999) The PTEN/MMAC1/TEP tumor suppressor gene

decreases cell growth and induces apoptosis and anoikis in breast cancer cells. *Oncogene,* **18**(50), 7034-7045.

Lundgren, C.H., Brown, S. L., Nordt, T. K., Sobel, B. E. and Fujii, S. (1996) Elaboration of type-1 plasminogen activator inhibitor from adipocytes. A potential pathogenetic link between obesity and cardiovascular disease. *Circulation,* **93**(1), 106-110.

Machinal, F., Dieudonne, M. N., Leneveu, M. C., Pecquery, R. and Giudicelli, Y. (1999) In vivo and in vitro ob gene expression and leptin secretion in rat adipocytes: Evidence for a regional specific regulation by sex steroid hormones. *Endocrinology,* **140**(4), 1567-1574.

Maeda, K., Okubo, K., Shimomura, I., Funahashi, T., Matsuzawa, Y. and Matsubara, K. (1996) cDNA cloning and expression of a novel adipose specific collagen-like factor, apM1 (AdiPose most abundant gene transcript 1). *Biochem. Biophys. Res. Commun.,* **221**(2), 286-289.

Maes, H.H., Neale, M. C. and Eaves, L. J. (1997) Genetic and environmental factors in relative body weight and human adiposity. *Behav. Genet.,* **27**(4), 325-351.

Maiti, B., Kundranda, M. N., Spiro, T. P. and Daw, H. A. (2009) The association of metabolic syndrome with triple-negative breast cancer. *Breast Cancer Res. Treat.,* .

Maki, K.C., Kanter, M., Rains, T. M., Hess, S. P. and Geohas, J. (2009) Acute effects of low insulinemic sweeteners on postprandial insulin and glucose concentrations in obese men. *Int. J. Food Sci. Nutr.,* **60 Suppl 3**, 48-55.

Mallmann, P., Diedrich, K., Mallmann, R., Koenig, U. D. and Krebs, D. (1991) Determination of TNF alpha, interferon alpha, interleukin 2 and reactivity in the leucocyte migration inhibition test in breast cancer patients. *Anticancer Res.,* **11**(4), 1509-1515.

Malmberg, S.E. and Adams, C. M. (2008) Insulin signaling and the general amino acid control response. two distinct pathways to amino acid synthesis and uptake. *J. Biol. Chem.,* **283**(28), 19229-19234.

Mantzoros, C., Petridou, E., Dessypris, N., Chavelas, C., Dalamaga, M., Alexe, D. M., Papadiamantis, Y., Markopoulos, C., Spanos, E., Chrousos, G. and Trichopoulos, D. (2004) Adiponectin and breast cancer risk. *J. Clin. Endocrinol. Metab.,* **89**(3), 1102-1107.

Manzella, D., Parillo, M., Razzino, T., Gnasso, P., Buonanno, S., Gargiulo, A., Caputi, M. and Paolisso, G. (2002) Soluble leptin receptor and insulin resistance as determinant of sleep apnea. *Int. J. Obes. Relat. Metab. Disord.,* **26**(3), 370-375.

Margetic, S., Gazzola, C., Pegg, G. G. and Hill, R. A. (2002) Leptin: A review of its peripheral actions and interactions. *Int. J. Obes. Relat. Metab. Disord.*, **26**(11), 1407-1433.

Masuzaki, H., Ogawa, Y., Sagawa, N., Hosoda, K., Matsumoto, T., Mise, H., Nishimura, H., Yoshimasa, Y., Tanaka, I., Mori, T. and Nakao, K. (1997) Nonadipose tissue production of leptin: Leptin as a novel placenta-derived hormone in humans. *Nat. Med.*, **3**(9), 1029-1033.

Mathew, A., George, P. S. and Ildaphonse, G. (2009) Obesity and kidney cancer risk in women: A meta-analysis (1992-2008). *Asian Pac. J. Cancer. Prev.*, **10**(3), 471-478.

Mattar, S.G., Velcu, L. M., Rabinovitz, M., Demetris, A. J., Krasinskas, A. M., Barinas-Mitchell, E., Eid, G. M., Ramanathan, R., Taylor, D. S. and Schauer, P. R. (2005) Surgically-induced weight loss significantly improves nonalcoholic fatty liver disease and the metabolic syndrome. *Ann. Surg.*, **242**(4), 610-7; discussion 618-20.

Mayer, J. (1955) Regulation of energy intake and the body weight: The glucostatic theory and the lipostatic hypothesis. *Ann. N. Y. Acad. Sci.*, **63**(1), 15-43.

McCawley, G.M., Ferriss, J. S., Geffel, D., Northup, C. J. and Modesitt, S. C. (2009) Cancer in obese women: Potential protective impact of bariatric surgery. *J. Am. Coll. Surg.*, **208**(6), 1093-1098.

McCubrey, J.A., Steelman, L. S., Abrams, S. L., Lee, J. T., Chang, F., Bertrand, F. E., Navolanic, P. M., Terrian, D. M., Franklin, R. A., D'Assoro, A. B., Salisbury, J. L., Mazzarino, M. C., Stivala, F. and Libra, M. (2006) Roles of the RAF/MEK/ERK and PI3K/PTEN/AKT pathways in malignant transformation and drug resistance. *Adv. Enzyme Regul.*, **46**, 249-279.

McCubrey, J.A., Steelman, L. S., Chappell, W. H., Abrams, S. L., Wong, E. W., Chang, F., Lehmann, B., Terrian, D. M., Milella, M., Tafuri, A., Stivala, F., Libra, M., Basecke, J., Evangelisti, C., Martelli, A. M. and Franklin, R. A. (2007) Roles of the Raf/MEK/ERK pathway in cell growth, malignant transformation and drug resistance. *Biochim. Biophys. Acta*, **1773**(8), 1263-1284.

McGinnis, J.M. and Foege, W. H. (1993) Actual causes of death in the united states. *JAMA*, **270**(18), 2207-2212.

McTiernan, A. (2005) Obesity and cancer: The risks, science, and potential management strategies. *Oncology (Williston Park)*, **19**(7), 871-81; discussion 881-2, 885-6.

Meier, R. and Hemmings, B. A. (1999) Regulation of protein kinase B. *J. Recept. Signal Transduct. Res.*, **19**(1-4), 121-128.

Mellinkoff, S.M., Frankland, M., Boyle, D. and Greipel, M. (1956) Relationship between serum amino acid concentration and fluctuations in appetite. *J. Appl. Physiol.,* **8**(5), 535-538.

Merajver, S.D., Pham, T. M., Caduff, R. F., Chen, M., Poy, E. L., Cooney, K. A., Weber, B. L., Collins, F. S., Johnston, C. and Frank, T. S. (1995) Somatic mutations in the BRCA1 gene in sporadic ovarian tumours. *Nat. Genet.,* **9**(4), 439-443.

Mercer, J.G. and Speakman, J. R. (2001) Hypothalamic neuropeptide mechanisms for regulating energy balance: From rodent models to human obesity. *Neurosci. Biobehav. Rev.,* **25**(2), 101-116.

Mercer, J.G., Hoggard, N., Williams, L. M., Lawrence, C. B., Hannah, L. T. and Trayhurn, P. (1996a) Localization of leptin receptor mRNA and the long form splice variant (ob-rb) in mouse hypothalamus and adjacent brain regions by in situ hybridization. *FEBS Lett.,* **387**(2-3), 113-116.

Mercer, J.G., Hoggard, N., Williams, L. M., Lawrence, C. B., Hannah, L. T., Morgan, P. J. and Trayhurn, P. (1996b) Coexpression of leptin receptor and preproneuropeptide Y mRNA in arcuate nucleus of mouse hypothalamus. *J. Neuroendocrinol.,* **8**(10), 733-735.

Metcalfe, K.A. (2009) Oophorectomy for breast cancer prevention in women with BRCA1 or BRCA2 mutations. *Womens Health. (Lond. Engl),* **5**(1), 63-68.

Mick, G.J., Wang, X. and McCormick, K. (2002) White adipocyte vascular endothelial growth factor: Regulation by insulin. *Endocrinology,* **143**(3), 948-953.

Milazzo, G., Giorgino, F., Damante, G., Sung, C., Stampfer, M. R., Vigneri, R., Goldfine, I. D. and Belfiore, A. (1992) Insulin receptor expression and function in human breast cancer cell lines. *Cancer Res.,* **52**(14), 3924-3930.

Miyoshi, Y., Funahashi, T., Kihara, S., Taguchi, T., Tamaki, Y., Matsuzawa, Y. and Noguchi, S. (2003) Association of serum adiponectin levels with breast cancer risk. *Clin. Cancer Res.,* **9**(15), 5699-5704.

Mizukami, M., Araki, G., Mihara, H., Tomita, T. and Fujinaga, R. (1972) Arteriographically visualized extravasation in hypertensive intracerebral hemorrhage. report of seven cases. *Stroke,* **3**(5), 527-537.

Moggs, J.G., Murphy, T. C., Lim, F. L., Moore, D. J., Stuckey, R., Antrobus, K., Kimber, I. and Orphanides, G. (2005) Anti-proliferative effect of estrogen in breast cancer cells that re-express ERalpha is mediated by aberrant regulation of cell cycle genes. *J. Mol. Endocrinol.,* **34**(2), 535-551.

Moghaddam, A.A., Woodward, M. and Huxley, R. (2007) Obesity and risk of colorectal cancer: A meta-analysis of 31 studies with 70,000 events. *Cancer Epidemiol. Biomarkers Prev.,* **16**(12), 2533-2547.

Mohamed-Ali, V., Goodrick, S., Rawesh, A., Katz, D. R., Miles, J. M., Yudkin, J. S., Klein, S. and Coppack, S. W. (1997) Subcutaneous adipose tissue releases interleukin-6, but not tumor necrosis factor-alpha, in vivo. *J. Clin. Endocrinol. Metab.,* **82**(12), 4196-4200.

Mokdad, A.H., Marks, J. S., Stroup, D. F. and Gerberding, J. L. (2004) Actual causes of death in the united states, 2000. *JAMA,* **291**(10), 1238-1245.

Molloy, C.A., May, F. E. and Westley, B. R. (2000) Insulin receptor substrate-1 expression is regulated by estrogen in the MCF-7 human breast cancer cell line. *J. Biol. Chem.,* **275**(17), 12565-12571.

Monaco, M.E. and Lippman, M. E. (1977) Insulin stimulation of fatty acid synthesis in human breast cancer in long term tissue culture. *Endocrinology,* **101**(4), 1238-1246.

Montague, C.T., Farooqi, I. S., Whitehead, J. P., Soos, M. A., Rau, H., Wareham, N. J., Sewter, C. P., Digby, J. E., Mohammed, S. N., Hurst, J. A., Cheetham, C. H., Earley, A. R., Barnett, A. H., Prins, J. B. and O'Rahilly, S. (1997) Congenital leptin deficiency is associated with severe early-onset obesity in humans. *Nature,* **387**(6636), 903-908.

Moore, L.L., Bradlee, M. L., Singer, M. R., Splansky, G. L., Proctor, M. H., Ellison, R. C. and Kreger, B. E. (2004) BMI and waist circumference as predictors of lifetime colon cancer risk in framingham study adults. *Int. J. Obes. Relat. Metab. Disord.,* **28**(4), 559-567.

Morelli, C., Garofalo, C., Bartucci, M. and Surmacz, E. (2003) Estrogen receptor-alpha regulates the degradation of insulin receptor substrates 1 and 2 in breast cancer cells. *Oncogene,* **22**(26), 4007-4016.

Moschos, S.J. and Mantzoros, C. S. (2002) The role of the IGF system in cancer: From basic to clinical studies and clinical applications. *Oncology,* **63**(4), 317-332.

Moxham, C.P. and Jacobs, S. (1992) Insulin/IGF-I receptor hybrids: A mechanism for increasing receptor diversity. *J. Cell. Biochem.,* **48**(2), 136-140.

Mueller, H., Flury, N., Liu, R., Scheidegger, S. and Eppenberger, U. (1996) Tumour necrosis factor and interferon are selectively cytostatic in vitro for hormone-dependent and hormone-independent human breast cancer cells. *Eur. J. Cancer,* **32A**(13), 2312-2318.

Murano, I., Barbatelli, G., Parisani, V., Latini, C., Muzzonigro, G., Castellucci, M. and Cinti, S. (2008) Dead adipocytes, detected as crown-like structures, are

prevalent in visceral fat depots of genetically obese mice. *J. Lipid Res.*, **49**(7), 1562-1568.

Nachlas, M.M. (1991) Irrationality in the management of breast cancer. I. the staging system. *Cancer*, **68**(4), 681-690.

Nakatani, K., Thompson, D. A., Barthel, A., Sakaue, H., Liu, W., Weigel, R. J. and Roth, R. A. (1999) Up-regulation of Akt3 in estrogen receptor-deficient breast cancers and androgen-independent prostate cancer lines. *J. Biol. Chem.*, **274**(31), 21528-21532.

Nakayama, S., Miyoshi, Y., Ishihara, H. and Noguchi, S. (2008) Growth-inhibitory effect of adiponectin via adiponectin receptor 1 on human breast cancer cells through inhibition of S-phase entry without inducing apoptosis. *Breast Cancer Res. Treat.*, **112**(3), 405-410.

Nakshatri, H., Bhat-Nakshatri, P., Martin, D. A., Goulet, R. J.,Jr and Sledge, G. W.,Jr (1997) Constitutive activation of NF-kappaB during progression of breast cancer to hormone-independent growth. *Mol. Cell. Biol.*, **17**(7), 3629-3639.

Nandi, A., Kitamura, Y., Kahn, C. R. and Accili, D. (2004) Mouse models of insulin resistance. *Physiol. Rev.*, **84**(2), 623-647.

Neel, J.V. (1962) Diabetes mellitus: A "thrifty" genotype rendered detrimental by "progress"? *Am. J. Hum. Genet.*, **14**, 353-362.

Negrini, M., Sabbioni, S., Haldar, S., Possati, L., Castagnoli, A., Corallini, A., Barbanti-Brodano, G. and Croce, C. M. (1994) Tumor and growth suppression of breast cancer cells by chromosome 17-associated functions. *Cancer Res.*, **54**(7), 1818-1824.

Nelson, L.R. and Bulun, S. E. (2001) Estrogen production and action. *J. Am. Acad. Dermatol.*, **45**(3 Suppl), S116-24.

Neville, M.C., McFadden, T. B. and Forsyth, I. (2002) Hormonal regulation of mammary differentiation and milk secretion. *J. Mammary Gland Biol. Neoplasia*, **7**(1), 49-66.

Nieto-Vazquez, I., Fernandez-Veledo, S., Kramer, D. K., Vila-Bedmar, R., Garcia-Guerra, L. and Lorenzo, M. (2008) Insulin resistance associated to obesity: The link TNF-alpha. *Arch. Physiol. Biochem.*, **114**(3), 183-194.

Nissen, S.E. (2005) Effect of intensive lipid lowering on progression of coronary atherosclerosis: Evidence for an early benefit from the reversal of atherosclerosis with aggressive lipid lowering (REVERSAL) trial. *Am. J. Cardiol.*, **96**(5A), 61F-68F.

Nkhata, K.J., Ray, A., Schuster, T. F., Grossmann, M. E. and Cleary, M. P. (2009) Effects of adiponectin and leptin co-treatment on human breast cancer cell growth. *Oncol. Rep.*, **21**(6), 1611-1619.

Nussey, S.S. and Whitehead, S.A. (2001) Endocrinology: An Integrated Approach, chapter 5: The gonad, London: Taylor & Francis, http://www.ncbi.nlm.nih.gov/bookshelf/br.fcgi?book=endocrin&part=A972, accessed on 6th December 2009.

O'brien, S.N., Welter, B. H. and Price, T. M. (1999) Presence of leptin in breast cell lines and breast tumors. *Biochem. Biophys. Res. Commun.*, **259**(3), 695-698.

Oda, A., Taniguchi, T. and Yokoyama, M. (2001) Leptin stimulates rat aortic smooth muscle cell proliferation and migration. *Kobe J. Med. Sci.*, **47**(3), 141-150.

Ogden, C.L., Carroll, M. D., McDowell, M. A. and Flegal, K. M. (2007) Obesity among adults in the united states--no statistically significant chance since 2003-2004. *NCHS Data Brief*, **(1)**(1), 1-8.

Ogden, C.L., Carroll, M. D., Curtin, L. R., McDowell, M. A., Tabak, C. J. and Flegal, K. M. (2006) Prevalence of overweight and obesity in the united states, 1999-2004. *JAMA*, **295**(13), 1549-1555.

Ogden, J. and Flanagan, Z. (2008) Beliefs about the causes and solutions to obesity: A comparison of GPs and lay people. *Patient Educ. Couns.*, **71**(1), 72-78.

Ogunwobi, O.O. and Beales, I. L. (2007) The anti-apoptotic and growth stimulatory actions of leptin in human colon cancer cells involves activation of JNK mitogen activated protein kinase, JAK2 and PI3 kinase/Akt. *Int. J. Colorectal Dis.*, **22**(4), 401-409.

Okobia, M.N., Bunker, C. H., Garte, S. J., Zmuda, J. M., Ezeome, E. R., Anyanwu, S. N., Uche, E. E., Kuller, L. H., Ferrell, R. E. and Taioli, E. (2008) Leptin receptor Gln223Arg polymorphism and breast cancer risk in nigerian women: A case control study. *BMC Cancer*, **8**, 338.

Okumura, M., Yamamoto, M., Sakuma, H., Kojima, T., Maruyama, T., Jamali, M., Cooper, D. R. and Yasuda, K. (2002) Leptin and high glucose stimulate cell proliferation in MCF-7 human breast cancer cells: Reciprocal involvement of PKC-alpha and PPAR expression. *Biochim. Biophys. Acta*, **1592**(2), 107-116.

Oliveira, C., Velho, S., Domingo, E., Preto, A., Hofstra, R. M., Hamelin, R., Yamamoto, H., Seruca, R. and Schwartz, S.,Jr (2005) Concomitant RASSF1A hypermethylation and KRAS/BRAF mutations occur preferentially in MSI sporadic colorectal cancer. *Oncogene*, **24**(51), 7630-7634.

Olszanecka-Glinianowicz, M., Zahorska-Markiewicz, B., Janowska, J. and Zurakowski, A. (2004) Serum concentrations of nitric oxide, tumor necrosis factor

(TNF)-alpha and TNF soluble receptors in women with overweight and obesity. *Metabolism,* **53**(10), 1268-1273.

O'Malley, D. and Harvey, J. (2004) Insulin activates native and recombinant large conductance ca(2+)-activated potassium channels via a mitogen-activated protein kinase-dependent process. *Mol. Pharmacol.,* **65**(6), 1352-1363.

O'Mara, B.A., Byers, T. and Schoenfeld, E. (1985) Diabetes mellitus and cancer risk: A multisite case-control study. *J. Chronic Dis.,* **38**(5), 435-441.

Osborne, C.K., Monaco, M. E., Lippman, M. E. and Kahn, C. R. (1978) Correlation among insulin binding, degradation, and biological activity in human breast cancer cells in long-term tissue culture. *Cancer Res.,* **38**(1), 94-102.

Osborne, C.K., Bolan, G., Monaco, M. E. and Lippman, M. E. (1976) Hormone responsive human breast cancer in long-term tissue culture: Effect of insulin. *Proc. Natl. Acad. Sci. U. S. A.,* **73**(12), 4536-4540.

Ottonello, L., Gnerre, P., Bertolotto, M., Mancini, M., Dapino, P., Russo, R., Garibotto, G., Barreca, T. and Dallegri, F. (2004) Leptin as a uremic toxin interferes with neutrophil chemotaxis. *J. Am. Soc. Nephrol.,* **15**(9), 2366-2372.

Ozbay, T. and Nahta, R. (2008) A novel unidirectional cross-talk from the insulin-like growth factor-I receptor to leptin receptor in human breast cancer cells. *Mol. Cancer. Res.,* **6**(6), 1052-1058.

Pagliacci, M.C., Fumi, G., Migliorati, G., Grignani, F., Riccardi, C. and Nicoletti, I. (1993) Cytostatic and cytotoxic effects of tumor necrosis factor alpha on MCF-7 human breast tumor cells are differently inhibited by glucocorticoid hormones. *Lymphokine Cytokine Res.,* **12**(6), 439-447.

Pai, R., Lin, C., Tran, T. and Tarnawski, A. (2005) Leptin activates STAT and ERK2 pathways and induces gastric cancer cell proliferation. *Biochem. Biophys. Res. Commun.,* **331**(4), 984-992.

Pan, H. and Djamgoz, M. B. (2008) Biochemical constitution of extracellular medium is critical for control of human breast cancer MDA-MB-231 cell motility. *J. Membr. Biol.,* **223**(1), 27-36.

Pandini, G., Frasca, F., Mineo, R., Sciacca, L., Vigneri, R. and Belfiore, A. (2002) Insulin/insulin-like growth factor I hybrid receptors have different biological characteristics depending on the insulin receptor isoform involved. *J. Biol. Chem.,* **277**(42), 39684-39695.

Pandini, G., Vigneri, R., Costantino, A., Frasca, F., Ippolito, A., Fujita-Yamaguchi, Y., Siddle, K., Goldfine, I. D. and Belfiore, A. (1999) Insulin and insulin-like growth factor-I (IGF-I) receptor overexpression in breast cancers leads to

insulin/IGF-I hybrid receptor overexpression: Evidence for a second mechanism of IGF-I signaling. *Clin. Cancer Res.*, **5**(7), 1935-1944.

Pandolfino, J.E., El-Serag, H. B., Zhang, Q., Shah, N., Ghosh, S. K. and Kahrilas, P. J. (2006) Obesity: A challenge to esophagogastric junction integrity. *Gastroenterology*, **130**(3), 639-649.

Panno, M.L., Salerno, M., Pezzi, V., Sisci, D., Maggiolini, M., Mauro, L., Morrone, E. G. and Ando, S. (1996) Effect of oestradiol and insulin on the proliferative pattern and on oestrogen and progesterone receptor contents in MCF-7 cells. *J. Cancer Res. Clin. Oncol.*, **122**(12), 745-749.

Papa, V., Milazzo, G., Goldfine, I. D., Waldman, F. M. and Vigneri, R. (1997) Sporadic amplification of the insulin receptor gene in human breast cancer. *J. Endocrinol. Invest.*, **20**(9), 531-536.

Papa, V., Gliozzo, B., Clark, G. M., McGuire, W. L., Moore, D., Fujita-Yamaguchi, Y., Vigneri, R., Goldfine, I. D. and Pezzino, V. (1993) Insulin-like growth factor-I receptors are overexpressed and predict a low risk in human breast cancer. *Cancer Res.*, **53**(16), 3736-3740.

Papa, V., Pezzino, V., Costantino, A., Belfiore, A., Giuffrida, D., Frittitta, L., Vannelli, G. B., Brand, R., Goldfine, I. D. and Vigneri, R. (1990) Elevated insulin receptor content in human breast cancer. *J. Clin. Invest.*, **86**(5), 1503-1510.

Parisot, J.P., Hu, X. F., DeLuise, M. and Zalcberg, J. R. (1999) Altered expression of the IGF-1 receptor in a tamoxifen-resistant human breast cancer cell line. *Br. J. Cancer*, **79**(5-6), 693-700.

Park, H.S., Park, J. Y. and Yu, R. (2005) Relationship of obesity and visceral adiposity with serum concentrations of CRP, TNF-alpha and IL-6. *Diabetes Res. Clin. Pract.*, **69**(1), 29-35.

Park, S.H., Choi, S. J., Lee, K. S. and Park, H. Y. (2009) Waist circumference and waist-to-height ratio as predictors of cardiovascular disease risk in korean adults. *Circ. J.*, **73**(9), 1643-1650.

Pasarica, M., Sereda, O. R., Redman, L. M., Albarado, D. C., Hymel, D. T., Roan, L. E., Rood, J. C., Burk, D. H. and Smith, S. R. (2009) Reduced adipose tissue oxygenation in human obesity: Evidence for rarefaction, macrophage chemotaxis, and inflammation without an angiogenic response. *Diabetes*, **58**(3), 718-725.

Pascual, D. and Borque, A. (2008) Epidemiology of kidney cancer. *Adv. Urol. 2008:782381 Epub*, , 782381.

Pasquali, R., Casimirri, F. and Vicennati, V. (1997) Weight control and its beneficial effect on fertility in women with obesity and polycystic ovary syndrome. *Hum. Reprod.*, **12 Suppl 1**, 82-87.

Payne, P.R. and Dugdale, A. E. (1977) A model for the prediction of energy balance and body weight. *Ann. Hum. Biol.,* **4**(6), 525-535.

Pearson, G., Robinson, F., Beers Gibson, T., Xu, B. E., Karandikar, M., Berman, K. and Cobb, M. H. (2001) Mitogen-activated protein (MAP) kinase pathways: Regulation and physiological functions. *Endocr. Rev.,* **22**(2), 153-183.

Pearson, O.H., Manni, A. and Arafah, B. M. (1982) Antiestrogen treatment of breast cancer: An overview. *Cancer Res.,* **42**(8 Suppl), 3424s-3429s.

Pechlivanis, S., Bermejo, J. L., Pardini, B., Naccarati, A., Vodickova, L., Novotny, J., Hemminki, K., Vodicka, P. and Forsti, A. (2009) Genetic variation in adipokine genes and risk of colorectal cancer. *Eur. J. Endocrinol.,* **160**(6), 933-940.

Pedersen, B.K., Steensberg, A., Keller, P., Keller, C., Fischer, C., Hiscock, N., van Hall, G., Plomgaard, P. and Febbraio, M. A. (2003) Muscle-derived interleukin-6: Lipolytic, anti-inflammatory and immune regulatory effects. *Pflugers Arch.,* **446**(1), 9-16.

Pennica, D., Nedwin, G. E., Hayflick, J. S., Seeburg, P. H., Derynck, R., Palladino, M. A., Kohr, W. J., Aggarwal, B. B. and Goeddel, D. V. (1984) Human tumour necrosis factor: Precursor structure, expression and homology to lymphotoxin. *Nature,* **312**(5996), 724-729.

Pera, M., Manterola, C., Vidal, O. and Grande, L. (2005) Epidemiology of esophageal adenocarcinoma. *J. Surg. Oncol.,* **92**(3), 151-159.

Perera, C.N., Chin, H. G., Duru, N. and Camarillo, I. G. (2008) Leptin-regulated gene expression in MCF-7 breast cancer cells: Mechanistic insights into leptin-regulated mammary tumor growth and progression. *J. Endocrinol.,* **199**(2), 221-233.

Pessin, J.E. and Saltiel, A. R. (2000) Signaling pathways in insulin action: Molecular targets of insulin resistance. *J. Clin. Invest.,* **106**(2), 165-169.

Peters, T., Ausmeier, K., Dildrop, R. and Ruther, U. (2002) The mouse fused toes (ft) mutation is the result of a 1.6-mb deletion including the entire iroquois B gene cluster. *Mamm. Genome,* **13**(4), 186-188.

Petridou, E., Papadiamantis, Y., Markopoulos, C., Spanos, E., Dessypris, N. and Trichopoulos, D. (2000) Leptin and insulin growth factor I in relation to breast cancer (greece). *Cancer Causes Control,* **11**(5), 383-388.

Pfeiler, G.H., Buechler, C., Neumeier, M., Schaffler, A., Schmitz, G., Ortmann, O. and Treeck, O. (2008) Adiponectin effects on human breast cancer cells are dependent on 17-beta estradiol. *Oncol. Rep.,* **19**(3), 787-793.

Phillips, L.H., Whisnant, J. P. and Reagan, T. J. (1977) Sudden death from stroke. *Stroke,* **8**(3), 392-395.

Pirola, L., Johnston, A. M. and Van Obberghen, E. (2004) Modulation of insulin action. *Diabetologia,* **47**(2), 170-184.

Pisani, P. (2008) Hyper-insulinaemia and cancer, meta-analyses of epidemiological studies. *Arch. Physiol. Biochem.,* **114**(1), 63-70.

Pischon, T., Lahmann, P. H., Boeing, H., Friedenreich, C., Norat, T., Tjonneland, A., Halkjaer, J., Overvad, K., Clavel-Chapelon, F., Boutron-Ruault, M. C., Guernec, G., Bergmann, M. M., Linseisen, J., Becker, N., Trichopoulou, A., Trichopoulos, D., Sieri, S., Palli, D., Tumino, R., Vineis, P., Panico, S., Peeters, P. H., Bueno-de-Mesquita, H. B., Boshuizen, H. C., Van Guelpen, B., Palmqvist, R., Berglund, G., Gonzalez, C. A., Dorronsoro, M., Barricarte, A., Navarro, C., Martinez, C., Quiros, J. R., Roddam, A., Allen, N., Bingham, S., Khaw, K. T., Ferrari, P., Kaaks, R., Slimani, N. and Riboli, E. (2006) Body size and risk of colon and rectal cancer in the european prospective investigation into cancer and nutrition (EPIC). *J. Natl. Cancer Inst.,* **98**(13), 920-931.

Pitt, H.A. (2007) Hepato-pancreato-biliary fat: The good, the bad and the ugly. *HPB (Oxford),* **9**(2), 92-97.

Platet, N., Cathiard, A. M., Gleizes, M. and Garcia, M. (2004) Estrogens and their receptors in breast cancer progression: A dual role in cancer proliferation and invasion. *Crit. Rev. Oncol. Hematol.,* **51**(1), 55-67.

Pliquett, R.U., Fuhrer, D., Falk, S., Zysset, S., von Cramon, D. Y. and Stumvoll, M. (2006) The effects of insulin on the central nervous system--focus on appetite regulation. *Horm. Metab. Res.,* **38**(7), 442-446.

Pondugula, K., Wani, S. and Sharma, P. (2007) Barrett's esophagus and esophageal adenocarcinoma in adults: Long-term GERD or something else? *Curr. Gastroenterol. Rep.,* **9**(6), 468-474.

Poston, W.S.,2nd and Foreyt, J. P. (1999) Obesity is an environmental issue. *Atherosclerosis,* **146**(2), 201-209.

Potischman, N., Hoover, R. N., Brinton, L. A., Siiteri, P., Dorgan, J. F., Swanson, C. A., Berman, M. L., Mortel, R., Twiggs, L. B., Barrett, R. J., Wilbanks, G. D., Persky, V. and Lurain, J. R. (1996) Case-control study of endogenous steroid hormones and endometrial cancer. *J. Natl. Cancer Inst.,* **88**(16), 1127-1135.

Prentice, A.M. (2005) Starvation in humans: Evolutionary background and contemporary implications. *Mech. Ageing Dev.,* **126**(9), 976-981.

Prentice, A.M. (2001) Obesity and its potential mechanistic basis. *Br. Med. Bull.,* **60**, 51-67.

Prentice, A.M., Hennig, B. J. and Fulford, A. J. (2008) Evolutionary origins of the obesity epidemic: Natural selection of thrifty genes or genetic drift following predation release? *Int. J. Obes. (Lond),* **32**(11), 1607-1610.

Pronk, G.J., Medema, R. H., Burgering, B. M., Clark, R., McCormick, F. and Bos, J. L. (1992) Interaction between the p21ras GTPase activating protein and the insulin receptor. *J. Biol. Chem.,* **267**(33), 24058-24063.

Punjabi, N.M., Sorkin, J. D., Katzel, L. I., Goldberg, A. P., Schwartz, A. R. and Smith, P. L. (2002) Sleep-disordered breathing and insulin resistance in middle-aged and overweight men. *Am. J. Respir. Crit. Care Med.,* **165**(5), 677-682.

Purohit, A. and Reed, M. J. (2002) Regulation of estrogen synthesis in postmenopausal women. *Steroids,* **67**(12), 979-983.

Purohit, A., Newman, S. P. and Reed, M. J. (2002) The role of cytokines in regulating estrogen synthesis: Implications for the etiology of breast cancer. *Breast Cancer Res.,* **4**(2), 65-69.

Pusztai, L., Lewis, C. E. and McGee, J. O. (1993) Growth arrest of the breast cancer cell line, T47D, by TNF alpha; cell cycle specificity and signal transduction. *Br. J. Cancer,* **67**(2), 290-296.

Putnam, J.J. and Allshouse, J.E. (1999) Food Consumption, Prices, and Expenditures, 1970-97, Food and Rural Economics Division, Economic Research Service, U.S. Department of Agriculture. Statistical Bulletin No. 965, http://www.ers.usda.gov/publications/sb965/sb965.pdf, accessed on 17th November 2009.

Qiao, L.Y., Goldberg, J. L., Russell, J. C. and Sun, X. J. (1999) Identification of enhanced serine kinase activity in insulin resistance. *J. Biol. Chem.,* **274**(15), 10625-10632.

Qiao, L.Y., Zhande, R., Jetton, T. L., Zhou, G. and Sun, X. J. (2002) In vivo phosphorylation of insulin receptor substrate 1 at serine 789 by a novel serine kinase in insulin-resistant rodents. *J. Biol. Chem.,* **277**(29), 26530-26539.

Raman, M. and Allard, J. (2006) Non alcoholic fatty liver disease: A clinical approach and review. *Can. J. Gastroenterol.,* **20**(5), 345-349.

Rausch, M.E., Weisberg, S., Vardhana, P. and Tortoriello, D. V. (2008) Obesity in C57BL/6J mice is characterized by adipose tissue hypoxia and cytotoxic T-cell infiltration. *Int. J. Obes. (Lond),* **32**(3), 451-463.

Ravussin, E., Valencia, M. E., Esparza, J., Bennett, P. H. and Schulz, L. O. (1994) Effects of a traditional lifestyle on obesity in pima indians. *Diabetes Care,* **17**(9), 1067-1074.

Ray, A., Nkhata, K. J. and Cleary, M. P. (2007) Effects of leptin on human breast cancer cell lines in relationship to estrogen receptor and HER2 status. *Int. J. Oncol.,* **30**(6), 1499-1509.

Reaven, G.M. (2006) The metabolic syndrome: Is this diagnosis necessary? *Am. J. Clin. Nutr.,* **83**(6), 1237-1247.

Reaven, G.M. (2005) The metabolic syndrome: Requiescat in pace. *Clin. Chem.,* **51**(6), 931-938.

Reaven, G.M. (1993) Role of insulin resistance in human disease (syndrome X): An expanded definition. *Annu. Rev. Med.,* **44**, 121-131.

Reaven, G.M. (1988) Banting lecture 1988. role of insulin resistance in human disease. *Diabetes,* **37**(12), 1595-1607.

Reddy, K.B., Nabha, S. M. and Atanaskova, N. (2003) Role of MAP kinase in tumor progression and invasion. *Cancer Metastasis Rev.,* **22**(4), 395-403.

Renehan, A.G., Frystyk, J. and Flyvbjerg, A. (2006) Obesity and cancer risk: The role of the insulin-IGF axis. *Trends Endocrinol. Metab.,* **17**(8), 328-336.

Renehan, A.G., Zwahlen, M., Minder, C., O'Dwyer, S. T., Shalet, S. M. and Egger, M. (2004) Insulin-like growth factor (IGF)-I, IGF binding protein-3, and cancer risk: Systematic review and meta-regression analysis. *Lancet,* **363**(9418), 1346-1353.

Revillion, F., Charlier, M., Lhotellier, V., Hornez, L., Giard, S., Baranzelli, M. C., Djiane, J. and Peyrat, J. P. (2006) Messenger RNA expression of leptin and leptin receptors and their prognostic value in 322 human primary breast cancers. *Clin. Cancer Res.,* **12**(7 Pt 1), 2088-2094.

Rinaldi, S., Peeters, P. H., Berrino, F., Dossus, L., Biessy, C., Olsen, A., Tjonneland, A., Overvad, K., Clavel-Chapelon, F., Boutron-Ruault, M. C., Tehard, B., Nagel, G., Linseisen, J., Boeing, H., Lahmann, P. H., Trichopoulou, A., Trichopoulos, D., Koliva, M., Palli, D., Panico, S., Tumino, R., Sacerdote, C., van Gils, C. H., van Noord, P., Grobbee, D. E., Bueno-de-Mesquita, H. B., Gonzalez, C. A., Agudo, A., Chirlaque, M. D., Barricarte, A., Larranaga, N., Quiros, J. R., Bingham, S., Khaw, K. T., Key, T., Allen, N. E., Lukanova, A., Slimani, N., Saracci, R., Riboli, E. and Kaaks, R. (2006) IGF-I, IGFBP-3 and breast cancer risk in women: The european prospective investigation into cancer and nutrition (EPIC). *Endocr. Relat. Cancer,* **13**(2), 593-605.

Rivas, M.A., Tkach, M., Beguelin, W., Proietti, C. J., Rosemblit, C., Charreau, E. H., Elizalde, P. V. and Schillaci, R. (2009) Transactivation of ErbB-2 induced by tumor necrosis factor alpha promotes NF-kappaB activation and breast cancer cell proliferation. *Breast Cancer Res. Treat.,* **122**(1), 111-124.

Rivas, M.A., Carnevale, R. P., Proietti, C. J., Rosemblit, C., Beguelin, W., Salatino, M., Charreau, E. H., Frahm, I., Sapia, S., Brouckaert, P., Elizalde, P. V. and Schillaci, R. (2008) TNF alpha acting on TNFR1 promotes breast cancer growth via p42/P44 MAPK, JNK, akt and NF-kappa B-dependent pathways. *Exp. Cell Res.*, **314**(3), 509-529.

Rivera, C.A. (2008) Risk factors and mechanisms of non-alcoholic steatohepatitis. *Pathophysiology*, **15**(2), 109-114.

Robertson, J.F. (2001) Faslodex (ICI 182, 780), a novel estrogen receptor downregulator--future possibilities in breast cancer. *J. Steroid Biochem. Mol. Biol.*, **79**(1-5), 209-212.

Rodriguez-Viciana, P., Warne, P. H., Dhand, R., Vanhaesebroeck, B., Gout, I., Fry, M. J., Waterfield, M. D. and Downward, J. (1994) Phosphatidylinositol-3-OH kinase as a direct target of ras. *Nature*, **370**(6490), 527-532.

Rollison, D.E., Newschaffer, C. J., Tao, Y., Pollak, M. and Helzlsouer, K. J. (2006) Premenopausal levels of circulating insulin-like growth factor I and the risk of postmenopausal breast cancer. *Int. J. Cancer*, **118**(5), 1279-1284.

Rose, D.P. and Vona-Davis, L. (2009) Influence of obesity on breast cancer receptor status and prognosis. *Expert Rev. Anticancer Ther.*, **9**(8), 1091-1101.

Rose, D.P., Komninou, D. and Stephenson, G. D. (2004) Obesity, adipocytokines, and insulin resistance in breast cancer. *Obes. Rev.*, **5**(3), 153-165.

Rosen, N., Yee, D., Lippman, M. E., Paik, S. and Cullen, K. J. (1991) Insulin-like growth factors in human breast cancer. *Breast Cancer Res. Treat.*, **18 Suppl 1**, S55-62.

Rotwein, P. (1991) Structure, evolution, expression and regulation of insulin-like growth factors I and II. *Growth Factors*, **5**(1), 3-18.

Rozen, F., Zhang, J. and Pollak, M. (1998) Antiproliferative action of tumor necrosis factor-alpha on MCF-7 breastcancer cells is associated with increased insulin-like growth factor binding protein-3 accumulation. *Int. J. Oncol.*, **13**(4), 865-869.

Ruderman, N.B., Keller, C., Richard, A. M., Saha, A. K., Luo, Z., Xiang, X., Giralt, M., Ritov, V. B., Menshikova, E. V., Kelley, D. E., Hidalgo, J., Pedersen, B. K. and Kelly, M. (2006) Interleukin-6 regulation of AMP-activated protein kinase. potential role in the systemic response to exercise and prevention of the metabolic syndrome. *Diabetes*, **55 Suppl 2**, S48-54.

SABiosciences, Frederick, MD, USA, Oligo GEArray System, The Pathway-Specific Microarray Gene Expression Profiling System (Part # 1018A, Version 3.2, 20th October 2006), http://sabiosciences.com/Manual/Oligo_Manual.pdf, accessed on 11th September 2009.

SABiosciences, Frederick, MD, USA, TrueLabeling-AMP 2.0 Kit User Manual, http://sabiosciences.com/Manual/TrueLabelAMP.pdf, accessed on 11th September 2009.

Sachdev, D. and Yee, D. (2007) Disrupting insulin-like growth factor signaling as a potential cancer therapy. *Mol. Cancer. Ther.,* **6**(1), 1-12.

Sachdev, D. and Yee, D. (2001) The IGF system and breast cancer. *Endocr. Relat. Cancer,* **8**(3), 197-209.

Sachdev, D., Li, S. L., Hartell, J. S., Fujita-Yamaguchi, Y., Miller, J. S. and Yee, D. (2003) A chimeric humanized single-chain antibody against the type I insulin-like growth factor (IGF) receptor renders breast cancer cells refractory to the mitogenic effects of IGF-I. *Cancer Res.,* **63**(3), 627-635.

Safe, S. (2001) Transcriptional activation of genes by 17 beta-estradiol through estrogen receptor-Sp1 interactions. *Vitam. Horm.,* **62**, 231-252.

Saha, A., Bairwa, N. K., Ranjan, A., Gupta, V. and Bamezai, R. (2003) Two novel somatic mutations in the human interleukin 6 promoter region in a patient with sporadic breast cancer. *Eur. J. Immunogenet.,* **30**(6), 397-400.

Salgado, R., Junius, S., Benoy, I., Van Dam, P., Vermeulen, P., Van Marck, E., Huget, P. and Dirix, L. Y. (2003) Circulating interleukin-6 predicts survival in patients with metastatic breast cancer. *Int. J. Cancer,* **103**(5), 642-646.

Saltiel, A.R. and Kahn, C. R. (2001) Insulin signalling and the regulation of glucose and lipid metabolism. *Nature,* **414**(6865), 799-806.

Samaras, K., Botelho, N. K., Chisholm, D. J. and Lord, R. V. (2010) Subcutaneous and visceral adipose tissue gene expression of serum adipokines that predict type 2 diabetes. *Obesity (Silver Spring),* **18**(5), 884-889.

Sanchez, I. and Dynlacht, B. D. (2005) New insights into cyclins, CDKs, and cell cycle control. *Semin. Cell Dev. Biol.,* **16**(3), 311-321.

Sansone, P., Storci, G., Tavolari, S., Guarnieri, T., Giovannini, C., Taffurelli, M., Ceccarelli, C., Santini, D., Paterini, P., Marcu, K. B., Chieco, P. and Bonafe, M. (2007) IL-6 triggers malignant features in mammospheres from human ductal breast carcinoma and normal mammary gland. *J. Clin. Invest.,* **117**(12), 3988-4002.

Santen, R.J., Song, R. X., McPherson, R., Kumar, R., Adam, L., Jeng, M. H. and Yue, W. (2002) The role of mitogen-activated protein (MAP) kinase in breast cancer. *J. Steroid Biochem. Mol. Biol.,* **80**(2), 239-256.

Sato, N., Kamino, K., Tateishi, K., Satoh, T., Nishiwaki, Y., Yoshiiwa, A., Miki, T. and Ogihara, T. (1997) Elevated amyloid beta protein(1-40) level induces CREB

phosphorylation at serine-133 via p44/42 MAP kinase (Erk1/2)-dependent pathway in rat pheochromocytoma PC12 cells. *Biochem. Biophys. Res. Commun.*, **232**(3), 637-642.

Saxena, N.K., Sharma, D., Ding, X., Lin, S., Marra, F., Merlin, D. and Anania, F. A. (2007a) Concomitant activation of the JAK/STAT, PI3K/AKT, and ERK signaling is involved in leptin-mediated promotion of invasion and migration of hepatocellular carcinoma cells. *Cancer Res.*, **67**(6), 2497-2507.

Saxena, N.K., Taliaferro-Smith, L., Knight, B. B., Merlin, D., Anania, F. A., O'Regan, R. M. and Sharma, D. (2008) Bidirectional crosstalk between leptin and insulin-like growth factor-I signaling promotes invasion and migration of breast cancer cells via transactivation of epidermal growth factor receptor. *Cancer Res.*, **68**(23), 9712-9722.

Saxena, N.K., Vertino, P. M., Anania, F. A. and Sharma, D. (2007b) Leptin-induced growth stimulation of breast cancer cells involves recruitment of histone acetyltransferases and mediator complex to CYCLIN D1 promoter via activation of Stat3. *J. Biol. Chem.*, **282**(18), 13316-13325.

Saye, J.A., Cassis, L. A., Sturgill, T. W., Lynch, K. R. and Peach, M. J. (1989) Angiotensinogen gene expression in 3T3-L1 cells. *Am. J. Physiol.*, **256**(2 Pt 1), C448-51.

Schaefer, E.J. and Asztalos, B. F. (2007) Increasing high-density lipoprotein cholesterol, inhibition of cholesteryl ester transfer protein, and heart disease risk reduction. *Am. J. Cardiol.*, **100**(11 A), n25-31.

Scheen, A.J. and Luyckx, F. H. (2002) Obesity and liver disease. *Best Pract. Res. Clin. Endocrinol. Metab.*, **16**(4), 703-716.

Scheid, M.P. and Woodgett, J. R. (2001a) Phosphatidylinositol 3' kinase signaling in mammary tumorigenesis. *J. Mammary Gland Biol. Neoplasia*, **6**(1), 83-99.

Scheid, M.P. and Woodgett, J. R. (2001b) PKB/AKT: Functional insights from genetic models. *Nat. Rev. Mol. Cell Biol.*, **2**(10), 760-768.

Scherer, P.E., Williams, S., Fogliano, M., Baldini, G. and Lodish, H. F. (1995) A novel serum protein similar to C1q, produced exclusively in adipocytes. *J. Biol. Chem.*, **270**(45), 26746-26749.

Schernhammer, E.S., Holly, J. M., Hunter, D. J., Pollak, M. N. and Hankinson, S. E. (2006) Insulin-like growth factor-I, its binding proteins (IGFBP-1 and IGFBP-3), and growth hormone and breast cancer risk in the nurses health study II. *Endocr. Relat. Cancer*, **13**(2), 583-592.

Schrauwen-Hinderling, V.B., van Loon, L. J., Koopman, R., Nicolay, K., Saris, W. H. and Kooi, M. E. (2003) Intramyocellular lipid content is increased after

exercise in nonexercising human skeletal muscle. *J. Appl. Physiol.,* **95**(6), 2328-2332.

Schuyer, M. and Berns, E. M. (1999) Is TP53 dysfunction required for BRCA1-associated carcinogenesis? *Mol. Cell. Endocrinol.,* **155**(1-2), 143-152.

Schwartz, G.J. (2000) The role of gastrointestinal vagal afferents in the control of food intake: Current prospects. *Nutrition,* **16**(10), 866-873.

Schwartz, M.W. and Seeley, R. J. (1997) The new biology of body weight regulation. *J. Am. Diet. Assoc.,* **97**(1), 54-8; quiz 59-60.

Schwartz, M.W., Sipols, A. J., Marks, J. L., Sanacora, G., White, J. D., Scheurink, A., Kahn, S. E., Baskin, D. G., Woods, S. C. and Figlewicz, D. P. (1992) Inhibition of hypothalamic neuropeptide Y gene expression by insulin. *Endocrinology,* **130**(6), 3608-3616.

Semenza, G.L. (1998) Hypoxia-inducible factor 1: Master regulator of O2 homeostasis. *Curr. Opin. Genet. Dev.,* **8**(5), 588-594.

Semenza, G.L. and Wang, G. L. (1992) A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. *Mol. Cell. Biol.,* **12**(12), 5447-5454.

Senn, J.J., Klover, P. J., Nowak, I. A., Zimmers, T. A., Koniaris, L. G., Furlanetto, R. W. and Mooney, R. A. (2003) Suppressor of cytokine signaling-3 (SOCS-3), a potential mediator of interleukin-6-dependent insulin resistance in hepatocytes. *J. Biol. Chem.,* **278**(16), 13740-13746.

Shaheen, N.J. and Richter, J. E. (2009) Barrett's oesophagus. *Lancet,* **373**(9666), 850-861.

Shapiro, J.A., Williams, M. A. and Weiss, N. S. (1999) Body mass index and risk of renal cell carcinoma. *Epidemiology,* **10**(2), 188-191.

Sharma, D., Saxena, N. K., Vertino, P. M. and Anania, F. A. (2006) Leptin promotes the proliferative response and invasiveness in human endometrial cancer cells by activating multiple signal-transduction pathways. *Endocr. Relat. Cancer,* **13**(2), 629-640.

Sheen-Chen, S.M., Chen, W. J., Eng, H. L. and Chou, F. F. (1997) Serum concentration of tumor necrosis factor in patients with breast cancer. *Breast Cancer Res. Treat.,* **43**(3), 211-215.

Shepherd, P.R. and Kahn, B. B. (1999) Glucose transporters and insulin action--implications for insulin resistance and diabetes mellitus. *N. Engl. J. Med.,* **341**(4), 248-257.

Shi, R., Yu, H., McLarty, J. and Glass, J. (2004) IGF-I and breast cancer: A meta-analysis. *Int. J. Cancer*, **111**(3), 418-423.

Shimizu, C., Hasegawa, T., Tani, Y., Takahashi, F., Takeuchi, M., Watanabe, T., Ando, M., Katsumata, N. and Fujiwara, Y. (2004) Expression of insulin-like growth factor 1 receptor in primary breast cancer: Immunohistochemical analysis. *Hum. Pathol.*, **35**(12), 1537-1542.

Shirai, T., Yamaguchi, H., Ito, H., Todd, C. W. and Wallace, R. B. (1985) Cloning and expression in escherichia coli of the gene for human tumour necrosis factor. *Nature*, **313**(6005), 803-806.

Shulman, G.I. (2000) Cellular mechanisms of insulin resistance. *J. Clin. Invest.*, **106**(2), 171-176.

Sierra-Honigmann, M.R., Nath, A. K., Murakami, C., Garcia-Cardena, G., Papapetropoulos, A., Sessa, W. C., Madge, L. A., Schechner, J. S., Schwabb, M. B., Polverini, P. J. and Flores-Riveros, J. R. (1998) Biological action of leptin as an angiogenic factor. *Science*, **281**(5383), 1683-1686.

Siiteri, P.K. (1978) Steroid hormones and endometrial cancer. *Cancer Res.*, **38**(11 Pt 2), 4360-4366.

Simon, M.A., Dodson, G. S. and Rubin, G. M. (1993) An SH3-SH2-SH3 protein is required for p21Ras1 activation and binds to sevenless and sos proteins in vitro. *Cell*, **73**(1), 169-177.

Simpson, L. and Parsons, R. (2001) PTEN: Life as a tumor suppressor. *Exp. Cell Res.*, **264**(1), 29-41.

Sisci, D., Morelli, C., Cascio, S., Lanzino, M., Garofalo, C., Reiss, K., Garcia, M., Russo, A., Ando, S. and Surmacz, E. (2007) The estrogen receptor alpha:Insulin receptor substrate 1 complex in breast cancer: Structure-function relationships. *Ann. Oncol.*, **18 Suppl 6**, vi81-5.

Skolnik, E.Y., Lee, C. H., Batzer, A., Vicentini, L. M., Zhou, M., Daly, R., Myers, M. J.,Jr, Backer, J. M., Ullrich, A., White, M. F. and Schlessinger, J. (1993) The SH2/SH3 domain-containing protein GRB2 interacts with tyrosine-phosphorylated IRS1 and shc: Implications for insulin control of ras signalling. *EMBO J.*, **12**(5), 1929-1936.

Skurk, T., Alberti-Huber, C., Herder, C. and Hauner, H. (2007) Relationship between adipocyte size and adipokine expression and secretion. *J. Clin. Endocrinol. Metab.*, **92**(3), 1023-1033.

Skurk, T., Herder, C., Kraft, I., Muller-Scholze, S., Hauner, H. and Kolb, H. (2005) Production and release of macrophage migration inhibitory factor from human adipocytes. *Endocrinology*, **146**(3), 1006-1011.

Slattery, M.L., Curtin, K., Baumgartner, R., Sweeney, C., Byers, T., Giuliano, A. R., Baumgartner, K. B. and Wolff, R. R. (2007) IL6, aspirin, nonsteroidal anti-inflammatory drugs, and breast cancer risk in women living in the southwestern united states. *Cancer Epidemiol. Biomarkers Prev.*, **16**(4), 747-755.

Smith, S.C.,Jr and Haslam, D. (2007) Abdominal obesity, waist circumference and cardio-metabolic risk: Awareness among primary care physicians, the general population and patients at risk--the shape of the nations survey. *Curr. Med. Res. Opin.*, **23**(1), 29-47.

Snoussi, K., Strosberg, A. D., Bouaouina, N., Ben Ahmed, S., Helal, A. N. and Chouchane, L. (2006) Leptin and leptin receptor polymorphisms are associated with increased risk and poor prognosis of breast carcinoma. *BMC Cancer*, **6**, 38.

Soma, D., Kitayama, J., Yamashita, H., Miyato, H., Ishikawa, M. and Nagawa, H. (2008) Leptin augments proliferation of breast cancer cells via transactivation of HER2. *J. Surg. Res.*, **149**(1), 9-14.

Somasundar, P., Yu, A. K., Vona-Davis, L. and McFadden, D. W. (2003) Differential effects of leptin on cancer in vitro. *J. Surg. Res.*, **113**(1), 50-55.

Soos, M.A., Whittaker, J., Lammers, R., Ullrich, A. and Siddle, K. (1990) Receptors for insulin and insulin-like growth factor-I can form hybrid dimers. characterisation of hybrid receptors in transfected cells. *Biochem. J.*, **270**(2), 383-390.

Soule, H.D., Maloney, T. M., Wolman, S. R., Peterson, W. D.,Jr, Brenz, R., McGrath, C. M., Russo, J., Pauley, R. J., Jones, R. F. and Brooks, S. C. (1990) Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10. *Cancer Res.*, **50**(18), 6075-6086.

Spalding, K.L., Arner, E., Westermark, P. O., Bernard, S., Buchholz, B. A., Bergmann, O., Blomqvist, L., Hoffstedt, J., Naslund, E., Britton, T., Concha, H., Hassan, M., Ryden, M., Frisen, J. and Arner, P. (2008) Dynamics of fat cell turnover in humans. *Nature*, **453**(7196), 783-787.

Speakman, J.R. (2008) Thrifty genes for obesity, an attractive but flawed idea, and an alternative perspective: The 'drifty gene' hypothesis. *Int. J. Obes. (Lond)*, **32**(11), 1611-1617.

Speakman, J.R. (2007) A nonadaptive scenario explaining the genetic predisposition to obesity: The "predation release" hypothesis. *Cell. Metab.*, **6**(1), 5-12.

Speakman, J.R. (2004) Obesity: The integrated roles of environment and genetics. *J. Nutr.*, **134**(8 Suppl), 2090S-2105S.

Speakman, J.R., Stubbs, R. J. and Mercer, J. G. (2002) Does body mass play a role in the regulation of food intake? *Proc. Nutr. Soc.*, **61**(4), 473-487.

Staal, S.P. (1987) Molecular cloning of the akt oncogene and its human homologues AKT1 and AKT2: Amplification of AKT1 in a primary human gastric adenocarcinoma. *Proc. Natl. Acad. Sci. U. S. A.,* **84**(14), 5034-5037.

Stacey, D.W. (2003) Cyclin D1 serves as a cell cycle regulatory switch in actively proliferating cells. *Curr. Opin. Cell Biol.,* **15**(2), 158-163.

Starr, R., Willson, T. A., Viney, E. M., Murray, L. J., Rayner, J. R., Jenkins, B. J., Gonda, T. J., Alexander, W. S., Metcalf, D., Nicola, N. A. and Hilton, D. J. (1997) A family of cytokine-inducible inhibitors of signalling. *Nature,* **387**(6636), 917-921.

Stattin, P., Soderberg, S., Biessy, C., Lenner, P., Hallmans, G., Kaaks, R. and Olsson, T. (2004) Plasma leptin and breast cancer risk: A prospective study in northern sweden. *Breast Cancer Res. Treat.,* **86**(3), 191-196.

Stefan, N., Machann, J., Schick, F., Claussen, C. D., Thamer, C., Fritsche, A. and Haring, H. U. (2005) New imaging techniques of fat, muscle and liver within the context of determining insulin sensitivity. *Horm. Res.,* **64 Suppl 3**, 38-44.

Steinberg, G.R. (2007) Inflammation in obesity is the common link between defects in fatty acid metabolism and insulin resistance. *Cell. Cycle,* **6**(8), 888-894.

Stephenson, G.D. and Rose, D. P. (2003) Breast cancer and obesity: An update. *Nutr. Cancer,* **45**(1), 1-16.

Steppan, C.M., Bailey, S. T., Bhat, S., Brown, E. J., Banerjee, R. R., Wright, C. M., Patel, H. R., Ahima, R. S. and Lazar, M. A. (2001) The hormone resistin links obesity to diabetes. *Nature,* **409**(6818), 307-312.

Stevens, J., Couper, D., Pankow, J., Folsom, A. R., Duncan, B. B., Nieto, F. J., Jones, D. and Tyroler, H. A. (2001) Sensitivity and specificity of anthropometrics for the prediction of diabetes in a biracial cohort. *Obes. Res.,* **9**(11), 696-705.

Stirewalt, D.L., Kopecky, K. J., Meshinchi, S., Appelbaum, F. R., Slovak, M. L., Willman, C. L. and Radich, J. P. (2001) FLT3, RAS, and TP53 mutations in elderly patients with acute myeloid leukemia. *Blood,* **97**(11), 3589-3595.

Straus, D.S. (1984) Growth-stimulatory actions of insulin in vitro and in vivo. *Endocr. Rev.,* **5**(2), 356-369.

Strauss, B.S. (1984) The interaction of u.v.- and methyl methanesulfonate-induced DNA repair synthesis: A role for poly(ADP-ribose)? *Carcinogenesis,* **5**(5), 577-582.

Strissel, K.J., Stancheva, Z., Miyoshi, H., Perfield, J. W.,2nd, DeFuria, J., Jick, Z., Greenberg, A. S. and Obin, M. S. (2007) Adipocyte death, adipose tissue remodeling, and obesity complications. *Diabetes,* **56**(12), 2910-2918.

Stuart Wood, I., de Heredia, F. P., Wang, B. and Trayhurn, P. (2009) Cellular hypoxia and adipose tissue dysfunction in obesity. *Proc. Nutr. Soc.,* **68**(4), 370-377.

Suganami, T., Nishida, J. and Ogawa, Y. (2005) A paracrine loop between adipocytes and macrophages aggravates inflammatory changes: Role of free fatty acids and tumor necrosis factor alpha. *Arterioscler. Thromb. Vasc. Biol.,* **25**(10), 2062-2068.

Sugumar, A., Liu, Y. C., Xia, Q., Koh, Y. S. and Matsuo, K. (2004) Insulin-like growth factor (IGF)-I and IGF-binding protein 3 and the risk of premenopausal breast cancer: A meta-analysis of literature. *Int. J. Cancer,* **111**(2), 293-297.

Suikkari, A.M., Koivisto, V. A., Koistinen, R., Seppala, M. and Yki-Jarvinen, H. (1989) Dose-response characteristics for suppression of low molecular weight plasma insulin-like growth factor-binding protein by insulin. *J. Clin. Endocrinol. Metab.,* **68**(1), 135-140.

Suk, S.H., Sacco, R. L., Boden-Albala, B., Cheun, J. F., Pittman, J. G., Elkind, M. S., Paik, M. C. and Northern Manhattan Stroke Study (2003) Abdominal obesity and risk of ischemic stroke: The northern manhattan stroke study. *Stroke,* **34**(7), 1586-1592.

Sun, M., Wang, G., Paciga, J. E., Feldman, R. I., Yuan, Z. Q., Ma, X. L., Shelley, S. A., Jove, R., Tsichlis, P. N., Nicosia, S. V. and Cheng, J. Q. (2001) AKT1/PKBalpha kinase is frequently elevated in human cancers and its constitutive activation is required for oncogenic transformation in NIH3T3 cells. *Am. J. Pathol.,* **159**(2), 431-437.

Surmacz, E. (2007) Obesity hormone leptin: A new target in breast cancer? *Breast Cancer Res.,* **9**(1), 301.

Surmacz, E. and Bartucci, M. (2004) Role of estrogen receptor alpha in modulating IGF-I receptor signaling and function in breast cancer. *J. Exp. Clin. Cancer Res.,* **23**(3), 385-394.

Surmi, B.K. and Hasty, A. H. (2008) Macrophage infiltration into adipose tissue: Initiation, propagation and remodeling. *Future Lipidol.,* **3**(5), 545-556.

Tait, L., Soule, H. D. and Russo, J. (1990) Ultrastructural and immunocytochemical characterization of an immortalized human breast epithelial cell line, MCF-10. *Cancer Res.,* **50**(18), 6087-6094.

Talavera, F., Reynolds, R. K., Roberts, J. A. and Menon, K. M. (1990) Insulin-like growth factor I receptors in normal and neoplastic human endometrium. *Cancer Res.,* **50**(10), 3019-3024.

Tartaglia, L.A. (1997) The leptin receptor. *J. Biol. Chem.,* **272**(10), 6093-6096.

Tartaglia, L.A., Dembski, M., Weng, X., Deng, N., Culpepper, J., Devos, R., Richards, G. J., Campfield, L. A., Clark, F. T., Deeds, J., Muir, C., Sanker, S., Moriarty, A., Moore, K. J., Smutko, J. S., Mays, G. G., Wool, E. A., Monroe, C. A. and Tepper, R. I. (1995) Identification and expression cloning of a leptin receptor, OB-R. *Cell*, **83**(7), 1263-1271.

Tatemoto, K., Hosoya, M., Habata, Y., Fujii, R., Kakegawa, T., Zou, M. X., Kawamata, Y., Fukusumi, S., Hinuma, S., Kitada, C., Kurokawa, T., Onda, H. and Fujino, M. (1998) Isolation and characterization of a novel endogenous peptide ligand for the human APJ receptor. *Biochem. Biophys. Res. Commun.*, **251**(2), 471-476.

Tauxe, R.V., Holmberg, S. D., Dodin, A., Wells, J. V. and Blake, P. A. (1988) Epidemic cholera in mali: High mortality and multiple routes of transmission in a famine area. *Epidemiol. Infect.*, **100**(2), 279-289.

Tchernof, A. (2007) Visceral adipocytes and the metabolic syndrome. *Nutr. Rev.*, **65**(6 Pt 2), S24-9.

Teras, L.R., Goodman, M., Patel, A. V., Bouzyk, M., Tang, W., Diver, W. R. and Feigelson, H. S. (2009) No association between polymorphisms in LEP, LEPR, ADIPOQ, ADIPOR1, or ADIPOR2 and postmenopausal breast cancer risk. *Cancer Epidemiol. Biomarkers Prev.*, **18**(9), 2553-2557.

Tessitore, L., Vizio, B., Pesola, D., Cecchini, F., Mussa, A., Argiles, J. M. and Benedetto, C. (2004) Adipocyte expression and circulating levels of leptin increase in both gynaecological and breast cancer patients. *Int. J. Oncol.*, **24**(6), 1529-1535.

Testa, J.R. and Bellacosa, A. (2001) AKT plays a central role in tumorigenesis. *Proc. Natl. Acad. Sci. U. S. A.*, **98**(20), 10983-10985.

Thomas, P., Pang, Y., Filardo, E. J. and Dong, J. (2005) Identity of an estrogen membrane receptor coupled to a G protein in human breast cancer cells. *Endocrinology*, **146**(2), 624-632.

Tian, Y.F., Chu, C. H., Wu, M. H., Chang, C. L., Yang, T., Chou, Y. C., Hsu, G. C., Yu, C. P., Yu, J. C. and Sun, C. A. (2007) Anthropometric measures, plasma adiponectin, and breast cancer risk. *Endocr. Relat. Cancer*, **14**(3), 669-677.

Toniolo, P.G., Levitz, M., Zeleniuch-Jacquotte, A., Banerjee, S., Koenig, K. L., Shore, R. E., Strax, P. and Pasternack, B. S. (1995) A prospective study of endogenous estrogens and breast cancer in postmenopausal women. *J. Natl. Cancer Inst.*, **87**(3), 190-197.

Trayhurn, P. and Wood, I. S. (2004) Adipokines: Inflammation and the pleiotropic role of white adipose tissue. *Br. J. Nutr.*, **92**(3), 347-355.

Trayhurn, P., Wang, B. and Wood, I. S. (2008) Hypoxia in adipose tissue: A basis for the dysregulation of tissue function in obesity? *Br. J. Nutr.,* **100**(2), 227-235.

Treeck, O., Lattrich, C., Juhasz-Boess, I., Buchholz, S., Pfeiler, G. and Ortmann, O. (2008) Adiponectin differentially affects gene expression in human mammary epithelial and breast cancer cells. *Br. J. Cancer,* **99**(8), 1246-1250.

Tremblay, F., Brule, S., Hee Um, S., Li, Y., Masuda, K., Roden, M., Sun, X. J., Krebs, M., Polakiewicz, R. D., Thomas, G. and Marette, A. (2007) Identification of IRS-1 ser-1101 as a target of S6K1 in nutrient- and obesity-induced insulin resistance. *Proc. Natl. Acad. Sci. U. S. A.,* **104**(35), 14056-14061.

Trempe, G.L. (1976) Human breast cancer in culture. *Recent Results Cancer Res.,* **(57)**(57), 33-41.

Trivers, K.F., Lund, M. J., Porter, P. L., Liff, J. M., Flagg, E. W., Coates, R. J. and Eley, J. W. (2009) The epidemiology of triple-negative breast cancer, including race. *Cancer Causes Control,* **20**(7), 1071-1082.

Tworoger, S.S., Eliassen, A. H., Kelesidis, T., Colditz, G. A., Willett, W. C., Mantzoros, C. S. and Hankinson, S. E. (2007) Plasma adiponectin concentrations and risk of incident breast cancer. *J. Clin. Endocrinol. Metab.,* **92**(4), 1510-1516.

University of Alberta, USA, http://flowcytometry.ualberta.ca/images/scatter.jpg, accessed on 10th September 2009

University of Berkeley, USA, http://biology.berkeley.edu/crl/flow_images/fig1.gif, accessed on 10th September 2009

Vague, J., Vague, P., Tramoni, M., Vialettes, B. and Mercier, P. (1980) Obesity and diabetes. *Acta Diabetol. Lat.,* **17**(2), 87-99.

Vaisse, C., Clement, K., Guy-Grand, B. and Froguel, P. (1998) A frameshift mutation in human MC4R is associated with a dominant form of obesity. *Nat. Genet.,* **20**(2), 113-114.

van der Burg, B., Rutteman, G. R., Blankenstein, M. A., de Laat, S. W. and van Zoelen, E. J. (1988) Mitogenic stimulation of human breast cancer cells in a growth factor-defined medium: Synergistic action of insulin and estrogen. *J. Cell. Physiol.,* **134**(1), 101-108.

van Herpen, N.A. and Schrauwen-Hinderling, V. B. (2008) Lipid accumulation in non-adipose tissue and lipotoxicity. *Physiol. Behav.,* **94**(2), 231-241.

van Landeghem, A.A., Poortman, J., Nabuurs, M. and Thijssen, J. H. (1985) Endogenous concentration and subcellular distribution of estrogens in normal and malignant human breast tissue. *Cancer Res.,* **45**(6), 2900-2906.

Varela, L.M., Stangle-Castor, N. C., Shoemaker, S. F., Shea-Eaton, W. K. and Ip, M. M. (2001) TNFalpha induces NFkappaB/p50 in association with the growth and morphogenesis of normal and transformed rat mammary epithelial cells. *J. Cell. Physiol.,* **188**(1), 120-131.

Vasan, R.S., Larson, M. G., Leip, E. P., Evans, J. C., O'Donnell, C. J., Kannel, W. B. and Levy, D. (2001) Impact of high-normal blood pressure on the risk of cardiovascular disease. *N. Engl. J. Med.,* **345**(18), 1291-1297.

Vaupel, P. (2004) Tumor microenvironmental physiology and its implications for radiation oncology. *Semin. Radiat. Oncol.,* **14**(3), 198-206.

Veldhuis, J.D. and Iranmanesh, A. (1996) Physiological regulation of the human growth hormone (GH)-insulin-like growth factor type I (IGF-I) axis: Predominant impact of age, obesity, gonadal function, and sleep. *Sleep,* **19**(10 Suppl), S221-4.

Verheus, M., Peeters, P. H., Rinaldi, S., Dossus, L., Biessy, C., Olsen, A., Tjonneland, A., Overvad, K., Jeppesen, M., Clavel-Chapelon, F., Tehard, B., Nagel, G., Linseisen, J., Boeing, H., Lahmann, P. H., Arvaniti, A., Psaltopoulou, T., Trichopoulou, A., Palli, D., Tumino, R., Panico, S., Sacerdote, C., Sieri, S., van Gils, C. H., Bueno-de-Mesquita, B. H., Gonzalez, C. A., Ardanaz, E., Larranaga, N., Garcia, C. M., Navarro, C., Quiros, J. R., Key, T., Allen, N., Bingham, S., Khaw, K. T., Slimani, N., Riboli, E. and Kaaks, R. (2006) Serum C-peptide levels and breast cancer risk: Results from the european prospective investigation into cancer and nutrition (EPIC). *Int. J. Cancer,* **119**(3), 659-667.

Vetto, J.T., Luoh, S. W. and Naik, A. (2009) Breast cancer in premenopausal women. *Curr. Probl. Surg.,* **46**(12), 944-1004.

Vgontzas, A.N., Bixler, E. O. and Chrousos, G. P. (2003) Metabolic disturbances in obesity versus sleep apnoea: The importance of visceral obesity and insulin resistance. *J. Intern. Med.,* **254**(1), 32-44.

Vgontzas, A.N., Papanicolaou, D. A., Bixler, E. O., Kales, A., Tyson, K. and Chrousos, G. P. (1997) Elevation of plasma cytokines in disorders of excessive daytime sleepiness: Role of sleep disturbance and obesity. *J. Clin. Endocrinol. Metab.,* **82**(5), 1313-1316.

Vgontzas, A.N., Papanicolaou, D. A., Bixler, E. O., Hopper, K., Lotsikas, A., Lin, H. M., Kales, A. and Chrousos, G. P. (2000) Sleep apnea and daytime sleepiness and fatigue: Relation to visceral obesity, insulin resistance, and hypercytokinemia. *J. Clin. Endocrinol. Metab.,* **85**(3), 1151-1158.

Vidal, H. (2001) Gene expression in visceral and subcutaneous adipose tissues. *Ann. Med.,* **33**(8), 547-555.

Visvader, J.E. (2009) Keeping abreast of the mammary epithelial hierarchy and breast tumorigenesis. *Genes Dev.,* **23**(22), 2563-2577.

von Gruenigen, V.E., Gil, K. M., Frasure, H. E., Jenison, E. L. and Hopkins, M. P. (2005) The impact of obesity and age on quality of life in gynecologic surgery. *Am. J. Obstet. Gynecol.,* **193**(4), 1369-1375.

Vona-Davis, L. and Rose, D. P. (2007) Adipokines as endocrine, paracrine, and autocrine factors in breast cancer risk and progression. *Endocr. Relat. Cancer,* **14**(2), 189-206.

Wagner, U., Burkhardt, E. and Failing, K. (1999) Evaluation of canine lymphocyte proliferation: Comparison of three different colorimetric methods with the 3H-thymidine incorporation assay. *Vet. Immunol. Immunopathol.,* **70**(3-4), 151-159.

Walter, M., Liang, S., Ghosh, S., Hornsby, P. J. and Li, R. (2009) Interleukin 6 secreted from adipose stromal cells promotes migration and invasion of breast cancer cells. *Oncogene,* **28**(30), 2745-2755.

Wang, B., Wood, I. S. and Trayhurn, P. (2008) Hypoxia induces leptin gene expression and secretion in human preadipocytes: Differential effects of hypoxia on adipokine expression by preadipocytes. *J. Endocrinol.,* **198**(1), 127-134.

Wang, G.L. and Semenza, G. L. (1993) General involvement of hypoxia-inducible factor 1 in transcriptional response to hypoxia. *Proc. Natl. Acad. Sci. U. S. A.,* **90**(9), 4304-4308.

Wang, Y., Cortez, D., Yazdi, P., Neff, N., Elledge, S. J. and Qin, J. (2000) BASC, a super complex of BRCA1-associated proteins involved in the recognition and repair of aberrant DNA structures. *Genes Dev.,* **14**(8), 927-939.

Weigle, D.S. (1994) Appetite and the regulation of body composition. *FASEB J.,* **8**(3), 302-310.

Weisberg, S.P., McCann, D., Desai, M., Rosenbaum, M., Leibel, R. L. and Ferrante, A. W.,Jr (2003) Obesity is associated with macrophage accumulation in adipose tissue. *J. Clin. Invest.,* **112**(12), 1796-1808.

Welborn, T.A. and Dhaliwal, S. S. (2007) Preferred clinical measures of central obesity for predicting mortality. *Eur. J. Clin. Nutr.,* **61**(12), 1373-1379.

Welcker, M. and Clurman, B. (2005) Cell cycle: How cyclin E got its groove back. *Curr. Biol.,* **15**(19), R810-2.

Weng, L.P., Smith, W. M., Dahia, P. L., Ziebold, U., Gil, E., Lees, J. A. and Eng, C. (1999) PTEN suppresses breast cancer cell growth by phosphatase activity-dependent G1 arrest followed by cell death. *Cancer Res.,* **59**(22), 5808-5814.

Weroha, S.J. and Haluska, P. (2008) IGF-1 receptor inhibitors in clinical trials--early lessons. *J. Mammary Gland Biol. Neoplasia,* **13**(4), 471-483.

Westphal, S.A. (2008) Obesity, abdominal obesity, and insulin resistance. *Clin. Cornerstone,* **9**(1), 23-29; discussion 30-1.

White, M.F. (2003) Insulin signaling in health and disease. *Science,* **302**(5651), 1710-1711.

White, M.F. and Kahn, C. R. (1994) The insulin signaling system. *J. Biol. Chem.,* **269**(1), 1-4.

White, M.F., Shoelson, S. E., Keutmann, H. and Kahn, C. R. (1988) A cascade of tyrosine autophosphorylation in the beta-subunit activates the phosphotransferase of the insulin receptor. *J. Biol. Chem.,* **263**(6), 2969-2980.

Whittaker, J., Okamoto, A. K., Thys, R., Bell, G. I., Steiner, D. F. and Hofmann, C. A. (1987) High-level expression of human insulin receptor cDNA in mouse NIH 3T3 cells. *Proc. Natl. Acad. Sci. U. S. A.*, **84**(15), 5237-5241.

Wilson, P.W., Cupples, L. A. and Kannel, W. B. (1991) Is hyperglycemia associated with cardiovascular disease? the framingham study. *Am. Heart J.,* **121**(2 Pt 1), 586-590.

Winter, Y., Rohrmann, S., Linseisen, J., Lanczik, O., Ringleb, P. A., Hebebrand, J. and Back, T. (2008) Contribution of obesity and abdominal fat mass to risk of stroke and transient ischemic attacks. *Stroke,* **39**(12), 3145-3151.

Woo, H.Y., Park, H., Ki, C. S., Park, Y. L. and Bae, W. G. (2006) Relationships among serum leptin, leptin receptor gene polymorphisms, and breast cancer in korea. *Cancer Lett.,* **237**(1), 137-142.

World Cancer Research Fund / American Institute for Cancer Research (2007) Food, Nutrition, Physical Activity, and the Prevention of Cancer: a Global Perspective. Washington DC.

World Health Organisation Expert Committee (1995) Physical Status: The Use and Interpretation of Anthropometry, WHO Technical Report Series No 854, Geneva http://whqlibdoc.who.int/trs/WHO_TRS_854.pdf, accessed on 4th November 2009.

World Health Organization (2000) Obesity: preventing and managing the global epidemic, Report of a WHO Consultation, WHO Technical Report Series No 894, Geneva, http://whqlibdoc.who.int/trs/WHO_TRS_894.pdf, accessed on 18th November 2009.

Wu, M.H., Chen, K. F., Lin, S. C., Lgu, C. W. and Tsai, S. J. (2007) Aberrant expression of leptin in human endometriotic stromal cells is induced by elevated levels of hypoxia inducible factor-1alpha. *Am. J. Pathol.,* **170**(2), 590-598.

Xu, H., Barnes, G. T., Yang, Q., Tan, G., Yang, D., Chou, C. J., Sole, J., Nichols, A., Ross, J. S., Tartaglia, L. A. and Chen, H. (2003) Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *J. Clin. Invest.*, **112**(12), 1821-1830.

Yager, J.D. (2000) Endogenous estrogens as carcinogens through metabolic activation. *J. Natl. Cancer. Inst. Monogr.*, **(27)**(27), 67-73.

Yan, J., Roy, S., Apolloni, A., Lane, A. and Hancock, J. F. (1998) Ras isoforms vary in their ability to activate raf-1 and phosphoinositide 3-kinase. *J. Biol. Chem.*, **273**(37), 24052-24056.

Yang, Q., Graham, T. E., Mody, N., Preitner, F., Peroni, O. D., Zabolotny, J. M., Kotani, K., Quadro, L. and Kahn, B. B. (2005) Serum retinol binding protein 4 contributes to insulin resistance in obesity and type 2 diabetes. *Nature*, **436**(7049), 356-362.

Ye, J., Gao, Z., Yin, J. and He, Q. (2007) Hypoxia is a potential risk factor for chronic inflammation and adiponectin reduction in adipose tissue of ob/ob and dietary obese mice. *Am. J. Physiol. Endocrinol. Metab.*, **293**(4), E1118-28.

Yee, D. and Lee, A. V. (2000) Crosstalk between the insulin-like growth factors and estrogens in breast cancer. *J. Mammary Gland Biol. Neoplasia*, **5**(1), 107-115.

Yeo, G.S., Farooqi, I. S., Aminian, S., Halsall, D. J., Stanhope, R. G. and O'Rahilly, S. (1998) A frameshift mutation in MC4R associated with dominantly inherited human obesity. *Nat. Genet.*, **20**(2), 111-112.

Yin, N., Wang, D., Zhang, H., Yi, X., Sun, X., Shi, B., Wu, H., Wu, G., Wang, X. and Shang, Y. (2004) Molecular mechanisms involved in the growth stimulation of breast cancer cells by leptin. *Cancer Res.*, **64**(16), 5870-5875.

Yokosuka, M., Xu, B., Pu, S., Kalra, P. S. and Kalra, S. P. (1998) Neural substrates for leptin and neuropeptide Y (NPY) interaction: Hypothalamic sites associated with inhibition of NPY-induced food intake. *Physiol. Behav.*, **64**(3), 331-338.

Yu, H. and Rohan, T. (2000) Role of the insulin-like growth factor family in cancer development and progression. *J. Natl. Cancer Inst.*, **92**(18), 1472-1489.

Yusuf, S., Hawken, S., Ounpuu, S., Dans, T., Avezum, A., Lanas, F., McQueen, M., Budaj, A., Pais, P., Varigos, J., Lisheng, L. and INTERHEART Study Investigators (2004) Effect of potentially modifiable risk factors associated with myocardial infarction in 52 countries (the INTERHEART study): Case-control study. *Lancet*, **364**(9438), 937-952.

Yusuf, S., Hawken, S., Ounpuu, S., Bautista, L., Franzosi, M. G., Commerford, P., Lang, C. C., Rumboldt, Z., Onen, C. L., Lisheng, L., Tanomsup, S., Wangai, P.,Jr, Razak, F., Sharma, A. M., Anand, S. S. and INTERHEART Study

Investigators (2005) Obesity and the risk of myocardial infarction in 27,000 participants from 52 countries: A case-control study. *Lancet*, **366**(9497), 1640-1649.

Zemel, M.B. and Shi, H. (2000) Pro-opiomelanocortin (POMC) deficiency and peripheral melanocortins in obesity. *Nutr. Rev.*, **58**(6), 177-180.

Zhang, G.J. and Adachi, I. (1999) Serum interleukin-6 levels correlate to tumor progression and prognosis in metastatic breast carcinoma. *Anticancer Res.*, **19**(2B), 1427-1432.

Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L. and Friedman, J. M. (1994) Positional cloning of the mouse obese gene and its human homologue. *Nature*, **372**(6505), 425-432.

Zhu, K., Bernard, L. J., Levine, R. S. and Williams, S. M. (1997) Estrogen receptor status of breast cancer: A marker of different stages of tumor or different entities of the disease? *Med. Hypotheses*, **49**(1), 69-75.

Zielinski, C.C., Mueller, C., Tyl, E., Tichatschek, E., Kubista, E. and Spona, J. (1990) Impaired production of tumor necrosis factor in breast cancer. *Cancer*, **66**(9), 1944-1948.

Zimmermann, S. and Moelling, K. (1999) Phosphorylation and regulation of raf by akt (protein kinase B). *Science*, **286**(5445), 1741-1744.

Zyromski, N.J., Mathur, A., Pitt, H. A., Wade, T. E., Wang, S., Nakshatri, P., Swartz-Basile, D. A. and Nakshatri, H. (2009) Obesity potentiates the growth and dissemination of pancreatic cancer. *Surgery*, **146**(2), 258-263.