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2020

Journal Pre-proof

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PII: S1383-5742(19)30045-6

DOI: https://doi.org/10.1016/j.mrrev.2019.108288

Reference: MUTREV 108288

To appear in: *Mutation Research-Reviews in Mutation Research*

Received Date: 1 July 2019

Revised Date: 29 October 2019

Accepted Date: 7 November 2019

Please cite this article as: Azqueta A, Ladeira C, Giovannelli L, Boutet-Robinet E, Bonassi S, Neri M, Gajski G, Duthie S, Del Bo' C, Riso P, Koppen G, Basaran N, Collins A, Møller P, Application of the comet assay in human biomonitoring: an hCOMET perspective, *Mutation Research-Reviews in Mutation Research* (2019), doi: https://doi.org/10.1016/j.mrrev.2019.108288

Application of the comet assay in human biomonitoring: an hCOMET perspective

Amaya Azqueta[a,b], Carina Ladeira[c,d], Lisa Giovannelli[e], Elisa Boutet-Robinet[f], Stefano Bonassi[g,h], Monica Neri[g], Goran Gajski[i], Susan Duthie[j], Cristian Del Bo'[k], Patrizia Riso[k], Gudrun Koppen[l], Nursen Basaran[m], Andrew Collins[n], Peter Møller[o*]

Affiliations

[a]Department of Pharmacology and Toxicology, University of Navarra, C/Irunlarrea 1, 31009 Pamplona, Spain.

[b]IdiSNA, Navarra Institute for Health Research, Spain.

[c]H&TRC - Health & Technology Research Center, ESTeSL- Escola Superior de Tecnologia da Saúde, Instituto Politécnico de Lisboa, Av. D. João II, lote 4.69.01, Parque das Nações, 1990-096 Lisboa, Portugal.

[d]Centro de Investigação e Estudos em Saúde Pública, Escola Nacional de Saúde Pública, ENSP, Universidade Nova de Lisboa, Portugal.

[e]Dept. NEUROFARBA, Section Pharmacology and Toxicology, University of Florence, Florence, Italy.

[f]Toxalim (Research Centre in Food Toxicology), Université de Toulouse, INRA, ENVT, INP-Purpan, UPS, Toulouse, France.

[g]Unit of Clinical and Molecular Epidemiology IRCCS San Raffaele Pisana, Rome, Italy.

[h]Department of Human Sciences and Quality of Life Promotion San Raffaele University, Rome, Italy.

[i]Mutagenesis Unit, Institute for Medical Research and Occupational Health, Ksaverska cesta 2, 10000 Zagreb, Croatia.

[j]School of Pharmacy and Life Sciences, The Robert Gordon University, Riverside East, Garthdee Road, Aberdeen, AB10 7GJ, United Kingdom.

[k]Università degli Studi di Milano, Department of Food, Environmental and Nutritional Sciences (DeFENS), Division of Human Nutrition, Via G. Celoria 2, 20133 Milano, Italy.

[l]Environmental Risk and Health unit, Flemish Institute for Technological Research (VITO-Health), Mol, Belgium.

[m]Hacettepe University, Faculty of Pharmacy , Department of Toxicology, 06100, Ankara, Turkey.

[n]Department of Nutrition, Institute for Basic Medical Sciences, University of Oslo, Sognsvannsveien 9, 0372 Oslo, Norway.

[o]Department of Public Health, Section of Environmental Health, University of Copenhagen, DK-1014 Copenhagen K, Denmark.

[*]Corresponding author

Abstract

The comet assay is a well-accepted biomonitoring tool to examine the effect of dietary, lifestyle, environmental and occupational exposure on levels of DNA damage in human cells. With such a wide range of determinants for DNA damage levels, it becomes challenging to deal with confounding and certain factors are inter-related (e.g. poor nutritional intake may correlated with smoking status). This review describes the effect of intrinsic (i.e. sex, age, tobacco smoking, occupational exposure and obesity) and extrinsic (season, environmental exposures, dietary factors, physical activity and alcohol consumption) factors on the level of DNA damage measured by the standard or enzyme-modified comet assay. Although each factor influences at least one comet assay endpoint, the collective evidence does not indicate single factors have a large impact. Thus, controlling for confounding may be necessary in a biomonitoring study, but none of the factors is strong enough to be regarded *a priori* as a confounder. Controlling for confounding in the comet assay requires a case-by-case approach. Inter-laboratory variation in levels of DNA damage and to some extent also reproducibility in biomonitoring studies are issues that have haunted the users of the comet assay for years. Procedures to collect specimens, and their storage, are not standardized. Likewise, statistical issues related to both sample-size calculation (before sampling of specimens) and statistical analysis of the results varies between studies. This review gives guidance to statistical analysis of the typically complex exposure, co-variate, and effect relationships in human biomonitoring studies.

Scheme

1. Introduction

Molecular epidemiology is based on the use of molecular biomarkers to identify or quantify disease effects or risk in epidemiological studies. Molecular epidemiology applied to humans has the advantage of being directly relevant, unlike animal or other experimental models that require extrapolation to humans. In epidemiological studies, biomarkers can be used in combination with health data to demonstrate an association between the body burden of pollutants and their health effects, or simply to test other research hypotheses [1,2].

Biomarkers are typically measured in samples that can be obtained (relatively) non-invasively, which generally means blood or urine. DNA damage is recognized as a useful biomarker, by virtue of its role in carcinogenesis. Many lesions occur in the DNA of every cell each day, inflicted by natural or artificial exogenous radiation sources or genotoxic chemicals, or by endogenous exposure to e.g. reactive oxygen species, or as a result of errors in replication. But it should be borne in mind that almost all DNA damage is repaired, and that even if damage is present when DNA is replicated, it doesn't necessarily cause mutations. DNA damage should be regarded as a marker of exposure to DNA-damaging agents, and in that sense it reflects disease risk, but whether it can be seen as a predictive marker of individual cancer risk is debatable, in the absence of compelling evidence.

The comet assay is the most popular method for measuring DNA damage in human cells. Essentially it measures DNA breaks, through their ability to relax the supercoiling of DNA loops which can then extend under (alkaline) electrophoresis to form comet-like images; the relative intensity of tail DNA fluorescence indicates the frequency of breaks (Figure 1). An additional step can be introduced, digesting the DNA with a lesion-specific enzyme, so that – for instance – oxidised bases are converted to breaks

and the comet tail intensity increases. This modification is particularly useful in biomonitoring, as oxidative stress – and consequent DNA oxidation – is a feature of various kinds of exposure and many human diseases.

The aim of this article is to describe the key issues that are relevant in a biomonitoring study in which the comet assay is applied to measure DNA damage. We describe how different co-variates may affect the outcome of the study. Some of the factors have been specifically assessed in systematic reviews, while others have not yet been assessed thoroughly. Some technical aspects related specifically to biomonitoring are also covered, such as sampling and storing of specimens, as well as statistical and ethical issues that should be addressed before and after the biomonitoring study. However, technical issues related specifically to variability in assay conditions and efforts to produce standard procedures have been reviewed elsewhere [4]. Intra-laboratory variation in DNA damage levels has been described since the early 2000s through ring-trials involving multiple laboratories [5-7]. Later ring-trials have shown reductions in inter-laboratory variation in DNA damage levels on cryopreserved cells by using standardization with reference standards and standardized comet assay procedures, although some variation persists even after such means of standardization [8-13].

In the context of the present review, exposure may refer to potentially hazardous agents of either physical (e.g. radiation) or chemical nature. Alternatively, an exposure may be positive in terms of health benefits - for example, certain dietary items and habits such as regular exercise. The present review encompasses studies that have assessed the effect of exposure on DNA damage. In this type of study, exposure (e.g. to a chemical agent) is the predictor and the level of DNA damage is the outcome. By design, the case-control study selects first the cases (and a matched control group) and then

assesses the exposure. In that sense, the level of DNA damage is the predictor, whereas the disease status is the outcome.

2. Selection of subjects

In human exposure studies, the predictor is either dichotomized into *no exposure* versus *exposure* (or *low* versus *high* exposure) or treated as a continuous variable. The outcome variable is usually treated as a continuous factor, although data reduction to categorical scale may occur in certain studies. The typical biomonitoring study assesses the effect of a certain predictor (here "exposure") on the outcome (here "DNA damage"). The trick is to obtain a gradient in the exposure, while at the same time avoiding selection of other factors that can influence the exposure-outcome relationship. This kind of disproportionate selection of certain factors in exposure groups is called confounding only if the factor is associated with both the predictor and the outcome. As the confounder *per se* is an independent risk factor for the outcome, it can be used ingeniously to gain better understanding of the complex relationship between exposure and outcome by for instance designing studies that utilize the information to test for interactions between the exposure and susceptibility factors. For example, age, sex and smoking are typically regarded as confounders, but a study can be carefully designed to assess the effect modification due to these factors by demonstrating that a certain exposure is only increasing the level of DNA damage in e.g. men, old people or smokers.

2.1. Confounders, effect modifiers and effect mediators in human biomonitoring studies

In molecular epidemiology, confounders, effect modifiers and effect mediators are factors that are used as co-variates in the statistical models. Below, age and sex are used as examples of factors that can be confounders, effect modifiers and effect mediators (further discussion of these variables in statistical modelling is provided in section 6. Statistical analysis). There is a wealth of information on effects of age and sex in biomonitoring studies because this information is frequently reported in the description of the subjects. However, only a few studies have been designed specifically to test effects of age and sex on DNA damage measured with the comet assay.

Figure 2 describes different ways that effects of age and sex can be (or have been) assessed in study designs and statistical analyses. In these examples age and sex are regarded as endogenous factors, whereas "exposure" is an external factor. Age and sex may be regarded as confounders, i.e. they are associated with both the exposure (i.e. predictor) and level of DNA damage (i.e. outcome). As confounders, age and sex are associated with exposure if the selection of subjects according to the exposure status has also introduced an unequal balance in the age and sex distribution (e.g. those who are exposed are also older or predominantly males). This implies that information on age and sex is "irrelevant" in the sense that the statistical analysis adjusts for the effect of the confounders. It should be noted that control for confounding is a statistical solution to a study design problem and the confounder is considered *a priori* to have an effect on the association between exposure and level of DNA damage. Other ways to prevent the effect of confounders are randomisation, restriction or matching of subjects. Age and sex may also be regarded as effect modifiers, i.e. the effect of an exposure will differ in strata of the dataset due to biological reasons (men and women, or old and young subjects, may have different responses to exposures). This is demonstrated by statistical analysis showing an interaction between the exposure and the modifier. Finally, the

effects of age and sex may be mediated through exposures or life-style factors. In this case age and sex do not have a direct effect on the outcome (i.e. DNA damage). The apparent effect of age (and sex) on the outcome is explained by intermediate steps in the pathway. In this case (figure 2C), it is incorrect to consider age and sex as confounders as they are not independent risk factors for the outcome.

2.2. Types of factors that influence the level of DNA damage in humans

The planning of human biomonitoring studies entails a careful assessment of possible contributing factors to the level of DNA damage as well as within-group variation. This pertains not only to dealing with confounders (i.e. avoiding a systematic error), but also to random factors that might give rise to outliers in the results. The latter may be difficult to control as it could compete with a smooth selection of subjects or matching criteria between groups of exposed and controls. Table 1 describes the four different types of factors, which are described in further detail in the subsequent sections.

2.2.1. Intrinsic and non-modifiable factors

Sex and age are factors that subjects cannot change in the same way that dietary habits, physical activity or specific chemical exposures can be altered. The impact of age and sex on levels of DNA damage is well-known as illustrated by the several biomonitoring studies that restrict the inclusion of subjects to specific age groups or sex. The present review encompasses only studies that have assessed age and sex differences in DNA damage levels as a primary purpose of the investigation. These are predominantly cross-sectional studies. A detailed analysis of the effect of age and sex on DNA damage levels measured with the comet assay is reported elsewhere [14]. Various types of exposure

studies have not been included because selection or matching of subjects in the reference group may have occurred. Cross-sectional studies are suitable for assessment of sex-specific differences in DNA damage levels, whereas they are not ideal for age-dependent differences because it is not possible to assess whether DNA damage levels increase with age. Cross-sectional studies can merely be used to show that subjects with different ages have different levels of DNA damage. The review only includes results from studies that have assessed levels of DNA damage in white blood cells (WBCs), isolated as either leukocytes, peripheral blood mononuclear cells (PBMCs) or lymphocytes.

2.2.1.1. Age

Studies published before 2000 have mainly shown that age is not a strong predictor for the basal levels of DNA strand breaks in WBCs, which may be due to the use of non-optimal comet assay descriptors such as tail length [15]. A meta-analysis of studies published up to 2005 has shown a positive association between age and levels of DNA strand breaks in WBCs in studies that had used %DNA in tail and visual classification as comet assay descriptors, whereas there was no difference in levels of Endonuclease III (EndoIII)- and Formamidopyrimidine DNA glycosylase (Fpg)-sensitive sites [16]. More recent studies, which specifically have assessed age-differences in levels of DNA strand breaks, have reported positive [17-23], null [24-28], or negative associations [29]. The discrepancy between studies may be related to multiple factors such as the age span, type of WBCs, analysis of fresh or frozen sample and the statistical analysis. The latter pertains to age treated as a categorical (i.e. age groups) or continuous (i.e. true age in years) variable. In addition, multivariate regression analysis seems to show little effect of age, which may be due to control for confounding or mediator effects. For

instance, a large cross-sectional study of 992 subjects showed a positive association between age and levels of DNA damage in PBMCs ($P<0.05$, linear regression, not reported in the original article), but this was not significant in analysis adjusted for sex and a number of metabolic risk factors [28]. This indicates that the presumed effect of age was mediated by various life-style factors.

Only a few studies have assessed associations between age and oxidatively damaged DNA in WBCs. The reports have been mixed with studies showing positive association [17, 19, 23] or no effect of age on levels of damage assessed with the Fpg- or hOGG1-modified comet assay [20, 25,28,30]. Although mixed results have been reported, age should be considered as a confounder in studies that do not have statistical power to control for the effect of mediating life-style factors.

2.2.1.2. Sex

A systematic review of the literature published up to 2005 did not show a difference between levels of DNA strand breaks, EndoIII- and Fpg-sensitive sites in WBCs between women and men [16]. This is in agreement with several more recent studies that have shown no or equivocal difference between sexes in studies using multivariate analysis [21,23, 25,26,28,31-34]. In fact, only a few studies have shown sex-differences in multivariate analysis; these reported a higher level of DNA strand breaks in women [35] or a higher level in men [27]. Studies without adjustment for potential confounders have either reported higher levels of DNA strand breaks in men [36], higher levels in women [24,32] or no effect of sex [37].

The relatively few studies on sex-dependent differences in levels of DNA damage in the Fpg-, hOGG1-, or EndoIII-modified comet assay have not revealed any difference between sexes [28,31,32,36].

2.2.2. Intrinsic and modifiable factors

In theory, intrinsic and modifiable factors are changeable. Examples of such factors are tobacco smoking, daily use of pain killers and occupational exposures. Quitting smoking or changing job is possible, but it is not likely to happen as part of a human biomonitoring study. Thus, the effect of smoking or daily use of pain killers is typically avoided by standard practices for control of confounding. Likewise, exclusion of subjects from certain occupations or job titles (e.g. blue collar workers) is possible in molecular epidemiology.

2.2.2.1. Tobacco smoking

The effect of tobacco smoking on DNA damage has been thoroughly studied since tobacco contains a great variety of genotoxic/carcinogenic agents. Thus, smoking has also been considered to be a confounding factor.

Kassie et al. published a review in which they found that tobacco smoking causes an increase in DNA strand breaks in several human studies; however, the extent of the damage did not correlate with the number of cigarettes [38]. Some years later, Faust et al. showed that only 9 reports, out of 29 studies, found that tobacco smoking significantly increases the DNA strand breaks in lymphocytes, while 16 reports did not find such an association [39]. Moreover, the relationship between DNA damage and the number of cigarettes smoked per day was still not clear; some studies reported that

smoking one or more cigarettes per day produces DNA damage in comparison with non-smokers, while others did not report any such effect even in those smoking 10 or more cigarettes per day. In the same paper it was reported that more than 55% of the studies specifically carried out to study the effect of tobacco smoking on DNA integrity using the comet assay did not find any association. Hoffmann et al. carried out a meta-analysis of 38 published studies and found a higher level of DNA strand breaks in smokers than in non-smokers [41]. However, the effect was very small when smoking was investigated as a potential confounding factor in occupational studies. In this direction, Collins et al. stated that smoking does not appear to be an important confounding factor in occupational studies [41]. It is worth mentioning that the design of a study in which smoking is considered just a confounding factor may not be powerful enough to detect differences between smokers and non-smokers.

The discrepancies observed in the different studies can be due to several factors such as the size of the sample (i.e., number of participants), the number of cigarettes smoked and the period over which they are smoked, the type of cigarettes (e.g., hand-rolled, with or without filter) and the brand. It has been noted that reports on the effect of smoking seemed to cluster in countries in the southern part of Europe and it is possible that brands of cigarettes with dark tobacco and high tar content are smoked more frequently in that region [15]. Kocygita et al. showed that the level of DNA strand breaks in mononuclear leukocytes were significantly higher in hand-rolled cigarette smokers than in filter-cigarette smokers [42]. Both groups showed a significantly higher level of DNA strand breaks compared to the never smoker group. Overall, smokers present, if anything, a slightly higher level of DNA damage compared with non-smokers; but this cannot be detected if the statistical power is weak [39].

Another factor that may contribute to the discrepancies observed in different studies is passive smoking in the reference group, which is normally overlooked [39]. In this respect, Lam et al. carried out a study in elevator factory workers potentially exposed to benzene and observed that tobacco smoking, both active and passive at home, was significantly associated with DNA damage [43]. However, passive smoking in the work place did not affect DNA damage.

In all these studies, the standard alkaline comet assay was used; however, the Fpg-modified assay (or the use of another enzyme detecting oxidized bases) can be more appropriate to detect the DNA damage induced by tobacco smoking. Studies in which the effect of tobacco smoking is assessed by using the enzyme-modified comet assay to measure oxidized lesions are scarce. Fracasso et al. showed a significant high level of DNA strand breaks in cells from active smokers compared with non-smokers or passive smokers [44]. The DNA damage observed when using Fpg was significantly higher than the level of strand breaks detected without using Fpg only in the group of active smokers. Surprisingly, the authors did not check for differences in the net Fpg-sensitive sites between groups. A review of 125 studies showed that smoking did not influence the level of Fpg- and EndoIII-sensitive sites [16]. It is worth mentioning that the objective of this study was to look for reference values for DNA lesions in blood cells and so not all the studies included smoking and non-smoking participants for the detection of Fpg- and EndoIII- sensitive sites.

Smokeless tobacco (electronic cigarettes) is also a source of genotoxic compounds. It should be emphasized that the effect of smokeless tobacco has not been assessed in human exposure studies. However, it has been demonstrated that this form also induces DNA strand breaks in peripheral blood samples [45]. Moreover, smokeless tobacco

induces a significantly higher level of DNA strand breaks in lymphocytes than does regular tobacco smoking [46].

2.2.2.2. Occupational exposure

The effect of occupational exposure on DNA damage has been the subject of a large number of studies for reasons such as workers' security and for regulatory purposes, as well as for the investigation of biological, physical and chemical occupational hazards.

A wide range of chemicals have mutagenic and/or carcinogenic properties, that can act as environmental hazards, and may also be exposure factors in specific occupational settings. For instance, besides the risks to the general public, atmospheric pollution can be considered as an occupational health hazard to professional groups, such as traffic police or professional drivers working in urban areas [47,48].

Biomonitoring of exposure to toxic chemicals in the workplace is a fundamental tool to evaluate human health risks, and to support strategies to establish a safe work environment. The comet assay has been used to detect and quantify DNA damage as a marker of exposure to genotoxic agents, in workers exposed to various occupational hazards including gases, chemicals and anticancer drugs. Its application in human safety and health risk monitoring is well established, particularly when assessing exposure to solvents, petrol by-products, heavy metals, mineral fibres and particulates. Several reviews summarize the wide-ranging use of the comet assay in occupational biomonitoring [39,41,49]. For instance, levels of Fpg-sites were significantly higher in WBCs and in buccal cells from “on the ground” airport workers, compared with controls selected from office workers at the same airport [50]. A similar finding was reported in a study of policeman in the Czech Republic [51]. Here, higher levels of both

oxidised purines and pyrimidines (Fpg- and EndoIII-sensitive sites) were measured in policemen working outdoors and exposed to traffic emissions when compared with colleagues working indoors. This effect was seasonal, and was only observed when air pollution, measured as particulate matter and polycyclic aromatic hydrocarbons (PAHs), was high. Oxidised DNA base damage correlated positively with recorded levels of environmental carcinogenic PAHs [51]. Genotoxic effects of occupational exposures to by-products of petrol, heavy metals, organic solvents etc. have been reviewed [47].

2.2.2.3. Body Mass Index (BMI)

The association between overweight/obesity (individuals with BMI $\geq$ 25 kg/m^2) and DNA strand breaks or oxidatively damaged DNA damage is well documented [52]. In contrast, studies evaluating the influence of BMI in a eutrophic population (BMI < 25 kg/m^2) are scarce. Two studies [53,54] have reported no correlation between normal BMI (< 25 kg/m^2) and DNA strand breaks. Thus, BMI within the normal range does not appear to influence DNA damage in healthy individuals.

2.2.3. Extrinsic and non-modifiable factors

Extrinsic and non-modifiable factors are characterized by being ubiquitous and random exposures that are virtually impossible to control in a molecular epidemiology study. Air pollution levels, ambient temperature and sunlight are such factors that vary over short (e.g. day-to-day) and long periods (e.g. seasons of the year). In addition, they may display a high degree of co-variability. For instance, high solar radiation correlates with temperature and ozone levels. As sunlight exposure is highly dependent on the time of the year, these exposures have typically been investigated in the context of seasonal

variation. Yet, air pollution is more complex as it may correlate inversely with temperature in some parts of the world (highest in the winter due to temperature inversion where cold air is trapped at ground level) and positively in warm parts (higher due to long-range dispersion of particles from wildland or forest fires).

2.2.3.1. Season

The impact of meteorology - and in a broader sense seasonal variations - on the level of base-line DNA damage measured via the comet assay, is studied in several papers. Topics relating to seasonal changes include variation in temperature, solar radiation (strength and duration), air pollution [ozone, particles, (semi-)volatile compounds], diet (antioxidants, cooking processes), allergy (pollen), physical exercise, time spent outdoors/in the sun, and wearing less skin-covering clothes. Recently, Gerić et al. [33] reported that sampling season and exposure to medical radiation were the two main variables in the results of comet assay tests on whole blood of 162 inhabitants of Zagreb (Croatia) recruited between 2008 and 2016. More specifically, this retrospective study showed that global sun radiation (J/cm^2), temperature, and daily insolation (h), were of influence. Their results confirmed most earlier cross-sectional and longitudinal studies. Increased DNA damage in warmer periods of the year was indeed also reported in studies in which healthy adults were sampled repetitively. In Greece, 40 non-smoking men, engaged in outdoor activities for 6 h each day during the summer, showed a higher level of DNA damage in mononuclear cells at the end of the summer (September) compared to the end of the winter (March) [55]. In Belgium, whole blood of 45 non-smoking office and laboratory workers was sampled four times, in all seasons of one year [56], and another group of 48 non-smoking office workers were sampled in winter and again in summer [57]. In both studies, DNA damage was correlated with the 8-

hour-average ozone concentrations and the average outdoor temperature in the week before sampling. The latter studies were done to confirm the findings of a cross-sectional study in 200 adolescents (17-18y) participating in the Flemish Environment and Health Survey. In that study, both the whole blood comet assay results and urinary 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) were affected by ozone concentrations and the average outdoor temperature, and by the hours of sunshine 3 days before sample collection [58]. The impact of solar radiation was intensively studied in another longitudinal study by Møller et al. [35]. Twenty-one office and laboratory workers were followed up about once a month, during a period of 14 months. DNA damage measured in the mononuclear cells was correlated to the average daily influx of sunlight during 3 to 6 days before blood sampling. The results suggested that sunlight penetrating the outer layer of the human epidermis (such as UVA and UVB) damaged DNA in PBMCs circulating in the vessels of the skin. Giovanelli et al. [25] observed in 79 office and laboratory workers sampled through the year a positive correlation of the individuals' DNA damage in mononuclear cells with the outdoor air temperature - and to a lesser extent with global solar radiation and air ozone levels. The frequency of DNA strand breaks showed a stronger seasonal trend as compared to the frequency of Fpg-sites, being higher in summer. UV radiation induces oxidative stress, and indeed specific photoproducts such as thymine dimers, that are converted into transient strand breaks upon DNA repair [25]. Interestingly, subjects with higher BMI (BMI>25 kg/m^2) appeared to have higher sensitivity to outdoor temperature variations, perhaps because of a less efficient heat dispersion [25].

On the other hand, it should be noted that also a few studies reported DNA damage being higher in blood cells collected during winter time, in polluted areas of e.g. Czech Republic [59] and Poland [60]. Local heavier air pollution in the colder period,

characterized by temperature inversion and increased fossil fuel burning, may explain this discrepancy.

Overall, higher sun radiation and outdoor temperatures seem to be important factors in explaining the observed higher DNA damage levels in warmer/sunnier seasons.

2.2.3.2. Environmental exposure

Anthropogenic pollution has become inherent to the modern environment. The rapid global increase in technogenic stress in the biosphere raises the question about possible consequences for biota, including humans, acknowledging that all forms of life are inter-connected and that human health is strongly linked to the ecosystem's health [61]. Environmental chemicals and contaminants are ubiquitous, occurring in water, air, food and soil. While some chemicals are short-lived in the environment and may elicit no harmful effects in humans, other chemicals bio-accumulate or persist for a long time in the environment or the human body due to frequent exposure, potentially leading to adverse health effects [62]. Various studies on comet assay endpoints in biomonitoring studies on environmental exposures have been reviewed elsewhere [63].

Among environmental exposures is air pollution, which is ubiquitous and difficult to assess without specific equipment, although the relevant literature describes both personal exposures as well as modelled data based on stationary monitoring station recordings of ambient air pollution components. Air pollution, primarily particulate matter from combustion processes, adversely affects human health, increasing both mortality and morbidity. The comet assay is being employed to establish the impact of air pollutants on DNA damage in different population and occupational groups (reviewed in Møller and Loft [64]).

Temporal and spatial variations in air pollution levels are very often used to obtain exposure gradients. In a study of people in Benin either living/working near to persistently heavy traffic, or in a rural environment, Fpg-sensitive sites in PBMCs increased according to location and personal exposure to benzene as a specific component in fuel as indicated by urinary S-phenylmercapturic acid (S-PMA), a biomarker of benzene exposure [65]. Examples of studies that have employed gradients in air pollution levels come from studies in Denmark where personal exposure to levels of ultrafine particles from traffic correlated with levels of Fpg-sensitive sites in PBMCs [66,67]. The same research group has reported that traffic-related air pollution in a chamber was associated with higher levels of DNA damage in PBMCs [68], whereas short-term exposure to even a high concentration of diesel exhaust (276 μg/m^3 for 3 hours) had no effect on DNA strand breaks or oxidatively damaged DNA in PBMCs [69]. Wood smoke is another important source of particulate matter, which is increasingly relevant as an environmental exposure with the increasing number of wildfires. Nevertheless, recent studies have shown mixed results - in chamber studies after short-term exposure [70,71], or a one-week stay in a reconstructed Viking Age house with an indoor open fire [72], or in wildfire firefighters [73]. Likewise, a recent chamber study showed that 5.5 h exposure to household dust (275 μg/m^3) did not alter levels of DNA strand breaks or oxidatively generated purine lesions in PBMCs [74]. The collective results indicate that traffic-related air pollution has a greater effect on the types of DNA lesions that are detected by the comet assay, compared with either wood smoke or dusts in household air. Also, gases such as carbon monoxide, ozone, nitrogen oxides, sulphur dioxide are examples of atmospheric pollutants that may lead to DNA damage and pose a serious threat to human health. Other applications of the comet assay regarding air pollution include exposure to formaldehyde and nitrogen dioxide in

children living near a chipboard factory in Italy [75], PAHs in the air in children in Mexico [76], and pollution containing cadmium, lead, dichlorodiphenyldichloroethylene (p,p'-DDE), hexachlorobenzene, polychlorinated biphenyls (PCBs) and benzene in residents in Flanders [77].

2.2.4. Extrinsic and modifiable factors

In daily life people are in contact with many agents with the potential to provoke or prevent mutagenic and carcinogenic effects [78]. In comparison with intrinsic and non-modifiable factors such as age and sex, these exposures are not constant as they may display day-to-day variation and may be avoided (e.g. by subjects disliking the taste of dietary items) or actively sought for personal reasons (e.g. regular physical activity). Such factors might not cause confounding in a study as they may be highly individualized. For instance, ingestion of certain dietary items may be associated with genotoxicity, but there are few subjects with the relevant dietary habit in the source population. Thus, in the rare event of such a person entering a biomonitoring study, the biomarker value is likely to be regarded as an outlier.

2.2.4.1. Dietary exposure to toxic substances

There are relatively few studies that have assessed the influence of dietary genotoxic compounds in humans. An example of such an investigation comes from a biomonitoring study on aflatoxin exposure in the Gambia [79]. However, genotoxic effects of food mutagens have been investigated in cell cultures or animal models. An interesting example of a hybrid approach is a study where the comet assay was used to

examine the negative influence of foods such as red meat, using cultured human colon cells exposed to human faecal waters [80].

2.2.4.2. Dietary macro-and micro-components

The comet assay is used extensively in human biomonitoring to assess the impact of whole foods (e.g. fruits and vegetables), specific nutrients (phytophenols and antioxidants) and supplements (e.g. folic acid, selenium, carotenoids) on biomarkers of DNA stability, including DNA strand breakage, and altered DNA bases (e.g. oxidation, alkylation and misincorporated uracil).

The ability of whole foods to protect against DNA damage has been tested extensively using the comet assay in observational studies. Historically, a high intake of various foodstuffs, such as fruits, vegetables and juices, that are rich in antioxidant vitamins and phytophenols, has been found to be positively associated with low levels of endogenous DNA strand breakage and oxidised DNA bases, and to protect against *ex vivo* generation of DNA damage (reviewed in Dusinska and Collins [81]). A recent cross-sectional study focused on habitual ingestion of fish, vegetables, fruits, salads, whole-grain bread and potatoes in a relatively large study of subjects (n = 973) in a high-income area of Copenhagen, Denmark [82]. This showed an inverse association between intake of fish and levels of Fpg-sensitive sites in women after adjustment for various other life-style factors. The same inverse association was also seen in univariate analysis in men, but it was not robust in adjusted analysis, whereas salad intake was inversely associated with levels of Fpg-sensitive sites in adjusted analysis. Although this is merely a cross-sectional study, it points especially to the intake of fish as a food category that may have a protective effect on levels of oxidatively damaged DNA in

humans. It is an observation that needs to be confirmed in a well-designed intervention trial.

Diet and dietary components have been shown to play an important role in the protection against DNA damage in well-designed intervention studies. The comet assay is used widely to investigate the influence of intervention with specific nutrients (primarily as supplements) and whole foods on biomarkers of genomic stability, including DNA strand breakage and base damage. In a ground-breaking study testing the antioxidant hypothesis of DNA damage and cancer, supplementing male smokers and non-smokers with vitamin C (100 mg), vitamin E (280 mg) and β-carotene (25 mg) daily for 20 weeks, substantially decreased endogenous DNA pyrimidine oxidation (measured using EndoIII) and significantly increased resistance to hydrogen-peroxide-induced DNA strand breakage *ex vivo* [83].

Several medium- to long-term intervention studies have shown reduced levels of DNA lesions after ingestion of bioactive-rich foods and/or food bioactives [84-89]. However, the protective effects have also been detected after the intake of single portions of bioactive-rich foods and/or components [90-93]. This modulation seems to be dependent on several factors such as the type of food product(s), the bioavailability of the constituent(s) and the length of time between food consumption and DNA damage evaluation.

There have been few studies evaluating the acute effect of bioactive-rich foods on the levels of oxidized purines and background DNA strand breaks, with conflicting results. Bakuradze and co-workers [94] found that the intake of 200 ml of coffee, every 2 hours until 8 hours, significantly decreased background DNA strand breaks levels in healthy volunteers. Brivida et al. [95] showed that the consumption of 1000 g of organically or conventionally grown apples, providing 308 and 321 µg/g fresh weight of total

polyphenols respectively, decreased the levels of EndoIII-sensitive sites at 24 hours after consumption. In contrast, Del Bo' and colleagues [91,96] reported no significant effect on DNA strand break levels and Fpg-sensitive sites following acute consumption of blueberries in smoking and non-smoking volunteers.

Folate deficiency very specifically results in the misincorporation of the RNA-associated base uracil into DNA, consequently inducing DNA single and double strand breakage and chromosomal damage [97]. One modification of the comet assay has allowed uracil explicitly to be measured in human lymphocyte DNA [98]. Here, nucleoids, post-lysis, are incubated with the bacterial DNA repair enzyme uracil DNA glycosylase, revealing misincorporated uracil in the DNA. In a randomised double-blind placebo-controlled intervention, mis-incorporated uracil, measured using the alkaline comet assay and uracil DNA glycosylase, was significantly decreased in healthy men and women supplemented with 1.2 mg folic acid each day for 3 months [99]. Moreover, it was also found that improved folate status (red cell, lymphocyte and plasma folate) strongly correlated with the reduction in DNA uracil mis-incorporation, highlighting the value of this modified comet assay in human biomonitoring [99].

A special application of the comet assay in dietary intervention studies has been the challenge assay where the resistance to *ex vivo* induced DNA damage by an oxidizing agent such as H_2O_2 has been measured. Protection has been reported following either an antioxidant cocktail of β-carotene, vitamin C and vitamin E [83] or more complex food sources such as carrot juice [100], tomato extract [101], kiwi fruits [102,103], broccoli [104,105], wild blueberry [106] and hazelnuts [107]. However, some studies reported no effect [108-110].

On the whole, bioactives should be recognized as potential confounding factors (see 2.2.3). This could represent a critical aspect within biomonitoring studies in which the

evaluation of DNA damage by the comet assay is performed in different subjects exposed to diverse conditions. On the other hand, evidence is very scarce or non-existent concerning the impact on DNA damage of the consumption of meals differing in macro and micronutrients or the impact of supplements and their possible antagonistic and/or synergistic effects. The dietary intervention studies have been instrumental for research on specific macro- or micronutrients. However, they also tend to be somewhat excessive in the exposure. For instance, it will be difficult to find a person who daily eats 300 g blueberry purée or drinks 500 mL of kiwifruit juice [91,93]. The results from the dietary intervention studies may not be directly applicable for biomonitoring guidelines. In that respect, it should be emphasized that certain intervention studies have selected specific groups of the population to avoid confounding, and the sampling of cells has been optimized to detect the effect on DNA damage levels. Generalization of the observations from dietary intervention studies to the general population is uncertain. Conservatively, it is recommended that the evaluation of DNA damage is performed on samples of blood that have been collected on a standardized time schedule (e.g. after an overnight fast). Such standardization of factors related to diet and overall behaviour could help to minimize potential bias and improve the comparison of data deriving from different studies.

2.2.4.3. Physical activity

The comet assay has been used to investigate the potential induction of DNA damage after physical exercise since it is known that this practice causes oxidative stress [111]. DNA instability has been assessed in two different scenarios; in volunteers just after performing exhaustive physical activity or in volunteers who regularly perform physical activity.

Hartmann et al. tested the effect of a short run with increasing speed in 3 healthy volunteers and found an increase in DNA strand breaks in WBCs just after the exercise [112]. The effect was also seen after 6 hours and it reached a maximum after 24 hours. DNA strand break levels were back to basal level after 72 hours. In the same study volunteers were subjected to a 45 minute run at fixed speed to ensure aerobic metabolism, and the aforementioned effect was not seen [112]. The same authors demonstrated how vitamin E supplements decreased the DNA damage detected after 24 hours of running on a treadmill until exhaustion [113]. They also showed that although trained and untrained volunteers exhibit a significant increase in the DNA damage of WBCs after exhaustive exercise, the increase was higher in untrained than in trained volunteers, suggesting an adaptation process [114]. Some years later, the same group performed a study in which they measured DNA damage in 6 athletes after a short-distance triathlon competition [115]. No effect was seen after the exercise; however a significant increase in DNA strand breaks was seen after 1 to 5 days in a biphasic pattern, with a small peak after 24 hours and a larger one after 3 days. After 5 days, the basal level was not reached. However, the Fpg-modified comet assay did not reveal any extra damage. Likewise, there were unaltered levels of Fpg-sensitive sites and DNA strand breaks after a triathlon race, whereas the levels of EndoIII-sensitive sites were slightly increased at day 5 post-exercise [116]. A shorter bout of exhaustive bicycle exercise also did not change the levels of DNA strand breaks, Fpg-sensitive sites and EndoIII-sensitive site when performed in normoxia, whereas there were increased levels of DNA strand breaks immediately after exercise in high altitude hypoxia [117]. The increase in DNA damage after a bout of exercise has also been seen in forty older adults and in young soccer players after performing physical activity [118,119]. The young soccer players did not recover the basal DNA damage level even after 45 days [11].

Esteves et al. showed how the level of DNA oxidation damage in dancers, measured using Fpg, significantly increased after the dancing season [120]. However, the pre-season level was lower than in a control group. In this case, the levels of DNA strand breaks were not significantly affected.

The effect of regular exercise on the basal level of DNA damage in volunteers who exercise regularly has also been tested. While some authors report a lower level of DNA damage in lymphocytes of people who perform regular exercise [19,120-122], others did not find any association between exercise and the level of DNA damage [37,123] and some even report a higher level [124]. In this last study, even the H_2O_2 resistance was lower in sportsmen doing regular exercise, namely rugby players. In a recent study, the DNA damage level found in lymphocytes was lower in the recreational group compared to sedentary and lifelong amateur endurance practice volunteers [125].

To sum up it is quite clear that DNA damage increases after a bout of exhaustive exercise but the effect is not so clear in people who regularly take part in sport. An adaptive response was proposed in the case of low levels of DNA damage found in sportsmen [114].

2.2.4.4. Alcohol consumption

Acetaldehyde, the first metabolite in alcohol degradation, induces crosslinks and forms adducts with DNA and proteins [126,127]. There are several human studies in which the effect of alcohol consumption on the DNA damage has been studied using the comet assay.

Studies performed in alcoholics showed that they present higher levels of DNA damage in blood cells than do control subjects [128,129]. A positive correlation between alcohol

intake and DNA damage has also been found in healthy individuals [27,130]. In contrast, Pool-Zobel and co-workers showed that DNA damage in rectal cells and lymphocytes, both DNA strand breaks and EndoIII-sensitive sites, was lower in male patients with alcohol abuse than in controls [131]. However, the number of participants, especially of the controls, was very low. Løhr et al. showed a significant positive association between alcohol intake and Fpg-sensitive sites in men but not in women [28]. The study was performed in 1,019 subjects (aged 18-93) with the aim of studying the association of the levels of oxidatively damaged DNA and metabolic risk factors. Milić et al. did not find any increase in DNA damage in blood cells of alcohol consumers compared to non-consumers [132].

Alcohol consumption has been included as co-variate in the statistical analysis (i.e. as a possible confounder) in several studies, showing conflicting results. Some studies have reported a positive association between alcohol consumptions and DNA damage in blood cells [133,134]. A lack of association between DNA damage in blood cells and alcohol consumption has been reported by several authors [135-139]. Moreover, the lack of association has also been reported in exfoliated buccal cells [140].

Conflicting results can be due to the amount or the type of alcohol consumed; many of the reports do not give this information. Also, the sample size can have a great impact on the reliability of results. Moderate alcohol consumption, especially wine drinking could also have beneficial effects on redox status and DNA stability. It has been hypothesized that regular consumption of moderate doses of wine could provide health benefits. Wine's beneficial effect has been attributed principally to its non-alcoholic portion, which has antioxidant properties [141-143]. An important issue to mention is that acetaldehyde induces DNA crosslinks, which can indeed decrease DNA migration.

This is a factor to take into account when interpreting results, and could be a cause of conflicting results.

3. Sample type and collection

Sample collection is an important step and variability needs to be reduced as much as possible in order to obtain reliable data. Each laboratory should set up standard collection procedures and adhere strictly to them throughout the experiments.

In particular, it is necessary to define the following during the planning phase: subject conditions at collection time (i.e. fasted or not for blood samples, timing during the day if relevant, etc.); collection modality; choice of anticoagulant for blood, cell maintenance medium for all cells, enzymatic treatment where required; temperature and maximal extent of bench time (from sample collection to comet slide preparation); container and tube types; sample identification and documentation.

3.1. Blood and blood-derived samples

3.1.1. Blood collection and anticoagulant

Blood is obviously the most suitable and widely used tissue in human biomonitoring, due both to the low invasiveness of the withdrawal and to the abundance of information available on blood composition deriving from routine clinical biochemistry. The majority of studies use venepuncture but blood collection with a lancet could also be suitable as only a small volume of blood is necessary and mean baseline levels of damage from pin-prick samples and samples collected by venepuncture have been described to be similar [144].

The choice of anticoagulant may be crucial, as an increase in plasma DNA (from damaged leukocytes) has been reported to occur beyond 6 h in samples collected with citrate or heparin [145]. However, whether the choice of anticoagulant has any effect on comet assay results has not been established, and EDTA, citrate and heparin are all currently used.

3.1.2. Different blood-derived preparations and cell types

Among blood cells, the most used so far are gradient-isolated mononuclear leukocytes, often referred to as "lymphocytes", although in this fraction monocytes are also present in a significant proportion (about ¼ of the mononuclear fraction). The reason to focus on this subpopulation, besides the fact that it is more homogeneous than total leukocytes, is that PBMCs circulate throughout the body, have a reasonably long life span, and can therefore serve as sentinel cells in biomonitoring studies [146]. However, it has been shown that the procedure of PBMC isolation is not devoid of consequences on the basal levels of DNA damage: strand breaks are increased in gradient-isolated PBMCs compared to non-isolated, although oxidized bases measured as Fpg-sensitive sites are not modified [147]. Bausinger and Speit reported that radiation- and alkylating agent-induced DNA damage is decreased in PBMCs compared to whole blood [148]. The response to *ex vivo* oxidative stress is also much higher in PBMCs than in whole blood [147]. This is actually an advantage of the isolated cells, especially in intervention studies where antioxidant/protective treatments are tested. In fact, the basal levels of DNA damage in blood cells are usually very low in healthy volunteers, and it would be difficult to measure a further decrease of these levels; instead, it is possible in this context to evaluate the protection from *ex vivo*-induced DNA damage, reflecting the antioxidant status [91,93].

An increasing number of human biomonitoring studies with the comet assay have used whole blood, thus avoiding the separation procedure [149-151]. In this case only nucleated cells, i.e. the leukocytes can be studied. Using whole blood is simpler, but since it contains the complete spectrum of leukocytes, results obtained with whole blood and with isolated PBMCs may not be comparable. The possibility has been described to score separately polymorphonuclear and mononuclear cells in whole blood slides, but the procedure is time consuming, and the visual distinction can be subjected to variability originating from both different operators and different employed assay conditions [147].

In biobanks (see section 5.3 Long-term storage of frozen samples), blood is usually stored either as such or as buffy coat, and the possibility to work with these samples can give access to large epidemiological retrospective studies. For this reason, buffy coat has recently received attention from researchers interested in the use of the comet assay in human biomonitoring. The preparation of the buffy coat involves a simple centrifugation of whole blood, producing a layer enriched in concentrated leukocytes at the top of the red cells) that can consequently be aspirated in a small volume. In the end, a blood preparation with 5-6 times concentrated leukocytes is obtained, in which the WBCs still benefit from the protective effects of plasma and red blood cell antioxidant power. Indeed, unpublished studies indicate that the basal levels of DNA damage measured in buffy coat preparations are not different from those measured in whole blood, supporting the hypothesis that this preparation can be equally useful. An advantage of buffy coat compared to whole blood is that it is often available in biobanks [152]. Still, it needs to be verified whether the presence of free radical-producing neutrophils can modify the basal levels of damage with time, particularly upon long-term storage, given the presence of protective red blood cells [153].

Although most of the studies use leukocytes from whole blood, there is increasing interest in assessing primary DNA damage in salivary leukocytes especially in terms of air pollution impact. These cells, directly exposed to air passage through the mouth, represent the target cells of this exposure and in that way are more appropriate than blood cells for the evaluation of its effects. Moreover, they are easy to retrieve, especially in children, using a simple and non-invasive method, which allows obtaining a substantial sample size. Despite that, some problems have been identified, such as the number of cells retrieved per sample, the interference caused by the buccal epithelial cells on the comet images, and the consequent time-consuming analysis of the slides [154-156].

3.2 Epithelial cells

Epithelial cells amenable for human biomonitoring are exfoliated cells from easily-reached epithelia, such as those in mouth and nose, which are also first-contact sites for environmental pollutants. Cell collection might be relatively easy, but the number of cells obtained is usually quite low, although this is generally not a problem with the comet assay, which can be run with very small numbers of cells. Another important issue with exfoliated cells is their physiological state (dead, necrotic/apoptotic, damaged or healthy) that can influence the measured levels of DNA damage: thus, the assessment of cell viability appears to be particularly important before proceeding to the measurement of DNA damage. Furthermore, in some cases there might be a need to separate cell aggregates through enzymatic digestion. For all these reasons, usually these cells display higher basal DNA damage levels than blood or cultured cells: however, it has been shown that it is still possible to detect increases in DNA damage in association with different environmental stressors. Sampling protocols, sample storage

and preparation for the most used cell types, and the adaptations of the method for each specific context, have been reviewed by Rojas et al [157].

The use of exfoliated buccal cells has been proposed and repeatedly used for genotoxicity testing in human biomonitoring both with the micronucleus test and with the comet assay [158]. These cells can be simply obtained by mouth wash or oral mucosa brushing. Increases in DNA damage in these cells have been reported in occupational settings [159], and in association with lifestyle and environmental factors such as tobacco smoking, pollutants in drinking water [160], or radiations [161]. It has also been proposed that these cells can be a useful tool in nutritional studies [158].

Nasal cells are obtained by gently brushing the turbinate epithelium [162,163]. This cell type has an obvious advantage over circulating leukocytes in studies on inhalation exposure to airborne pollutants in the ambient air or workplace. In some cases of air pollution exposure, the results obtained in nasal cells were different from those obtained in peripheral leukocytes, indicating that these epithelial cells can be a better marker of damage, at least for some types of exposure or pathology [164]. Although this type of cell is very relevant for respiratory exposure, the brushing procedure, although minimally invasive, is reported to be unpleasant for the donor, and probably for this reason it has not gained as much popularity as it might have.

Tear duct epithelial cells are also exposed to environmental pollutants, and can be simply recovered from tears. Up to date, only one study has used them in human biomonitoring [165], finding a DNA-damaging effect of urban atmospheres with high ozone concentrations.

An interesting new application of the comet assay involves cells from surgically removed tissues from the human eye, including corneal epithelium and endothelium,

lens capsule, iris and retinal pigment epithelium [166]. This cell type is interesting due to the direct contact of airborne DNA damaging agents with the epithelial cells of the eye, although the technique needs to be further developed and validated.

Exfoliated bladder cells can be recovered from urine and used for routine urine cytology, and they have also been used for DNA damage measurement with the comet assay. In rubber workers at risk of bladder cancer, comet assay results showed a better concordance with cytoscopy than with urine cytology [167].

3.3. Biopsies

The comet assay is routinely used to detect DNA damage on tissue samples in genotoxicology studies. In fact, the OECD guideline for the *in vivo* comet assay (TG 489) refers specifically to the use of tissue samples in animal models [168]. However, this application of the comet assay is rarely used in research projects on healthy humans. A rare example is the use of the comet assay, including the enzyme-modified comet assay, on muscle biopsies from healthy human volunteers who were exposed to high altitude hypoxia [169]. In clinical contexts, cells from biopsies, such as colon biopsies have also been used [131].

4. Sample collection timing

Sampling conditions such as sampling time can be an important factor in the comet assay because DNA damage can increase, if environmental conditions are not optimal. It is suggested that seasonal variations and sampling time play a more important role in the comet assay than in other cytogenetic assays such as micronuclei and chromosomal aberrations [170]. In biomonitoring studies, it is advisable to take blood samples at the

same time from fasting subjects (usually in the morning hours) and to transport them in cool conditions. However, it is more important to collect samples from all exposed and control subjects at the same time of the day to reduce the possibility of time variations that can influence the results [171]. If a study extends over more than a year, samples should be taken in the same season in each year. When blood was sampled in the warmer period of the year, comet assay descriptors were found to be higher [33,81].

5. Sample processing and storage

5.1. Bench time

Ideally, the comet assay is performed immediately following peripheral blood collection. However, in large human studies or studies requiring sampling far from laboratories, these optimal conditions are not feasible. In this context, storage conditions of whole blood should be optimised before performing the comet assay. It can also be a good strategy to cryopreserve samples in studies with repeated sampling from the same subjects such as dietary interventions or environmental exposures (e.g. air pollution). The reason is that the exposure contrast may produce less response (i.e. delta-value between exposure and control period) than the inter-day assay variation.

In some cases, samples can be processed within several hours, and the comet assay is performed on fresh cells. Koppen et al. reported a weak decline of DNA strand breaks with increasing storage time on the bench, for samples processed within 6 hours of sampling. Turbulence and shearing during blood drawing might have induced mechanical damage upon sampling, being restored during the time after collection [172]. The same was also described by Dusinska and Collins [81] in PBMCs processed within 4 hours after sampling. On the other hand, in both studies, an increase in Fpg-

sensitive sites was observed when blood samples were stored at room temperature during those time periods.

Anderson *et al.* reported that storage for up to 4 days at either room temperature or 4°C did not induce any DNA strand breaks in human whole blood measured with the comet assay [173]. Similar conclusions that samples can be stored for up to 4 days at both room temperature and 4°C without affecting the level of DNA strand breaks were found in the study from Collins [174]. Chang and Hu have also shown that DNA strand breakage in whole blood was stable up to 4 hours at 4°C after isolation [175]. In contrast, the study from Narayanan et al. described an increase in DNA damage when human blood was stored at room temperature or 4°C for 24 hours, with a further increase after 48 hours of storage [176]. More recently, Al-Salmani and co-workers also reported that overnight storage (12 hours) of a large volume of blood (5 mL) at 4°C tended to induce an increase of damage [144].

The temperature, volume and duration of blood storage appear to be crucial parameters to consider when processing samples for the comet assay. Altogether, these studies suggest that some components of whole blood can be considered as preservative agents but these effects are limited, and temperature, volume and time dependent. Moreover, some components and reactions can also alter the DNA, e.g. lysis of red blood cells and presence of neutrophils that can undergo an oxidative burst.

Due to inconsistent results from all these studies, short time storage (less than 4 hours), at 4°C or room temperature is advised in order to avoid any DNA damage between whole blood collection and processing for the comet assay.

5.2. Effect of transport

Shipping of whole blood, kept in an isolated package, to keep the blood at room temperature (ca. 15-35°C) is a feasible option based on the results of Anderson et al. [173] and Koppen et al [172]. They showed that whole blood samples stored at room temperature up to respectively 4 and 3 days, did not show an increase in DNA strand breaks.

5.3 Long-term storage of frozen samples

Storage of samples represents a common practice in biomonitoring studies where, for logistic reasons, the comet assay is not generally performed on fresh material. In fact, the majority of human data on DNA damage are derived from analyses performed on cryopreserved cells, but in some cases, there is no mention of the storage process (e.g. method of cryopreservation, duration of storage). Both whole blood and isolated PBMCs can be frozen and stored at -80°C or in liquid nitrogen. However, the freezing method, the medium used for cryopreservation, storage conditions and the thawing process, could all represent potential critical factors affecting cell viability, DNA damage levels, and cellular responses (such as DNA repair). At the moment, there are various approaches to sample cryopreservation. For whole blood, some researchers suggest flash-freezing small volumes (max 250 µL) of samples in order to avoid crystal formation [144]; others report that diluting whole blood with an equal volume of cell culture medium containing 20% DMSO prevents damage during freezing [177]. Regarding PBMCs, some studies suggest suspending cells in PBS or medium with 10% DMSO [41], while others report suspending cells in 50% foetal bovine serum, 40% medium and 10% DMSO [13]. The number of cells stored could be an important factor to be standardized. For example, to improve cell viability it is recommended to freeze PBMCs at concentrations of $\leq 3\times10^7$ cells/mL [178]. For optimal cryoprotection of

PBMCs, slow freezing is recommended (ideally 1°C/min) using a commercial 'Mr Frosty' freezing unit with isopropyl alcohol, or a well-insulated expanded polystyrene box.

A different approach was used by Zhang et al. where DNA was successfully preserved in freeze-dried somatic cells using trehalose as protectant and storage at or below 4°C [179]. Furthermore, solid tissues for later comet assay analysis are typically stored via snap freezing in liquid nitrogen, keeping the tissue deep-frozen until they are rapidly homogenized and embedded in agarose [180].

Regarding the effect of long-term storage on DNA damage, the information available is limited and insufficient to exclude the existence of a significant impact. Several studies have reported an increase in DNA damage with the freeze-thaw process compared with that found in different fresh cells [181-183], while others reported no effect on the levels of DNA damage [144,184,185]. These conflicting results could be attributed to the time and storage temperature of cryopreservation used. For example, Del Bo' et al. documented an increase in the levels of background DNA damage and oxidized purines after 1 year of PBMC storage at -80°C compared with fresh samples [186]. In contrast, Duthie and co-workers showed that the levels of oxidized pyrimidines, oxidized purines and uracil were similar in both freshly isolated and frozen lymphocytes [185], implying that the DNA damage that is already present in the DNA of fresh cells is maintained throughout the isolation and storage procedure. However, the samples were stored only for 2 months. Pu and colleagues showed that the storage of lymphocytes at -80°C up to 28 days did not affect the DNA damage, while the storage at -20°C for 14 and 28 days increased both DNA strand breaks and oxidatively damaged DNA compared to fresh cells [181]. Regarding the use of whole blood, the same authors found no significant changes in samples stored at -20°C for 1 day or 7 days, or at -80°C for up to 28 days

compared to fresh samples, while they documented significant increases in both DNA strand breaks and DNA base oxidation in whole blood samples stored at -20°C for 14 or 28 days [181]. Akor-Dewu and colleagues showed that freezing whole blood at -80°C is a suitable method for storing samples, at least for periods of a few weeks or months, for subsequent analysis of DNA strand breaks and Fpg-sensitive sites [184]. However, the ability of H_2O_2 to induce DNA strand breaks is severely attenuated in whole blood compared with freshly isolated or frozen PBMCs. Leukocytes isolated from frozen blood showed higher basal levels of damage (strand breaks/alkali-labile sites) and an abnormal resistance to damage by H_2O_2 compared to PBMCs isolated from fresh blood [184], though after repeated washing (to remove residual erythrocyte components) their sensitivity to H_2O_2 resembled that of PBMCs.

The ultimate in long-term storage is the biobank. Biobanks are repositories storing biosamples, in -80°C freezers or in or above liquid nitrogen, generally for research and/or clinical purposes. They represent a potentially valuable resource for DNA damage analysis, but only if appropriate freezing and storage conditions have been followed. Cryopreservation protocols are available for PBMCs [185,187,188] as well as whole blood [144,152,172]. Protocols and try-outs for preservation of DNA in buffy coat samples were recently tested [152,153,189].

Assuming an appropriate preservation method was used, any type of sample from a biobank can be used for assessment of DNA damage using the comet assay. European biobanks can be identified through the biobank directory of the European Research Infrastructure Consortium: Biobanking and BioMolecular resources Research Infrastructure (BBMRI-ERIC) (http://www.bbmri-eric.eu/). The platform describes the nature, origin, destination, and the biobank lifecycle of each biospecimen (time of collection, pre-registration, receipt, processing, quality control, storage conditions,

request for use, retrieval, and distribution). Experience with the comet assay on samples stored for extended periods is limited, and careful evaluation of results will be needed, at least until optimal conditions for cryopreservation and for reducing adverse effects of storage have been defined.

6. Statistical analysis

Due to the epidemiological nature of biomonitoring studies, a critical evaluation of statistical methods for the analysis of comet assay results should take into consideration the study design and the control of external variables. Therefore, critical issues include a proper estimation of the sample size, the detection and control of bias during study design, and strategies to control for confounding and to search for effect modification. As a matter of fact, the question to be answered by a biomonitoring study is not if an effect of the condition under evaluation is present or not (p value), but how large is this effect (relative to the unexposed controls), and how reliable is this estimate (confidence interval).

6.1. Statistical considerations

The comet assay used in biomonitoring studies shares some basic features with other biomarkers used for the measurement of early pathogenetic events, such as high technical variability and poor agreement between scorers, the departure from normality, the need to define a fixed number of cells to be scored, etc. On the other hand, this biomarker is characterized by a number of peculiar features. For example, several endpoints can be evaluated for the same physical outcome (i.e. migration of DNA in the agarose gel). Even when statistical analyses include only the four most frequently used endpoints (i.e., tail intensity, tail length, tail moment and damage index), a problem of

multiple comparison may originate in many cases, because more than one endpoint is often measured in the same study.

The possible approaches for design and analysis of experimental studies using the comet assay have been described in several papers [190]. Much less literature is available for the use of this biomarker in biomonitoring studies. A critical evaluation of the most used statistical approaches has been published by Collins et al. [41]. The most common statistical procedures entailed univariate parametric and non-parametric techniques, while regression analysis was carried out in nearly one third of the 50 studies reviewed. Standard approaches used in epidemiologic studies, such as controlling of confounding and testing for interaction, were seldom applied. The use of the p-value to compare study groups was common, while quantitative measures of effect (point and interval) were used occasionally.

To describe the endpoints of the comet assay, measures of central tendency are commonly computed, usually the arithmetic mean and the median. This is generally reasonable when the sample size is large and the frequency distribution is unimodal or not markedly asymmetric. In this case, for descriptive purposes only, reporting arithmetic mean and standard deviation is correct. However, if these conditions are violated, the range of minimum/maximum values and some significant percentiles, for instance 25-*th*, 50-*th* (median) and 75-*th*, should be used instead, as they are less sensitive to the above-mentioned violations [191].

With appropriate transformations, it is possible to approximate the distribution of various endpoints of the comet assay to the *normal distribution*, which exhibits important statistical properties. In many cases it is suitable to use the original value logarithm to improve the distribution normality [192]. Whenever the data distribution does not differ from a normal distribution, the levels of DNA damage can be compared

between two groups by applying the Student's *t*-test. When the analysis is aimed at comparing three or more exposure groups, a natural extension of the *t*-test is the *analysis of variance* (*ANOVA*) [192]. When the sample size is small, and/or log-transformation does not work, a non-parametric approach is preferred. The *Mann-Whitney test* is an efficient non-parametric approach to compare two unpaired groups. If the data are paired or matched, then the *Wilcoxon matched pairs test* provides a valid alternative to the paired *Student's t-test*. The *Kruskal-Wallis test* can be applied as a non-parametric alternative to the *ANOVA*.

In observational investigations of human populations, individuals may differ in several conditions, including lifestyle, exposure to occupational and environmental toxic agents, or genomic profile. If the distribution of conditions affecting DNA damage is unbalanced in the study groups, the association under study can be *confounded* by the action of these factors. *Effect modification* is another important feature, since it identifies subpopulations that respond differentially to the condition under study. While the confounding effect of external variables can be taken into account in the statistical analysis of data, the presence of effect modification can be merely identified and separate analyses for relevant subpopulations should be performed. The most straightforward, efficient and powerful way of reducing confounding and assessing effect modification is *statistical modelling* [41, 191]. With the use of statistical modelling it is possible to quantify the presence of a relationship between the outcome and all considered variables (categorical or continuous) to generate estimates of association adjusted for the effect of confounders, and to assess the presence of effect modification. In the field of biomarkers, most statistical models are fitted to data to assess whether a variable predicts an outcome, and they are generally known as *regression models* [193].

In general, to build up regression model, it is necessary to define the way through which the linear predictor (*LP)* acts on the mean response (μ), and to identify a probability distribution for the response variable (*Y*), essential to estimate the parameters included in the regression equation using the *maximum likelihood* (*ML*) method. An extensive discussion on the probability distribution of data from biomonitoring studies using the comet assay can be found in Lovell and Omori [190], confirming that in most cases a normal or a log normal distribution can be assumed.

In its simplest form, that is the multiple normal (*additive*) regression, the coefficient of regression expresses the variation of μ per each unit increase of each linear predictor when the other predictors are held constant. The effect of the variables analysed on the level of DNA damage is measured in the same unit as the outcome, e.g., μm for the tail length or % for tail intensity. This feature may be misleading in the presence of a large interlaboratory variability since, for example, an increase of 2 μm in tail length may be a small variation in some laboratories while in others it may represent an important change. The shift to log transformed data to approximate the normal distribution, implies two important modelling characteristics, i.e., expected values (μ) are non-negative (due to the antilog function), and measures of effect are *ratios* (*multiplicative scale*) and not *differences* (*additive scale*). Thus, as is shown in the following equation, the measure of the association between exposure and outcome is represented by the *ratio of expected mean values* (*MR*) of exposed (E=1) to unexposed (E=0) individuals, once confounding is removed:

$$\log\left(\mu_{E=1}\right)-\log\left(\mu_{E=0}\right)=\log\left(\frac{\mu_{E=1}}{\mu_{E=0}}\right)=\beta_1 \qquad \rightarrow \qquad \frac{\mu_{E=1}}{\mu_{E=0}}=e^{\beta_1}=MR$$

Recently, traditional approaches to statistical analysis of data from biomonitoring studies have been implemented with advanced techniques, in many cases derived from

the analysis of clinical trials. For instance, multicentre biomonitoring studies may exhibit a hierarchical structure with multiple levels: if several laboratories are involved in different areas, individual DNA damage levels represent the first level of the hierarchy, while laboratories and areas represent the following levels. This type of data organization implicitly assumes that each level is nested in the higher level and represents a homogeneous *cluster* of observations. The purpose of the *multilevel regression* modelling [194] is to verify whether the hierarchical structure affects the measurements of interest. Ignoring the multilevel structure of data means that an important component of within-cluster correlation may be missed, underestimating the standard errors of the parameters evaluated. In this framework, two classes of regression parameters can be estimated using the *multilevel regression*. The first class is represented by the *fixed effects parameters* (β) which return the mean effect of covariates (exposure in particular) within or across levels. The second class are defined as the *random effects* (*REs*) *parameters* and reflect the degree of heterogeneity within each level.

6.2. Number of subjects and statistical power

Sample size evaluation is a preliminary step of study design. The study population size should be large enough to reach statistical significance if the observed effect (i.e., level of DNA damage in exposed *vs.* unexposed subjects) is as large as expected or even larger. Since the level of statistical power and the chance of the type 1 error are quite standardized, i.e., 80% and $\alpha<0.05$, the definition of the sample size is essentially based on the magnitude and the variability of the expected effect [191]. Under mild statistical conditions (normality and homoscedastic data assumption), sample size calculation can be derived from the following formula [192]:

$$N > 2\left(S\frac{z_\alpha + z_\beta}{\delta} \right)^2$$

where:

- z_α and z_β are the standardized normal deviates corresponding to α and β error rates [e.g. 1.96 (corresponding to z_α = 5%) and 0.84 (corresponding to z_β = *80%)]*;
- $\delta = \overline{y}_1 - \overline{y}_0$ is the anticipated difference in mean DNA damage between exposed and unexposed subjects respectively;
- *S* = standard deviation obtained from pilot studies or from the literature;
- *N* is the number of subjects in each group.

There are several user-friendly statistical packages available in the internet for the optimal sample size calculation. The presence of several possible endpoints, a peculiarity of the comet assay, is not a major concern, since all of them follow approximately a normal distribution (even if within a fixed interval, in the case of the damage index).

The choice of the reference value is a little more complicated. This value is the mean DNA damage that would be observed in the study population if the study factor were not present. Given the large inter-laboratory variability of the assay results, it may be unreliable to use reference values from studies performed in other laboratories or from the literature. It seems advisable to use data from previous studies conducted in the same laboratory, or even better from a pilot study performed using the same conditions as in the final study.

In some cases, it may be difficult to have a reliable estimate of the standard error, and the equation could be resolved for the ratio of the anticipated mean difference between exposed and unexposed to its variability (δ / S). Obviously, the study size will grow

larger as the expected difference between the means of unexposed and exposed individuals is smaller or its variability is larger.

7. Ethical issues

Ethical issues regarding biomonitoring studies conducted with the comet assay are not different from ethics requirements typical of other molecular epidemiology studies, because the *reasons* that justify conducting biomonitoring studies with this assay are similar to those for using other tests. The debate on ethical issues in molecular epidemiological research dates from the beginning of this discipline [195]. Often these studies do not provide direct and immediate benefits to participants; however, they may have scientific and public interest, as long as they can measure the effects of an intervention or improve our knowledge of occupational or environmental risks, allowing the implementation of preventive measures.

A desirable, distinctive feature of the comet assay is the possibility of using a variety of non-invasive procedures for biosample collection, which supports a highly favourable balance between health benefits and risks and involves minimal interference with the participants. Sampling methods, nature and quantity of the biosamples may have both ethical and practical implications, particularly when dealing with vulnerable populations [196,197].

Guidelines exist about sample collection and biorepository management, and they generally include substantive chapters on ethical issues (e.g. [198-200], inspired by the principles of the Declaration of Helsinki [201]. These guidelines are constantly updated and modified, as the scientific and social debate progresses, while technical evolution poses new ethical and legal questions and challenges. In addition, ethical and data

protection rules may vary greatly across laboratories and countries, an aspect that should be attentively considered when planning multicentric studies and international collaborations [198]. The need for a more harmonized and consistent socio-ethical and legal approach in human biomonitoring studies has been pinpointed some time ago, at least at the European level, with the aim of guaranteeing more equality in the protection of individual rights and increasing data comparability [202]. The European Cooperation in Science and Technology (COST) is a funding organization for research networks – called COST Actions. The network on human biomonitoring using the comet assay (hCOMET) strives to improve the quality of the technique in studies on human exposures and diseases. Preparation of specific guidelines with Standard Operating Procedures for human biomonitoring studies with the comet assay is one of the goals of hCOMET. The guidelines will cover all major aspects of planning and execution of this kind of research, including ethical issues (http://www.hcomet.org).

Ethical issues are governed by law in most countries. A study cannot start before submission to the concerned *ethics committee* (EC) at an institutional, regional or national level for "consideration, comment, guidance and approval" [201]. The committee must be transparent, independent and qualified.

The ECs generally include experts from several fields (e.g. lawyers, statisticians, toxicologists, psychologists) and evaluate the study design, sample collection procedures, questionnaires and informed consent documents. In addition, insurance arrangements may be required when the specimen collection can pose a risk to the participants (e.g. blood samples), and many countries also require clearance from the agencies which oversee data safety [203].

Informed consent has a central role for the respect of autonomy. It must be obtained before collecting any biological sample or clinical, social and occupational data from

the subjects participating in the study. Comprehensible information must be given to all participants on the nature and implications of the study, on potential risks, discomforts and benefits for them. It must be clearly stated that they enter voluntarily in the study and they have the right to withdraw with no consequence at any moment. Incentives or compensations, when permitted by local law, must be described in detail. Particular attention must be paid when vulnerable subjects are concerned, as is the case of adults with intellectual disabilities or children [196].

Future studies are being mentioned with increasing frequency in the informed consent forms, due to the rapid technological progress in sample analysis, the diffusion of large biorepositories, the drive towards analyses of pooled data and the spread of epidemiological study designs such as historical cohorts and nested case-control studies. Maximizing the amount of information which is obtainable from limited samples, while minimizing the burden imposed to participants is *per se* an ethical issue, and allows saving of time and resources. *Data sharing and material transfer* represent a major asset of modern molecular epidemiology; however they need to be governed by specific transfer agreements. The subjects should have the possibility to agree or not, freely and consciously, to the use of their biological data for future study questions and by other researchers in unforeseeable, unspecified assays or follow up studies [203].

Protection of *privacy* and confidentiality of data stored or processed for research purposes is the object of increasing attention by the regulatory bodies. Any biological material should be coded, and the identities of subjects participating in the study should be protected in databases through safe coding, encrypting or anonymizing procedures. Proper data management must be secured, and specific databases, not available to outsiders, may be used for storage of their personal data [200]. However, a strictly anonymous collection of samples may be almost useless for molecular epidemiological

research, which is based on linkage of laboratory results with other personal data of the subjects (e.g. occupation, health-related conditions, lifestyle, genetic profile, clinical follow up; [204]).

Finally, each subject has the right to decide whether *to be informed or not* of the results of the study, and this should be explicitly mentioned in the informed consent form. In human biomonitoring studies communication is preferably given at group level: information on individual risk might generate stress or stigmatization, and in any case the ability of the comet assay to assess individual risk is still far from being clear, when extrapolated from the research context. The development of a communication plan is recommended and may be of great help, particularly in case of reporting of unexpected results with unknown clinical significance [205].

8. Strengths, weaknesses and limitations of the comet assay in human biomonitoring

As a biomarker assay in human studies, the comet assay has these advantages:

- It can be applied to blood samples, which are obtained in a relatively non-invasive way; it is assumed that blood cells, circulating throughout the body, reflect the overall body burden of DNA damage;
- It is simple and economical to perform;
- It can be performed on samples stored frozen (though whether there is a limit on the period of storage needs to be established);
- It requires only a small number of cells (though for measurement of DNA repair, more material is needed);

- There is no need to stimulate the cells to divide;
- Other tissues can be used under certain circumstances (for example, tissue obtained after surgery for cancer);
- The measurement of oxidation damage to DNA using enzymes (i.e., Fpg or EndoIII) is relevant not just to cancer but to other diseases involving oxidative stress, whether as a cause or an effect.

There are some disadvantages and limitations:

- Circulating WBCs are not 'target' cells for carcinogenesis in solid tissues;
- Preparation of single cells is needed in the case of solid tissues, which may increase the background level of DNA damage;
- The assay shows rather high levels of variability, especially when comparing results between laboratories;
- Some forms of damage are not detected with the comet assay (bulky adducts), or cannot be distinguished (double- and single-strand breaks), or require complicated modifications (inter-strand cross-links);
- DNA damage measured in human cells is a marker of exposure to damaging agents, but there is no evidence as yet that it can be used as a predictive marker of cancer risk;
- The assay is saturated (i.e. the % tail DNA approaches 100) at a level of damage of a few thousand lesions per cell, and so its detection range is less than that of other assays. However, this range easily covers physiologically relevant levels of damage (levels that human cells can survive).

- Results with Fpg should be interpreted with caution; Fpg detects some alkylated as well as oxidised bases.
- The EndoIII-modified comet assay does not have an assay control, which implies that the results on this type of DNA damage are somewhat uncertain. Efforts should be made to develop a reliable positive assay control for the EndoIII-modified comet assay.

9. Application and interpretation of comet assay results in human biomonitoring studies

The comet assay is often used in biomonitoring studies on environmental, occupational and dietary exposures as a biomarker of exposure to genotoxic agents (or protective agents in the case of phytochemicals). This includes cross-sectional, panel (i.e. repeated sampling from the same subjects) and controlled exposures studies (e.g. intervention trials). Considering the diversity of study designs, it is not meaningful to list specific recommendations for enhancing the application and interpretation of comet assay results, although certain issues are noteworthy. The study design, way of controlling confounding factors, collection of samples and the choice of statistical analysis must be assessed on a case-by-case basis. Using one type of study design will affect the way confounding can be controlled and the choice of statistical analysis. As a biomarker of exposure to genotoxic agents, there are certain considerations relevant to users of the comet assay:

- Dealing with confounders is a challenging effort. A “one size fits all” solution does not exist as there are different ways to deal with confounders, depending on the sample population. It should also be stressed that control for confounding is not a “the more the

merrier" situation. Very strong control of confounding might introduce attenuation bias due to over-matching of exposed and unexposed subjects. At present there is not concrete knowledge on which factors are consistent and strong determinants for the level of DNA damage and which have just been associated with DNA damage in certain studies. There is relatively large inter-study variation in the effect of potential confounders as well as environmental, occupational and dietary exposures. The inter-laboratory variation may be due to differences in sampling of cells, comet assay procedures (i.e. differences in baseline levels of DNA damage), populations (i.e. susceptibility to DNA damage by the exposure), and control for confounding factors in the study design or statistical analysis. It is important to note that control for presumed confounders by restriction might yield results that cannot be generalized. For instance, if the presumed confounder is actually an effect modifier, there will be inter-study variation in the effect of the exposure between two studies that have assessed the association in the different strata of the general population.

- The sample collection depends on feasibilities and economic issues (e.g. it may only be possible to sample at one time point, which may have been selected to analyse other biomarkers with narrower window of effect). Likewise, the effect of cryopreservation and differences in DNA damage between subpopulations of leukocytes are issues that would be easy to standardize (e.g. by recommending the use of cryopreserved PBMCs), but would require adoption by all researchers.

- The proper statistical analysis is determined by the study design and control for confounding or effect modifiers. General recommendations on the statistical analysis cannot be made in the same way as for instance studies on animals or cells. However, biomonitoring studies are rarely so simple that Student's t-test, Mann-Whitney U test or simple correlation tests are appropriate statistical methods.

- The use of internal assay controls is crucial in biomonitoring studies. Assay controls should always be analysed in studies that use samples directly in the comet assay without cryopreservation because they provide information about the assay variation over time. In principle, it is not necessary to include assay control in controlled exposure studies on cryopreserved samples if they are analysed in the same comet assay run; but it increases the validity of the study and in the enzyme-modified comet assay it provides important information about the activity of the enzyme. However, it is important to note that inclusion of assay controls does not reduce the inter-laboratory variation in basal levels of DNA damage or differences in susceptibility due to effect modifiers.

10. Further development needs

This review has revealed many aspects of the comet assay where further development is necessary or desirable. Variability between laboratories would be greatly reduced if a standard protocol were adhered to; with this in mind, standard operating procedures are being devised within the hCOMET COST Action. The use of reference standards - i.e. cells with a known amount of DNA damage - should be mandatory in biomonitoring studies, serving two purposes; first, as an internal laboratory check on the performance of the comet assay on the day, and second, to facilitate comparisons between laboratories ('normalising' all data so that they are expressed as functions of the reference standard value). Ideally, reference standards could be provided by a central laboratory. There is a strong case for calibrating the assay (using X- or gamma-irradiated cells, since the frequency of breaks induced per Gy is known), so that all results could be expressed in terms of breaks per 10^9 Da, or per 10^6 base-pairs, for example.

While modifications of the comet assay to measure altered DNA bases (oxidised purines, oxidised pyrimidines, and uracil) are now used routinely to assess the impact of occupational and environmental exposures on genomic stability, new variations are being developed to further improve the specificity and effectiveness of the assay in human biomonitoring. A modified comet assay using specific restriction endonucleases (HpaII, HhaI and MspI) has been developed with the aim of measuring the global methylation status of DNA in single human cells [206,207]. Aberrant DNA methylation, both hypo- and hyper-methylation, is strongly linked with carcinogenesis *in vivo* and in humans. Adapting the comet assay to specifically detect changes in DNA methylation patterns in human subjects will undoubtedly advance understanding of how epigenetics influences human cancer risk.

DNA repair is an important facet of individual response to DNA damage effects, whether it is intrinsically determined, or inducible by exposure. As a biomonitoring tool it is somewhat neglected, as techniques for measuring it are laborious and/or require large numbers of cells. A miniaturized version of the DNA repair assay using a smaller number of cells would be very useful.

The question of whether DNA damage can be a predictive marker of cancer risk will only be answered by a prospective or retrospective trial, in which DNA damage is measured in a large number of healthy subjects who are subsequently monitored over many years for the occurrence of disease or death. The possibility of using stored blood or buffy coat samples from existing prospective studies should be explored.

There is an ongoing debate over the best method for short- and long-term storage of human blood and isolated blood cells. Many of us were surprised by the finding that small volumes of blood could be snap-frozen without any special precautions such as are routinely used to preserve larger volumes [144,184]. Validated standard procedures

would be very useful. There is also a need for further comparative study of the kinds and levels of damage in different blood cell fractions, and the correlation (or otherwise) between them.

As described above, disparate results have been reported concerning effects on DNA damage levels of smoking, various exposures, season of sampling, age, sex etc. In an attempt to resolve these issues, a pooled analysis of data from a large number of studies (involving almost 20,000 individual subjects) is being undertaken by hCOMET. Using a database this large, it may be possible to rank the effect of potential confounders and thus distinguish important confounders from those that have had little or inconsistent effect on DNA damage levels measured with the comet assay.

11. Final considerations

The comet assay for DNA damage and repair is now very well established as a biomonitoring tool, in various contexts: in cross-sectional studies, prospective cohort studies, case-control studies, and intervention trials. It has been applied to studies of occupational and environmental exposure, nutrition, disease risk, or simply used to investigate comparative levels of DNA damage/repair in different groups (for instance men, women and children, or different age groups, or vegetarians and omnivores). It is perhaps surprising that it is used successfully in spite of the excessive inter-laboratory variability that has been demonstrated in various ring studies; it would certainly be reassuring if this variability could be reduced or eliminated. It is probably too much to expect that laboratories will adopt a standard procedure, but an appreciation of which experimental variables are crucially important and which do not matter would help. It should be mandatory to include reference standards, and to report in publications the

critical experimental variables. The use of appropriate statistical methods in analysing comet assay data is essential, but to date this has too often been neglected. It should be clear that a well-performed comet assay experiment (i.e. with high technical skills in the laboratory) may be wasted if little attention has been paid to epidemiological principles including proper study design and statistical analysis. Simple statistical tests such as the Student's t-test or the Mann-Whitney U test are applicable in biomonitoring studies on binary exposures categories if there is adequate control of confounders. However, the situation is typically complex as most exposures and co-variates are continuous variables and even controlled exposure studies (e.g. intervention trials) may have subjects dropping out of the study or loss of samples. This "loss to follow-up" may lead to disproportionate distribution of confounders in exposure groups. Statistical planning to ameliorate the impact of such events should be considered as a good investment for biomonitoring studies using the comet assay.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflict of interest: None

Acknowledgements

The authors are grateful to the COST Action CA15132, 'hCOMET', for support.

Amaya Azqueta thanks the Ministry of Economy, Industry and Competitiveness

('Ramón y Cajal' programme, RYC-2013-14370) of the Spanish Government for personal support. The work of Stefano Bonassi and Monica Neri was supported by grants funded by the Italian Ministry of Health [Institutional Research 2017-2018], and by AIRC (Associazione Italiana per la Ricerca sul Cancro), IG2015/17564.

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Legends figures and tables

Figure 1. Scheme of the comet assay. Different parameters are used to describe each comet, for example % DNA in tail, tail length or tail moment, the last one being the most used parameter [3]. The mean or the median of at least 100 comets is normally used to describe each sample.

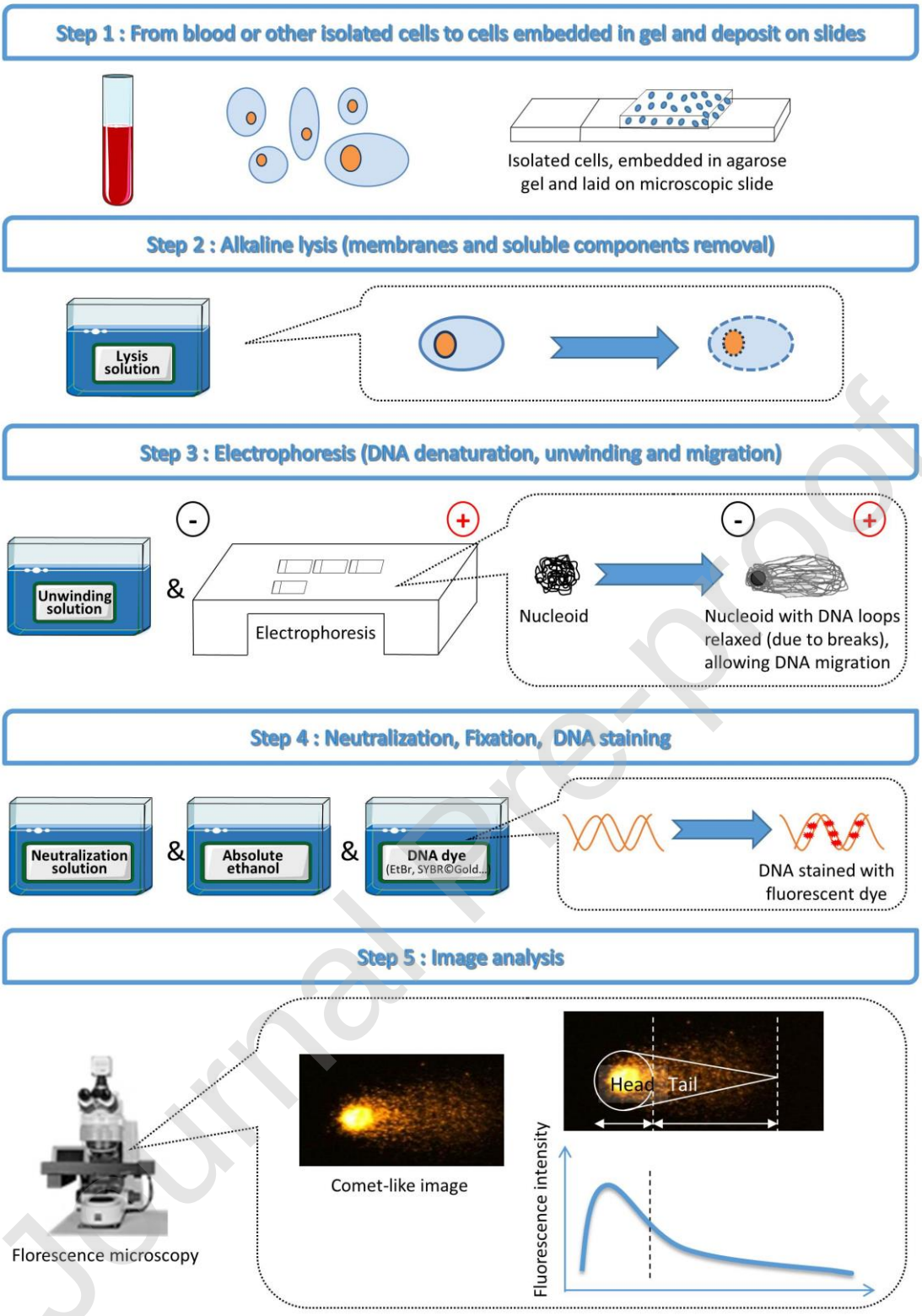

Figure 2. Effect of age and sex on levels of DNA damage. As endogenous factors, age and sex may be regarded as classic confounders, i.e. they are associated with both the exposure and level of DNA damage (A). They may also affect the level of DNA damage

through the exposure (B). As effect mediators, age and sex are observed to have an effect on DNA damage (e.g. in different strata of men and women, or old and young subjects), but this is mediated by underlying differences in exposures or life-style factors (C). Solid arrows refer to direct ("causal") relationships, whereas stippled arrows are associations ("perceived relationships"). The non-modifiable extrinsic factors are ubiquitous exposures (i.e. affecting whole populations rather than being relevant for certain individuals. These may be obtained by sampling on different days or in different seasons (i.e. seasonal variation).

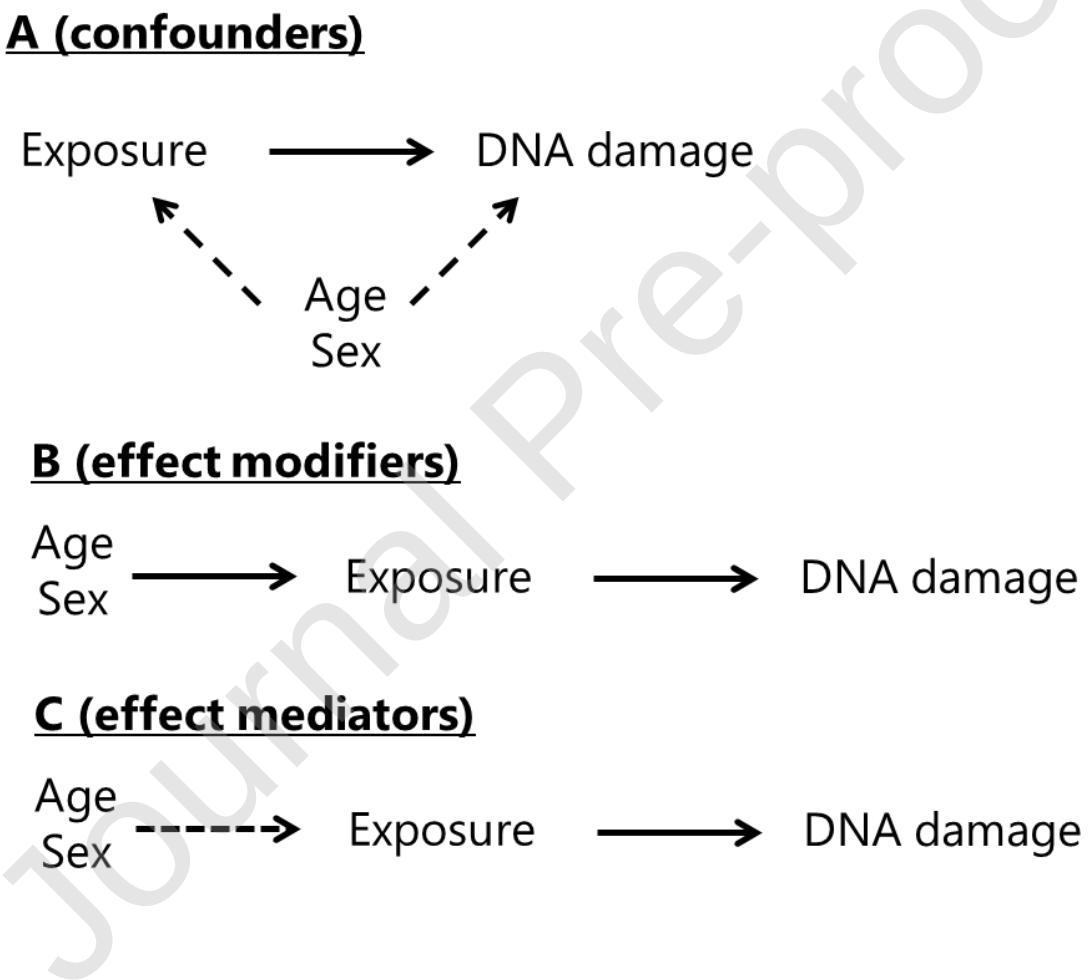

Table 1. Types of factors that can affect the level of DNA damage in biomonitoring studies

Type of factor	**Description**	**Examples**	**Controlling the influence**
Intrinsic (fixed)			
Non-modifiable	Factor that is fixed within each individual of the study. Subjects do not control the factor	Age, sex, prescribed medication	Can only be avoided by selection of subjects or statistical adjustment
Modifiable	Factor that *de facto* is fixed within each individual of the study. Subjects can change the factor, but are not able or willing to do so	Tobacco smoke, occupational exposure, obesity, over the counter drugs ("pain killers")	Can only be avoided by selection of subjects (i.e. occupational exposure) or statistical adjustment
Extrinsic (random)			
Non-modifiable	Factor that is difficult to avoid and may vary over time. The subjects do not control the source of agent, although they may control their own exposure	Air pollution, ambient temperature, sunlight	Can be avoided by sampling in short periods and having equal sampling of exposed and controls on the same days
Modifiable	Factor that varies within each individual, although it may fluctuate little due to personal habits. Subjects control the exposure	Diet, physical activity, alcohol consumption	Can be avoided by adherence to strict protocols (e.g. abstaining from exhaustive physical activity)