Effects of astaxanthin in animal models of obesity-associated diseases: a systematic review and meta-analysis.

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ABSTRACT

Background and aim: Obesity is a major risk factor for several diseases, including metabolic syndrome (MetS), non-alcoholic fatty liver disease (NAFLD) and type 2 diabetes (T2D). The use of natural products, such as astaxanthin (ASX), a potent antioxidant compound produced by the freshwater green microalga *Haematococcus pluvialis,* has gained particular interest to reduce oxidative stress and inflammation, and to improve redox status, often associated with obesity. A systematic review and meta-analysis was performed to comprehensively examine the effects of ASX in animal models of diet induced obesity-associated diseases in order to inform the design of future human clinical studies for ASX use as supplement or nutraceutical.

Methods: Cinahl, Cochraine, MEDLINE, Scopus and Web of Science were searched for English-language manuscripts published between January 2000 and April 2020 using the following key words: astaxanthin, obesity, non-alcoholic fatty liver disease, diabetes mellitus type 2, NAFLD and metabolic.

Results: Seventeen eligible articles, corresponding to 21 animal studies, were included in the final quantitative analysis. ASX, at different concentrations and administered for different length of time, induced a significant reduction in adipose tissue weight ($P=0.05$) and systolic blood pressure ($P<0.0001$) in control animals. In animal models of T2D, ASX significantly reduced serum glucose levels ($P=0.04$); whereas it improved several disease biomarkers in the blood (e.g. cholesterol, triglycerides, ALT and AST, $P<0.10$), and reduced liver ($P=0.0002$) and body weight ($P=0.11$), in animal models of NAFLD.

Conclusions: Supplementation of ASX in the diet has positive effects on symptoms associated with obesity related diseases in animals, by having lipid-lowering, hypo-insulin and hypoglycaemic capacity, protecting organs from oxidative stress and mitigating the immune system, as suggested in this review.

Keywords: Astaxanthin; meta-analysis; metabolic syndrome; non-alcoholic fatty liver disease; obesity; type 2 diabetes.

1. INTRODUCTION

Obesity is considered one of the most serious health problems in the world. The abundance and the use of energy-dense and high calories foods, smoke, stress and a sedentary lifestyle, lead to obesity, with 2 billion of people in the world considered obese and/or overweight (WHO) [1]. Obesity is also considered a major risk factor for metabolic syndrome (MetS), characterised by hyperinsulinemia, hyperglycaemia, hyperlipidaemia and hepatic disorders, such as non-alcoholic fatty liver disease (NAFLD) [2].

Oxidative stress (OS) plays an important role in the development of obesity associated diseases and obese individuals are characterised by higher levels of oxidative stress compared to lean people [3] and lower anti-oxidant defences [4]. An excess of reactive oxygen species (ROS) combined with a low anti-oxidant capacity in the cells has been suggested to promote the development of obesity-induced metabolic diseases [5]. In metabolic diseases, OS is caused by different factors including mitochondrial dysfunction, activation of ROS and nitrogen species (RNS) producing enzyme, accumulation of glucose, lipids and protein oxidation products [6]. Moreover, metabolic diseases are also associated with chronic low-grade inflammation (CLGI) [7], producing abnormal pro-inflammatory cytokines, and activating inflammatory signalling pathways [8]. Inflammation is promoted by the presence, in the enlarged adipose tissue, of macrophages and immune cells, such as lymphocytes T [9]. Adipocytes and T cells have similar roles in complementary activation of inflammatory pathways and production of inflammatory cytokines: in fact, adipocyte precursors can be transformed into macrophage-like cell thanks to the phagocytic capacity under specific stimuli [10]. Some of the most important molecules involved in obesity-derived inflammation processes are tumour necrosis factor α (TNF-α) [11], interleukin 1β and 6 (IL1β, IL-6) [12] [13], leptin, adiponectin and Janus kinase 3 (JAK3) [14] [15].

Due to the high number of obese people, different strategies and new protocols to fight the onset of this obesity epidemic and the increased incidence of associated co-morbidities are required. A balanced diet and proper physical activity are the basis of these strategies, however improving redox

status in obese people is of paramount importance. Healthy foods, rich in antioxidant and anti-inflammatory molecules have a role; yet, it is necessary to consider supplements or nutraceuticals that can increase biological activity against ROS and inflammatory state, and improve redox status.

Astaxanthin (ASX), called also 3,3'-dihydroxy-β, β'-carotene-4,4'-dione, is a secondary carotenoid belonging to xanthophyll family [16] [17]. ASX is ubiquitous in nature, in fact it can be produced by plants, bacteria and yeast [18], but one of the highest producer is *Haematococcus pluvialis,* a unicellular freshwater green microalga [19]. ASX structure is characterised by keto and hydroxyl group at the end of the molecule, which make ASX one of the most powerful antioxidant compounds. ASX, as antioxidant, has ten times higher activity than other carotenoids (e.g. β-carotene, lutein and zeaxanthin) and hundred times than α-tocopherol [16,17]. Furthermore, ASX differs from carotenoids in its metabolism: ASX is absorbed by the intestinal mucosa through passive diffusion and is carried to the liver *via* the lymphatic and blood system, enclosed in chylomicrons [20]. The difference between carotenoids and ASX mainly lies in the type of lipoprotein that carries them once metabolized by the liver. Carotenoids are redistributed in plasma through low-density lipoproteins (LDL), whereas ASX is equally divided between LDL lipoproteins and high-density lipoproteins (HDL) [21]. Very few studies have been conducted on the pharmacokinetics of ASX. Choi et al. reports that ASX is unstable to gastric juices and that oral absorption is dose-independent and follows a flip-flop model, unlike intravenous, which is dose-dependent [22]. Given the high instability of ASX, Ødeberg et al. suggests the use of lipid formulations to improve its absorption for potential use in clinical trials [23].

Few studies have been conducted on ASX and its effect on human metabolic disease. Mashhaid et al. reported, in their studies, that ASX plays an important role in reducing level of triglycerides, cholesterol and blood pressure in type 2 diabetes (T2D) patients [24]; Choi et al. showed that ASX improved oxidative stress biomarker activity in obese adults [25] and Chen et al. reported that ASX had anti-coagulant effects in T2D patients reducing level of plasminogen activator inhibitor (PAI)-1 and anticoagulant factor VII (FVII) [26]. Furthermore, ASX has been shown to have some effects

against obesity associated diseases in animal models and descriptive results and potential mechanisms of action have been reviewed by Bonet et al. [27]; however no systematic analysis of all the available data has been carried out to date. A systematic review and meta-analysis of animal studies would, therefore, provide useful information for the design of subsequent human clinical studies for the use of ASX as a supplement or nutraceutical. This systematic review and meta-analysis aimed to comprehensively examine the effects of ASX in animal models (mice or rats) of diet induced obesity-associated diseases, focusing specifically on MetS, NAFLD and T2D.

2. METHODS

A systematic search for English-language manuscripts, published between January 2000 and April 2020, was made using five databases: Cinahl, Cochraine, MEDLINE, Scopus and Web of Science. The key words "Astaxanthin, obesity, non-alcoholic fatty liver disease, nonalcoholic fatty liver disease, diabetes, diabetes mellitus, type 2, NAFLD and metabol*" were used in each database and the exact strings used for each data base are reported in Table 1. The results are reported in accordance with PRISMA guidelines [28].

Table 1: Search string used for retrieving studies in selected databases.

Database	Search string
Cinahl	Astaxanthin WITH (obesity or "diabetes mellitus" or "diabetes mellitus, type 2" or diabetes or "nonalcoholic fatty liver disease" or "non-alcoholic fatty liver disease" or NAFLD or "metabolic syndrome x" or "metabolic syndrome")
Cochraine	Astaxanthin AND (obesity or "diabetes mellitus" or "diabetes mellitus, type 2" or diabetes or "nonalcoholic fatty liver disease" or "non-alcoholic fatty liver disease" or NAFLD or "metabolic syndrome x" or "metabolic syndrome")
MEDLINE	(TX "Astaxanthin") AND ((MH "obesity") or (TX "obesity") or (MH "diabetes mellitus") or (MH "diabetes mellitus, type 2") or (TX "diabetes") or (MH "non-alcoholic fatty liver disease") or (TX "non-alcoholic fatty liver disease") or (TX" nonalcoholic fatty liver disease) or (TX "non alcoholic fatty liver disease) or (TX "NAFLD") or (TX "metabol*"))
Scopus	Astaxanthin AND (obesity or diabetes or "non-alcoholic fatty liver disease" or "non alcoholic fatty liver disease" or "nonalcoholic fatty liver disease" or NAFLD or metabol*)
Web of Science	Astaxanthin AND (obesity or diabetes or "non-alcoholic fatty liver disease" or "non alcoholic fatty liver disease" or "nonalcoholic fatty liver disease" or NAFLD or metabol*)

114 *2.1 Inclusion and exclusion criteria:* Published studies were included if they met the following
115 criteria: the study i) was carried out in mice or rats; ii) reported data on clinical conditions (e.g.
116 obesity, T2D, MetS and NAFLD) induced by diet or in animal models of the disease (e.g. db/db
117 mouse, ob/ob mouse, KK-A^y mouse); iii) provided data on organs injured by metabolic diseases; iv)
118 included a control group formed by the same animal model; and v) used natural ASX that was
119 administered through diet.
120 Published studies were excluded by the following exclusion criteria: the study i) was carried out on
121 human or other animal species; ii) reported data from animals in which T2D was induced by drugs;
122 iii) used ASX derived from yeast or fungi, or synthetic ASX, iv) used ASX combined with other
123 compounds, or injected in vein or in stomach; v) included a control group formed by a different
124 animal model. All study selection and exclusion procedures were carried out by two independent
125 investigators (RPR and GB). If there was discordance, a third independent reviewer, GM would make
126 the final decision.
127 Outcome measurements: Outcome measures considered in each study for this systematic review
128 included: final body weight (BW), and specific blood, liver and adipose tissue biomarkers as reported
129 in table 2. Selected studies were divided in three groups based on the different diseases analysed:
130 MetS, T2D and NAFLD (Table 2)
131
132 *2.2 Assessment of risk of bias in included studies and publication bias.*
133 To determine the methodological quality of individual studies, the SYRCLE's risk of bias tool for
134 animal studies was used [29] . Two authors (RPR and GB) independently evaluated the risk of bias
135 of the included studies, according to the following domains with three different outcomes ("low risk",
136 "high risk", "unclear risk"): random sequence generation (selection bias), baseline characteristics
137 (selection bias), allocation concealment (selection bias), random housing (performance bias),
138 blinding (performance bias), blinding of participants and personnel (performance bias), random
139 outcome assessment (detection bias), blinding of outcome assessment (detection bias), incomplete

outcome data (attrition bias) and selective reporting (reporting bias). A third author (GM) resolved any discrepancies on the risk of bias.

Finally, a graphical funnel plot was used to investigate whether publication bias was present in the studies included in the review [30].

2.3 Data synthesis

A meta-analysis was performed using Review Manager 5.4 software. A random-effect model was used for the analysis and the standard mean difference (SMD) was considered. To evaluate the effect of treatment on each parameter, 95% confidence interval (CI) was used and significance set at $P<0.10$. Heterogeneity values were also calculated to determinate if included studies were suitable for meta-analysis. I^2 has been used to quantify heterogeneity and $I^2>50$ was considered substantial and significant if $P<0.10$. Where studies compared multiple concentrations with a single control group, each comparison was made by dividing the total number of control animals by the number of concentration treatments (N of total control/ N of treatment group). Sensitivity analysis was also performed to assess the influence of individual studies on SMD and 95% CI by excluding each study in turn, for each of the parameters considered. Heterogeneity of the study results were further explored by assessing if T2D or NAFLD were confounders on the effect of ASX on body weight or blood glucose levels. Diseases were considered confounding if they were found to be significantly associated with changes in body weight or glucose levels $P<0.10$ on univariate analysis.

159 **Table 2:** Summary of included studies

Study	Animal model	Sex	Age (weeks)	Weight (g)	N per group	Dose or Concentration	Duration of intervention (weeks)	Outcome
METABOLIC SYNDROME								
Gao et al. 2020 [31]	C57BL/6J mice fed HFD	M	6	20-22	10	50mg/kg bw/day	8	Glc; INS; gene expression analysis
Nishida et al. 2020 [32]	C57BL/6J mice fed HFD	M	5	N/A	N/A	0.02%	8 16 24	BW; TC; TG; Glc; ALT; AST; INS; HbA1c: SBP
Bhuvaneswari et al. 2014 [33]	Mus musculus albino mice of Swiss strain fed HFFD	M	N/A	25-30	6	2mg/kg bw/day	8	Gene expression analysis
Arunkumar et al. 2012 [34]	Mus musculus albino mice of Swiss strain fed HFFD	M	N/A	25-35	6	6mg/kg/day	8	BW: eWAT; Glc; INS; TNF; IL6
Preuss et al. 2011 [35]	Sprague Dawley rat	M	N/A	252-324	8	LowASX: 25mg/kg MedASX: 50mg/kg HiASX: 100mg/kg	8 32	BW; TC; TG; Glc; ALT; AST; SBP
Bhuvaneswari et al. 2010 [36]	Mus musculus albino mice of Swiss strain fed HFFD	M	N/A	25-35	6	6mg/kg bw/day	8	BW; TC; TG; Glc; INS; ALT; AST;
Preuss et al. 2009 [37]	Zucker Fatty Rats	N/A	N/A	434-624 388-520	12	LowASX: 5mg/kg HiASX: 25mg/kg	8 10	BW; eWAT; TC; TG; Glc; ALT: AST; SBP
Ikeuchi et al. 2007 [38]	ddY mice	F	4	N/A	10	1.2mg/kg bw 6mg/kg bw 30mg/kg bw	8	TG
TYPE 2 DIABETES								
Chen et al. 2020 [39]	C57BL/KsJ mice db/db mice	F	8	N/A	12	30mg/kg	3	BW; Glc; INS; TC; LDL; HDL; TG; MDA;
Kumar et al- 2016 [40]	KK-A^{y} mice	M	4	N/A	7	0.1%	4	BW; eWAT; TC; LDL; HDL; Glc;
Kimura et al. 2014 [41]	OLETF rats	M	25	579	6	0.2%	6	BW; eWAT; TC; LDL; HDL; TG; Glc;
Uchiyama et al. 2002 [42]	db/db mice	F	N/A	N/A	8	1mg/mouse/day	12 18	BW; Glc;
NAFLD								
Kim et al. 2017 [43]	C57BL/6J mice fed HF/HS	M	8	23.3	9	0.03%	30	BW; eWAT; TC; TG; Glc; ALT; AST; gene expression analysis
Kobori et al. 2017 [44]	C57BL/6J mice fed HFD	M	7	N/A	N/A	0.02%	12	Gene analysis

Jia et al. 2016 [45]	C57BL/6J mice fed HFD	M	8	18-20	10	6mg/kg bw 30mg/kg bw	8	eWAT; TC; TG; Glc; ALT; AST; gene expression analysis
Ni et al. 2015 [46]	C57BL/6J mice ob/ob mice fed HFD	M	7 5	N/A	8	0.02%	12	BW; TC: TG; ALT; AST; Gene analysis
Yang et al. 2014 [47]	C57BL/6J mice fed HFD	M	6	39	8	0.03% w/w	12	BW; eWAT; TC; TG; ALT;AST; Gene analysis

Alanine transaminase (ALT), Aspartate transaminase (AST), Body weight (BW), Epididymal white adipose tissue (eWAT), Glucose level (Glc), Glycated haemoglobin (HbA1c), High-density lipoprotein (HDL), High fat/high sucrose diet (HF/HS), High fat diet (HFD), High fat fructose diet (HFFD), Insulin level (INS), Inerleukin-6 (IL6), Low-density lipoprotein (LDL), Serum malondialdehyde level (MDA), Systolic blood pressure (SBP), Total serum cholesterol (TC), Total serum triglycerides (TG), Tumor necrosis factor (TNF).

3. RESULTS

3.1 Search results

A total of 506 articles (Cinahl 107; Cochraine 27; MEDLINE 153; Scopus 116; WOS 103) were found and, after removing duplicates, 312 articles were selected for the next step. By screening title and abstract of the selected articles, reviews, cell studies and human studies were removed and 39 articles were selected for full text screening. Based on inclusion and exclusion criteria as described above, 17 articles were selected for inclusion in the review (Figure 1), which included 21 animal studies. Eight articles reported findings from 10 studies on MetS [31–38], four articles [39–42] from 5 studies on T2D (including one paper/study on gestational diabetes) [33–36] and five articles from 6 studies on NAFLD [43–47].

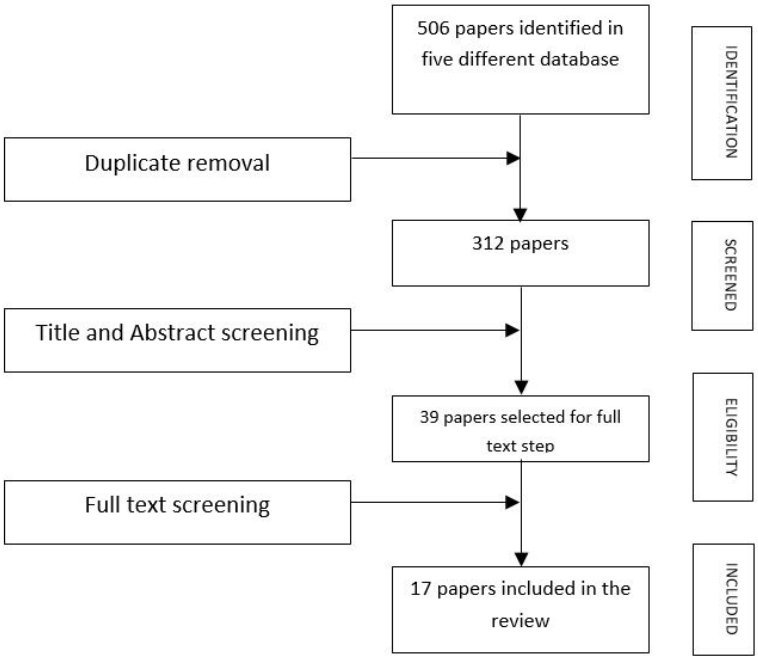


Figure 1: Flow diagram of study search process

3.2 Risk of bias in included studies and publication bias

The SYRCLE's risk of bias tool [25] for animal studies was used to assess the risk of bias in the included 17 articles. The risk of bias for each included study is summarized in Figure 2. The studies included in this review contained insufficient reporting of the experimental details and, as a result,

several studies were judged as having 'unclear risk of bias'. Allocation concealment, random housing, blinding, blinding of participants and personnel, random outcome assessment and blinding of outcome assessment were incompletely described in all the studies. However random sequence generation, baseline characteristic, incomplete outcome data and selective reporting were factors associated with a low risk of bias. Only one study disclosed not to report all the data and, therefore, associated to high risk of bias.

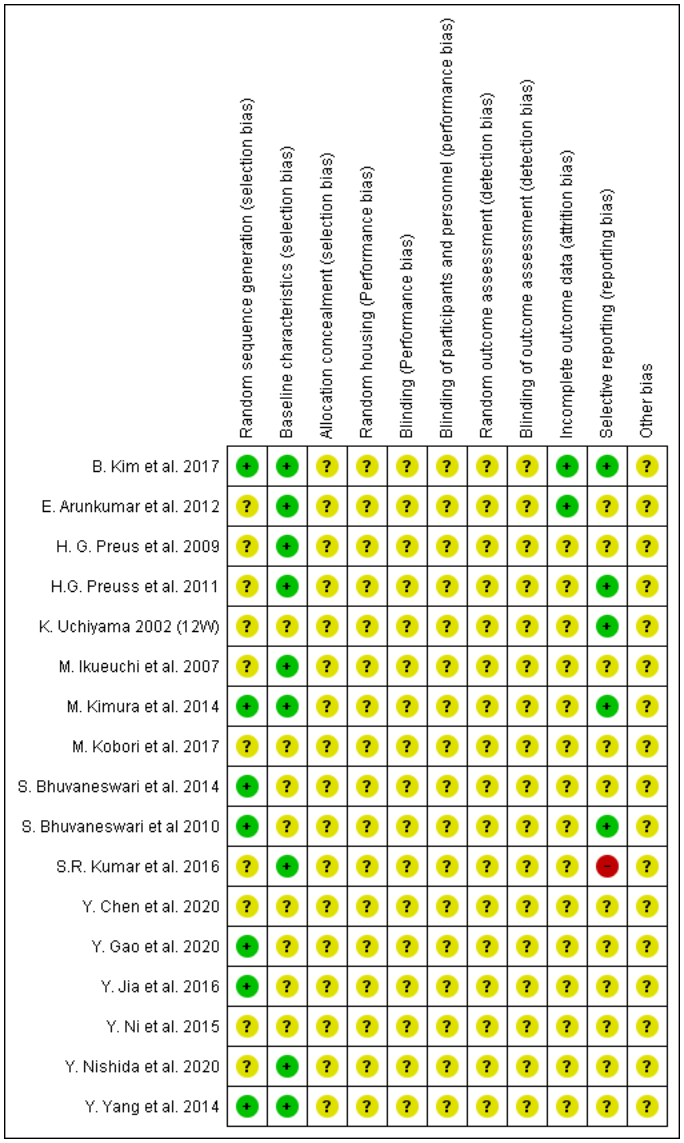


Figure 2: Risk of bias summary for the included studies.

The risk of publication bias is shown in a funnel plot graph (Figure 3). The result of the analyses carried out on SMD values for glucose levels, common biomarker to the three diseases examined,

showed an asymmetry, indicating the presence of publication bias. This can be explained by the fact that studies carried out on animals are characterized by small samples size per group influencing, therefore, the results of the analyses that can be over- or underestimated. Moreover, studies reporting a negative treatment effect are not commonly published.

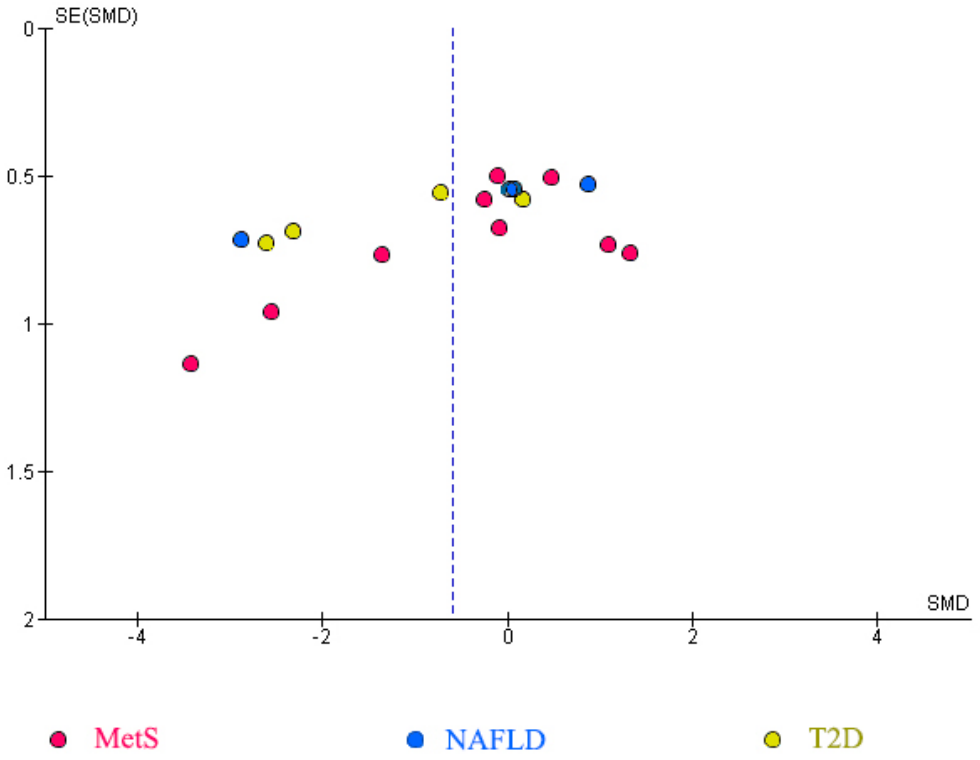


Figure 3: Funnel plot for publication bias. MetS: metabolic syndrome; NAFLD: non-alcoholic fatty liver disease; T2D: type 2 diabetes.

3.3. Metabolic syndrome

3.3.1 Body and tissue weight: The effect of ASX on final BW was considered in seven articles [31–38] selected for MetS; however, no numerical data were reported in three studies [31,32,38]. Three studies reported no effect of ASX on BW [32,35,37], whereas BW was reduced by ASX treatment when compared with the group fed a high fat and fructose diet (HFFD) groups only in two studies [34,36]. Liver weight was not affected by ASX treatment in animal fed with a control diet, as meta-analysis showed (SMD=0.23, 95% CI: -0.49 to 0.95, P=0.54 and heterogeneity χ^2=2.84, P=0.24, I^2=29%) (Figure 4). Epididymal white adipose tissue (eWAT) weight was analysed by Arunkumar et

al. [34] and Preuss et al. [37] and both reported a significant reduction in weight in ASX group independently from ASX concentration (SMD=-1.87, 95% CI: -3.70 to -0.04, P=0.05 and heterogeneity χ^2=10.62, P=0.005, I^2=81%) (Figure 4).

Sensitivity analysis was performed to determine whether any particular study had a greater degree of influence on the effect of ASX on tissue weight. Omission of each study one at a time and analysis of SMD for the rest of the studies, did not influence the effect of ASX in reducing liver weight significantly. For eWAT, sensitivity analysis showed that omitting values from Arunkumar et al. study [34] or values from LowASX treatment in Preus et al. study [37], the significant reduction in eWAT in ASX group was lost (SMD=-1,29, 95% CI: -3,33 to 0,75, P=0,22, and heterogeneity χ^2= 6,16, P=0,01; and SME=-1,69, , 95% CI: -4,67 to 1,29, P=0,27, and heterogeneity χ^2= 7,27, P=0,007, respectively).

3.3.2 Blood parameters: The effect of ASX was analysed not only in animals with MetS (i.e. fed a high fat diet (HFD) or HFFD) but also in animals fed a control diet, and seven articles reported values for blood parameters [25-31]. ASX treatment improved, significantly, serum total cholesterol (TC) levels in control group animals, and these findings were confirmed by meta-analysis (SMD=0.67, 95% CI: 0.15 to 1.20, P=0.01) (Figure 4); heterogeneity was not significant (χ^2=11.58, P=0.17, I^2=31%). Moreover, Bhuvaneswari et al. [36] and Nishida et al. [32] reported cholesterol levels to be reduced in ASX treated groups compared with HFFD and HFD group, respectively. Sensitivity analysis on the effect of ASX on cholesterol level showed that none of the study reversed the positive effect identified by the meta-analysis.

Triglycerides (TG) levels were analysed only in 3 studies [35–37], with Preuss et al. [35,37] testing 4 different ASX concentrations in their 3 studies. ASX induced an increase in TG levels in treated group compared to animals fed a control diet, even if not significantly (SMD=0.34, 95% CI: -0.83 to 1.50, P=0.57) and with substantial and significant heterogeneity (χ^2=44.70, P<0.00001, I^2=82%) (Figure 4). Only 3 studies [32,36,38] reported that ASX treatment significantly reduced TG levels in

234 animals with MetS, however a meta-analysis was not possible as numerical data were only provided
235 for one study [36].
236 Considering glucose level, ASX had no effect in control animals (normal diet group) (SMD=-0.36,
237 95% CI: -1.16 to 0.45, P=0.39 and heterogeneity χ^2=25.13, P=0.001, I^2=68%) (Figure 4). Only 2
238 studies [32,36] reported that ASX treatment significantly reduced glucose levels in animals fed with
239 HFD or HFFD, respectively. Alanine transaminase (ALT) level was improved by ASX treatment in
240 control animals (SMD=0.43, 95% CI: -0.03 to 0.90, P=0.07, Figure 4) even if heterogeneity was not
241 relevant and significant (χ^2=9.82, P=0.28, I^2=18%). Only 1 study [36] reported ASX treatment to
242 significantly reduce ALT levels in animals fed with HFFD. In the same way, ASX had a significant
243 effect on aspartate transaminase (AST) level, increasing it in treated groups (SMD=1.57, 95% CI:
244 0.63 to 2.51, P=0.001 and heterogeneity χ^2=26.06, P=0.001, I^2=69%). Similarly to ALT levels, only
245 one study [36] reported that ASX treatment significantly reduced AST levels in animals fed with
246 HFFD. Preuss et al. [35,37] reported in their studies that ASX reduced significantly systolic blood
247 pressure (SBP) in control animals (normal diet) (SMD=-3.80, 95% CI: -5.65 to -1.94, P<0.0001 and
248 heterogeneity χ^2=15.67, P=0.003, I^2=74%, (Figure 4); whereas Nishida et al. [32] showed a
249 significant reduction in SBP in animals fed with HFD and treated with ASX.
250 Sensitivity analysis on the effect of ASX on TG, glucose and AST levels, and SBP did not modify
251 the changes observed, whereas the significant increase in ALT levels after ASX treatment was lost
252 when omitting HiASX values from Preus et al. study [37] (SMD=0,27, 95% CI: -0,18 to 0,72, P=0,24,
253 and heterogeneity χ^2=6,42, P=0,49; I^2=0%), and values from MedASX at 32 weeks (SMD=0,33, 95%
254 CI: -0,10 to 0,76, P=0.13, and heterogeneity χ^2=6,46 (P=0,49); I^2=0%), and at 8 weeks (SMD=0,42,
255 95% CI: -0,10 to 0,95, P=0.12, and heterogeneity χ^2=9,78, P=0,20; I^2=28%). from Preuss et al. study
256 [35].
257
258 *3.3.3 Liver parameters:* Bhuvaneswari et al. [36] reported that ASX reduced liver TC, TG and lipids
259 level in animals with MetS (HFFD+ASX vs HFFD), whereas superoxide dismutase (SOD), catalase

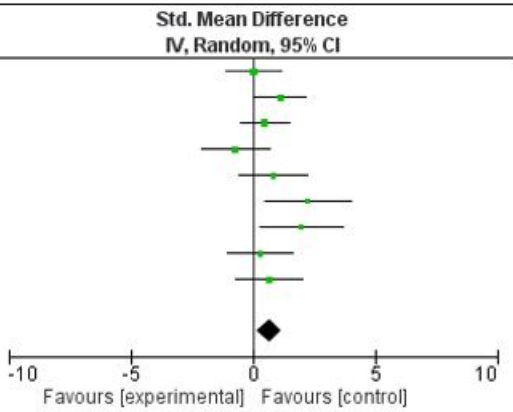
(Cat) and glutathione peroxidase (GPx) activities were improved by ASX. Lipid peroxidation was analysed in 2 articles: Bhuvaneswari et al. [36] and Preuss et al [37] reported a no significant reduction in lipid peroxidation in lean control group by ASX (SMD=-1.30, 95% CI: -3.15 to 0.54, P=0.17) even if the heterogeneity was substantial and significant (χ^2=13.33, P=0.001, I^2=85%, Figure 4), whereas ASX reduced lipid peroxidation in animals fed HFFD [36]. Sensitivity analysis showed that omitting values from Bhuvaneswari et al. study [36], ASX had a significant effect in reducing lipid peroxidation (SMD=-2,14, 95% CI:-3,91 to -0,38, P=0.02, and heterogeneity χ^2=3,60, P=0,06; I^2=72%).

METABOLIC SYNDROME

Serum cholesterol

Study or Subgroup	Control + Astaxanthin Mean	SD	Total	Control Mean	SD	Total	Weight	Std. Mean Difference IV, Random, 95% CI
Bhuvaneswari et al. 2010	100	58	6	97	54	6	13.5%	0.05 [-1.08, 1.18]
Preus et al. 2009 (HiASX)	314	13.6	12	295	20.4	6	14.5%	1.13 [0.06, 2.19]
Preus et al. 2009 (LowASX)	308	27.8	12	295	20.4	6	15.7%	0.48 [-0.51, 1.48]
Preuss et al. 2011 (32W) (HiASX)	131	4	8	135	8.2	3	10.4%	-0.70 [-2.07, 0.68]
Preuss et al. 2011 (32W) (LowASX)	142	7.2	8	135	8.2	3	10.1%	0.86 [-0.54, 2.26]
Preuss et al. 2011 (32W) (MedASX)	149	4.7	8	135	8.2	3	7.0%	2.26 [0.48, 4.03]
Preuss et al. 2011 (8W) (HiASX)	130	4.4	8	117	9.6	3	7.6%	1.99 [0.31, 3.68]
Preuss et al. 2011 (8W) (LowASX)	120	8.4	8	117	9.6	3	10.8%	0.32 [-1.02, 1.65]
Preuss et al. 2011 (8W) (MedASX)	122	5.8	8	117	9.6	3	10.4%	0.67 [-0.70, 2.04]
Total (95% CI)			**78**			**36**	**100.0%**	**0.67 [0.15, 1.20]**

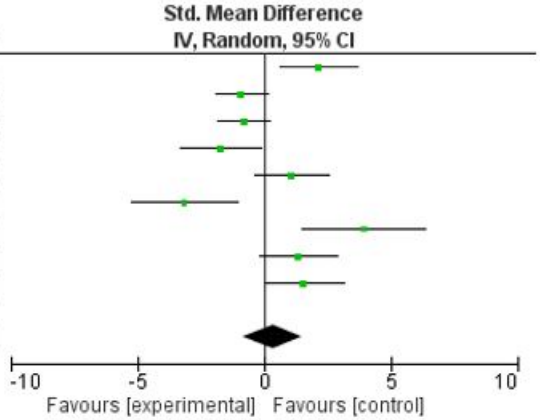
Heterogeneity: Tau² = 0.20; Chi² = 11.58, df = 8 (P = 0.17); I² = 31%
Test for overall effect: Z = 2.53 (P = 0.01)



Serum triglyceride

Study or Subgroup	Control + ASX Mean	SD	Total	Control Mean	SD	Total	Weight	Std. Mean Difference IV, Random, 95% CI
Bhuvaneswari et al. 2010	46.9	2.65	6	40.71	2.65	6	11.3%	2.16 [0.61, 3.70]
Preus et al. 2009 (HiASX)	1,172	51.2	12	1,226	70.5	6	12.6%	-0.89 [-1.92, 0.15]
Preus et al. 2009 (LowASX)	1,167	71.6	12	1,226	70.5	6	12.7%	-0.79 [-1.81, 0.23]
Preuss et al. 2011 (32W) (HiASX)	206	7.5	8	222	11.5	3	11.1%	-1.71 [-3.31, -0.11]
Preuss et al. 2011 (32W) (LowASX)	246	21.7	8	222	11.5	3	11.5%	1.10 [-0.34, 2.55]
Preuss et al. 2011 (32W) (MedASX)	185	10.5	8	222	11.5	3	9.6%	-3.15 [-5.27, -1.04]
Preuss et al. 2011 (8W) (HiASX)	190	6.1	8	162	7.7	3	8.7%	3.94 [1.50, 6.39]
Preuss et al. 2011 (8W) (LowASX)	182	14.6	8	162	7.7	3	11.4%	1.37 [-0.14, 2.87]
Preuss et al. 2011 (8W) (MedASX)	178	9.7	8	162	7.7	3	11.2%	1.57 [0.01, 3.13]
Total (95% CI)			**78**			**36**	**100.0%**	**0.34 [-0.83, 1.50]**

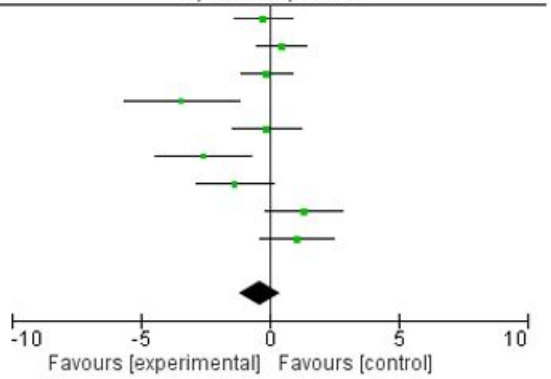
Heterogeneity: Tau² = 2.52; Chi² = 44.70, df = 8 (P < 0.00001); I² = 82%
Test for overall effect: Z = 0.57 (P = 0.57)



Serum glucose

Study or Subgroup	control + ASX Mean	SD	Total	Control Mean	SD	Total	Weight	Std. Mean Difference IV, Random, 95% CI
Arunkumar et al. 2012	82.66	4.74	6	83.91	4.52	6	12.7%	-0.25 [-1.39, 0.89]
Preus et al. 2009 (HiASX)	327	14.2	12	320	14.6	6	13.5%	0.47 [-0.53, 1.46]
Preus et al. 2009 (LowASX)	317	30.6	12	320	14.6	6	13.6%	-0.11 [-1.09, 0.87]
Preuss et al. 2011 (32W) (HiASX)	107.1	1.9	8	114.3	2	3	7.4%	-3.42 [-5.65, -1.20]
Preuss et al. 2011 (32W) (LowASX)	114.1	1.9	8	114.3	2	3	11.6%	-0.10 [-1.42, 1.23]
Preuss et al. 2011 (32W) (MedASX)	108.5	2.1	8	114.3	2	3	8.8%	-2.55 [-4.43, -0.67]
Preuss et al. 2011 (8W) (HiASX)	110.1	2.7	8	114.1	2.7	3	10.7%	-1.35 [-2.86, 0.15]
Preuss et al. 2011 (8W) (LowASX)	117.8	2.5	8	114.1	2.7	3	10.7%	1.33 [-0.17, 2.83]
Preuss et al. 2011 (8W) (MedASX)	117.3	2.7	8	114.1	2.7	3	11.0%	1.08 [-0.36, 2.53]
Total (95% CI)			**78**			**36**	**100.0%**	**-0.36 [-1.16, 0.45]**

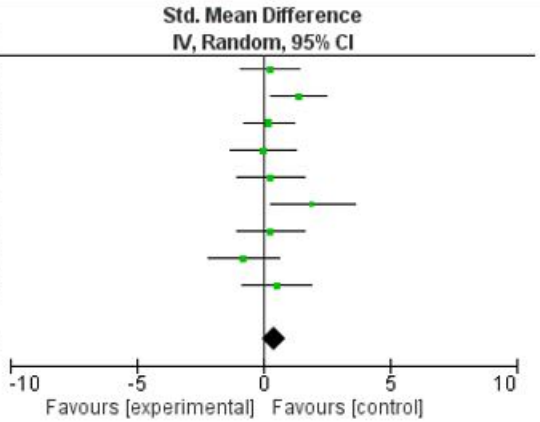
Heterogeneity: Tau² = 0.99; Chi² = 25.13, df = 8 (P = 0.001); I² = 68%
Test for overall effect: Z = 0.87 (P = 0.39)



Serum ALT

Study or Subgroup	Control + ASX Mean	SD	Total	Control Mean	SD	Total	Weight	Std. Mean Difference IV, Random, 95% CI
Bhuvaneswari et al. 2010	52.11	3.43	6	51.2	2.81	6	13.1%	0.27 [-0.87, 1.41]
Preus et al. 2009 (HiASX)	86.2	13.7	12	68.3	8	6	13.7%	1.40 [0.29, 2.50]
Preus et al. 2009 (LowASX)	70.9	12.4	12	68.3	8	6	16.4%	0.22 [-0.76, 1.20]
Preuss et al. 2011 (32W) (HiASX)	79	4.6	8	79	3.6	3	10.3%	0.00 [-1.33, 1.33]
Preuss et al. 2011 (32W) (LowASX)	80	2.9	8	79	3.6	3	10.1%	0.30 [-1.04, 1.63]
Preuss et al. 2011 (32W) (MedASX)	88	4.4	8	79	3.6	3	6.9%	1.94 [0.27, 3.61]
Preuss et al. 2011 (8W) (HiASX)	67	3.5	8	66	3	3	10.2%	0.27 [-1.07, 1.60]
Preuss et al. 2011 (8W) (LowASX)	63	3.6	8	66	3	3	9.5%	-0.79 [-2.18, 0.60]
Preuss et al. 2011 (8W) (MedASX)	68	3.4	8	66	3	3	9.9%	0.55 [-0.81, 1.91]
Total (95% CI)			**78**			**36**	**100.0%**	**0.43 [-0.03, 0.90]**

Heterogeneity: Tau² = 0.09; Chi² = 9.82, df = 8 (P = 0.28); I² = 18%
Test for overall effect: Z = 1.82 (P = 0.07)

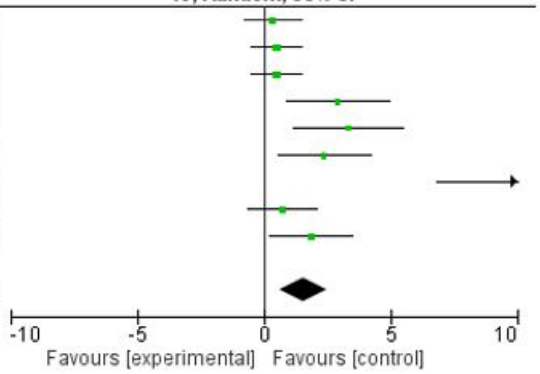


271

Serum AST

Study or Subgroup	Control + ASX Mean	SD	Total	Control Mean	SD	Total	Weight	Std. Mean Difference IV, Random, 95% CI
Bhuvaneswari et al. 2010	48.62	2.61	6	47.61	2.42	6	14.2%	0.37 [-0.78, 1.52]
Preus et al. 2009 (HiASX)	106.1	21	12	95.3	16.9	6	15.0%	0.52 [-0.48, 1.52]
Preus et al. 2009 (LowASX)	105.8	20.9	12	95.3	16.9	6	15.0%	0.51 [-0.49, 1.50]
Preuss et al. 2011 (32W) (HiASX)	118	4.6	8	102	6.2	3	9.8%	2.93 [0.90, 4.95]
Preuss et al. 2011 (32W) (LowASX)	120	4.5	8	102	6.2	3	9.1%	3.34 [1.15, 5.53]
Preuss et al. 2011 (32W) (MedASX)	116	5.1	8	102	6.2	3	10.8%	2.39 [0.57, 4.21]
Preuss et al. 2011 (8W) (HiASX)	180	5.3	8	98	4.6	3	1.4%	14.55 [6.85, 22.25]
Preuss et al. 2011 (8W) (LowASX)	102	5	8	98	4.6	3	13.0%	0.74 [-0.64, 2.13]
Preuss et al. 2011 (8W) (MedASX)	108	5	8	98	4.6	3	11.6%	1.86 [0.22, 3.50]
Total (95% CI)			**78**			**36**	**100.0%**	**1.57 [0.63, 2.51]**

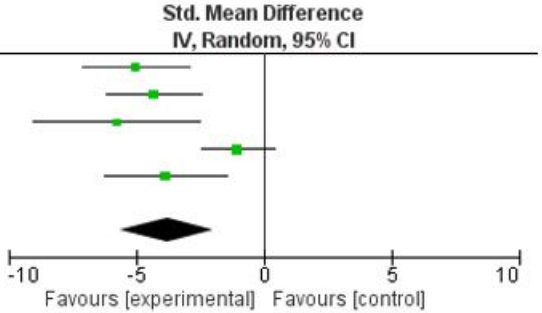
Heterogeneity: Tau² = 1.27; Chi² = 26.06, df = 8 (P = 0.001); I² = 69%
Test for overall effect: Z = 3.28 (P = 0.001)



Systolic Blood Pressure

Study or Subgroup	Control + ASX Mean	SD	Total	Control Mean	SD	Total	Weight	Std. Mean Difference IV, Random, 95% CI
Preus et al. 2009 (HiASX)	132	1.8	12	142	2.1	6	20.5%	-5.02 [-7.11, -2.92]
Preus et al. 2009 (LowASX)	134	1.6	12	142	2.1	6	21.7%	-4.30 [-6.17, -2.43]
Preuss et al. 2011 (32W) (HiASX)	142.1	1	8	149.6	1.7	3	14.9%	-5.75 [-9.04, -2.47]
Preuss et al. 2011 (32W) (LowASX)	147.6	1.8	8	149.6	1.7	3	23.9%	-1.03 [-2.46, 0.40]
Preuss et al. 2011 (32W) (MedASX)	143.4	1.4	8	149.6	1.7	3	19.0%	-3.85 [-6.26, -1.44]
Total (95% CI)			**48**			**21**	**100.0%**	**-3.80 [-5.65, -1.94]**

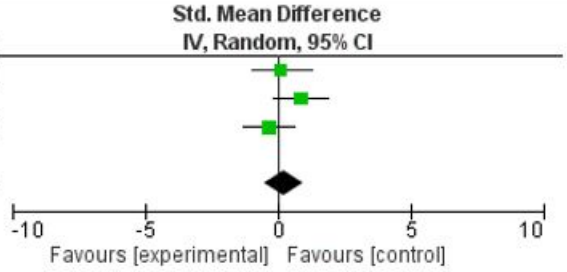
Heterogeneity: Tau² = 3.22; Chi² = 15.67, df = 4 (P = 0.003); I² = 74%
Test for overall effect: Z = 4.01 (P < 0.0001)



Liver weight

Study or Subgroup	Control + ASX Mean	SD	Total	Control Mean	SD	Total	Weight	Std. Mean Difference IV, Random, 95% CI
Bhuvaneswari et al. 2010	1.27	0.07	6	1.26	0.06	6	29.8%	0.14 [-0.99, 1.28]
Preus et al. 2009 (HiASX)	43.3	2.3	12	41.2	2.1	6	34.0%	0.89 [-0.14, 1.93]
Preus et al. 2009 (LowASX)	40.4	2.4	12	41.2	2.1	6	36.2%	-0.33 [-1.32, 0.66]
Total (95% CI)			**30**			**18**	**100.0%**	**0.23 [-0.49, 0.95]**

Heterogeneity: Tau² = 0.12; Chi² = 2.84, df = 2 (P = 0.24); I² = 29%
Test for overall effect: Z = 0.62 (P = 0.54)



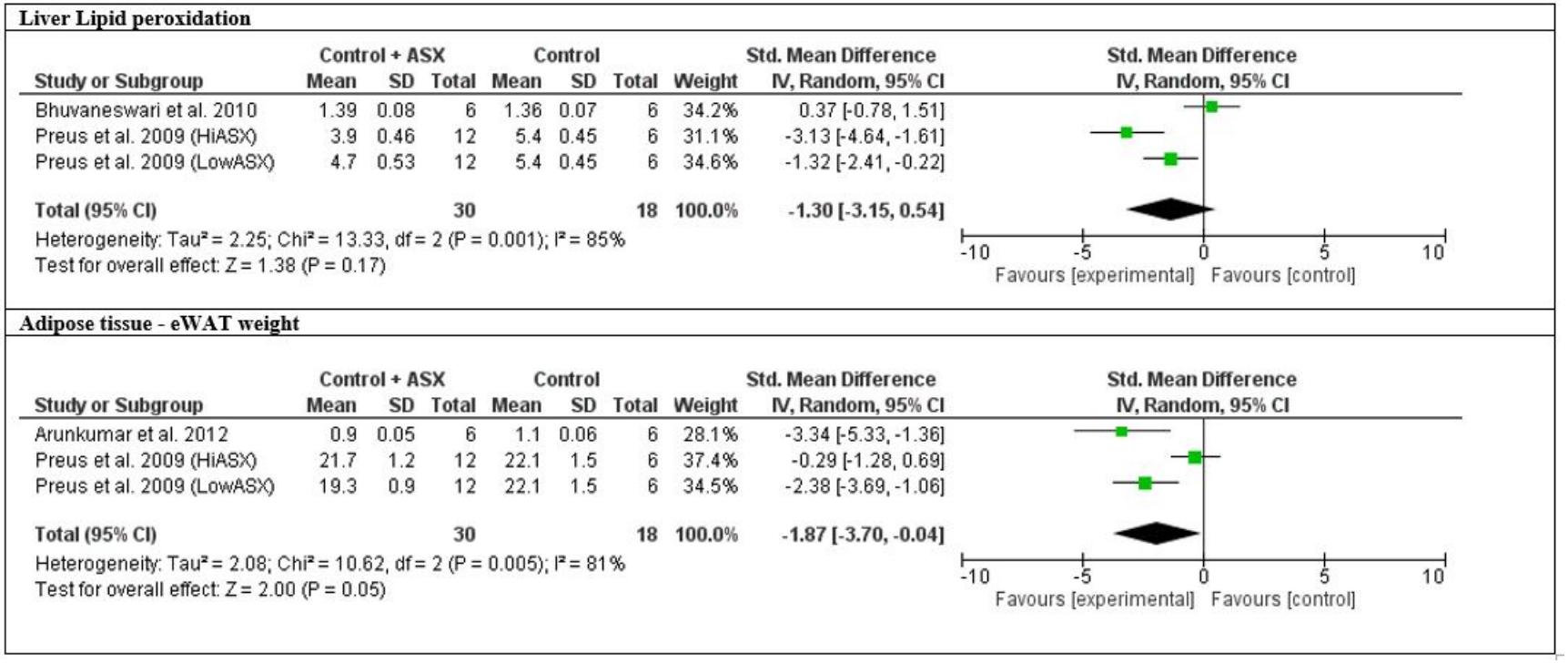


Figure 4: Forest plot comparing different parameters between treatment and control groups in animal models of metabolic syndrome. High astaxanthin concentration (HiASX), medium astaxanthin concentration (MedASX), low astaxanthin concentration (LowASX). For Preuss et al. 2011: 8 weeks of treatment (8W) and 32 weeks of treatment (32W)

277 *3.4 Type 2 Diabetes*

278 *3.4.1 Body and tissue weight*: Four studies reported the effect of ASX on final BW in animal model
279 of diabetes (db/db or KK-Y^A) [40–42]. ASX had no significant effect on BW (SMD=0.67, 95% CI: -
280 1.16 to 2.50, P=0.48) although heterogeneity was substantial (χ^2=24.80, P<0.0001, I^2=88%) (Figure
281 5). Sensitivity analysis did not modify the effect observed on BW. Only one study reported a
282 significant reduction of liver weight in animal fed with ASX comparing with the control group [40];
283 whereas 2 studies [40,41] analysed the effect of ASX on adipose tissue. ASX had no effect on
284 epidydimal white adipose tissue (eWAT) and on retroperitoneal adipose weight [40][41] but ASX
285 reduced adipocytes size in treated group [41].

286

287 *3.4.2 Blood parameters*: All the studies tested ASX on normal fed animal models for T2D. TC, low-
288 density lipoprotein (LDL) and high-density lipoprotein (HDL) were analysed only in 2 papers. Both
289 papers reported how ASX increased TC, LDL and HDL level in treated groups (T2D animal + ASX)
290 compared with control (T2D animal) [40,41]. Considering glucose level, analysed in four studies [40–
291 42], ASX reduced it significantly (SMD=-1.31, 95% CI: -2.58 to -0.04, P=0.04 and heterogeneity
292 χ^2=12.59, P=0.006, I^2=76%) (Figure 5). Sensitivity analysis showed that omitting values for animals
293 treated with ASX for 18 weeks or 12 week from Uchiyama et al. study [42], the significant reduction
294 in glucose levels in ASX group was lost (SMD=-0,99, 95% CI=-2,47 to 0,48, P=0.19, and
295 heterogeneity χ^2=8,88, P=0,01, I^2=77%; and SMD=-0,91, 95% CI:-2,26 to 0,43, P=0.18, and
296 heterogeneity χ^2=7,65, P=0,02, I^2=74%, respectively).
297 Uchiyama et al. reported a significant reduction of intraperitoneal glucose tolerance test (ipGTT) and
298 a significant increase in serum insulin level [42].

299

300 *3.4.3 Liver parameters:* Only one article analysed liver parameters: Kumar et al. [40] reported that
301 ASX increased SOD, Cat and GPx activity but, at the same time, reduced oxidized glutathione
302 (GSSG) and reduced glutathione (GSH) level in treated group.

303

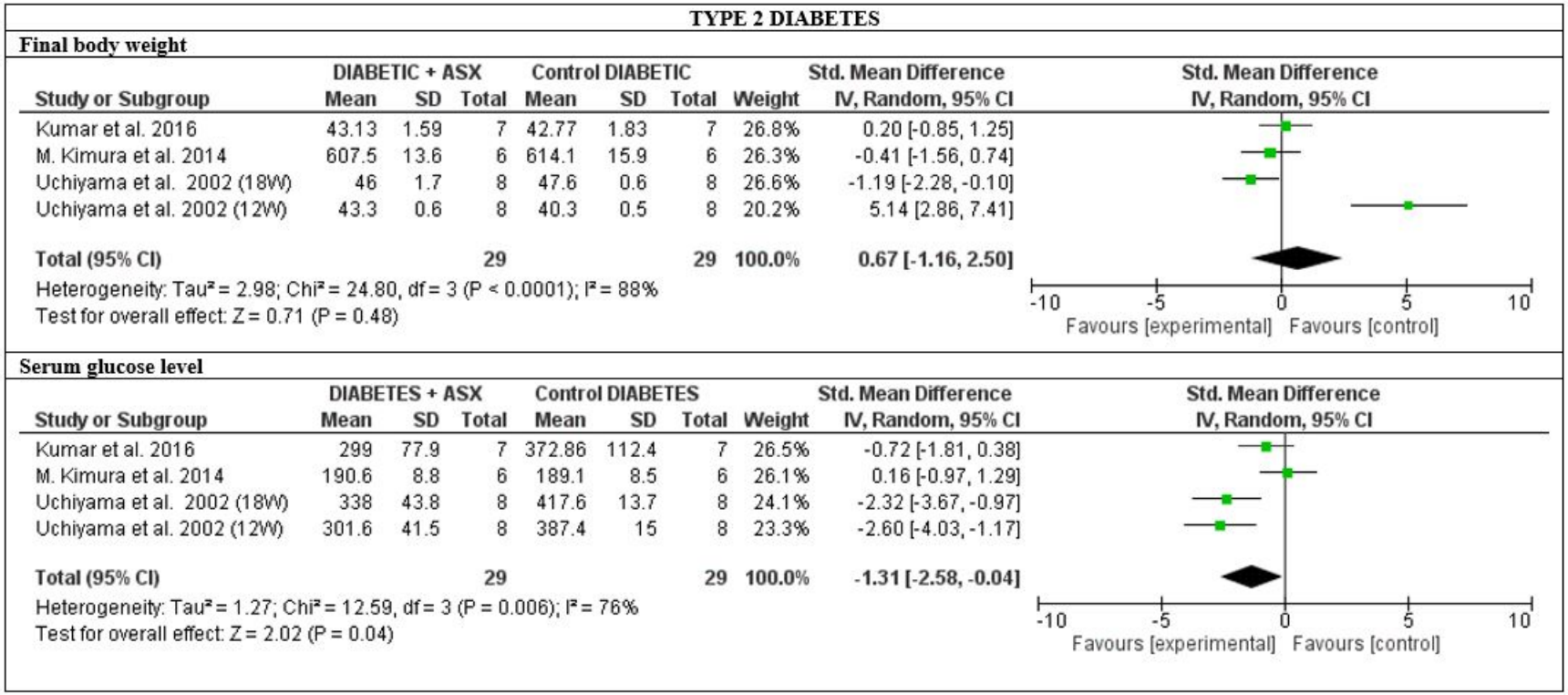


Figure 5: Forest plot comparing different parameters between treatment and control groups in animal models of type 2 diabetes
For Uchiyama et al. 2002: 18 weeks of treatment (18W) and 12 weeks of treatment (12W)

308 *3. 5 Non-alcoholic fatty liver diseases*

309 *3.5.1 Body and tissue weight:* The effect of ASX on final BW in animals fed with HFD was considered
310 in 4 studies [43,45–47]. Ni et al. [46] reported findings from two different animal sets: data obtained
311 from 20-week old fasted mice and 32-week old fasted mice after 12 weeks of treatment. ASX reduced
312 BW almost significantly (SMD=-0.89, 95% CI: -1.98 to 0.20, P=0.11) and heterogeneity was
313 substantial and significant (χ^2=10.57, P=0.01, I^2=72%) (Figure 6). Five studies analysed ASX effect
314 on liver weight [43,45,46]. ASX had a significant effect on liver weight (SMD=-0.91, 95% CI: -1.40
315 to -0.43, P=0.0002) but data were not heterogeneous (χ^2=3.74, P=0.44, I^2=0%) (Figure 6). For BW,
316 sensitivity analysis showed that omitting values from Yang et al. study [47], the reduction in BW
317 became significant (SMD=-1,35, 95% CI:-2,12 to -0,57, P=0.0006, and heterogeneity χ^2=2,89,
318 P=0,24, I^2=31%), whereas omission of each study one at a time and analysis of SMD for the rest of
319 the studies, did not influence the effect of ASX in significantly reducing liver weight. ASX treatment
320 had no effect on eWAT and retroperitoneal adipose weight as reported by Kim et al. [43] and Yang
321 et al. [47]. On the contrary, only Jia et al. [45] discovered ASX to reduce eWAT weight in treated
322 animals (HFD+ASX).

323

324 *3.5.2 Blood parameters:* How ASX acted on blood parameters was analysed in five studies [43,45–
325 47]. Considering TC, ASX reduced significantly TC levels in animal fed with HFD+ASX compared
326 with HFD group (SMD=-2.90, 95% CI: -4.82 to -0.98, P=0.003 and heterogeneity χ^2=31.32,
327 P<0.00001, I^2=87%); only Yang et al. [47] found no difference between the two groups. ASX had
328 also an effect on TG level, reducing it significantly (SMD=-3.14, 95% CI: -3.87 to -2.42, P<0.00001)
329 but heterogeneity was not significant and substantial (χ^2=3.49, P=0.48, I^2=0%). Glucose level was
330 analysed in four studies [43,45,47]: ASX had no effect in treated animals (HFD+ASX) (SMD=-0.41,
331 95% CI: -1.82 to 0.99, P=0.56) even if heterogeneity was substantial and significant (χ^2=18.34,
332 P=0.0004, I^2=84%). ALT was reduced significantly in all five studies [43,45,46] by ASX treatment
333 (SMD=-2.11, 95% CI: -4.00 to -0.21, P=0.03 and heterogeneity χ^2=36.58, P<0.00001, I^2=89%). In

the same way, ASX reduced AST level in four studies [45,46] (SMD=-2.17, 95% CI: -4.49 to 0.15, P=0.07) and this was also confirmed by heterogeneity (χ^2=30.30, P<0.00001, I^2=90%) (Figure 6). Sensitivity analysis on the effect of ASX on TC, TG and glucose levels did not modify the changes observed; whereas the significant reduction in ALT levels after ASX treatment was lost when omitting values from animals treated for 20 weeks from Ni et al. study [46] (SMD=-1,29, 95% CI:-2,97 to 0,40, P=0.13, and heterogeneity χ^2=21,83, P<0,0001, I^2=86%). The significant reduction in AST levels after ASX treatment was also lost when omitting values from animals treated for 20 or 32 weeks from Ni et al. study [46] (SMD=-1,45, 95% CI:-3,78 to 0,88, P=0.22, and heterogeneity χ^2=17,81, P=0,0001, I^2=89%; and SMD=-1,25, 95% CI: -3,38 to 0,88, P=0.25, and heterogeneity χ^2=15,98, P=0,0003, I^2=87%, respectively).

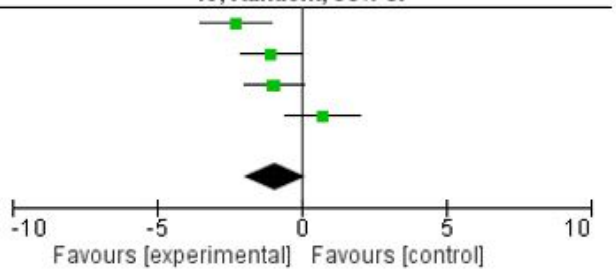
3.5.3 Liver parameters: Jia et al. [45] and Yang et al. [47] discovered that ASX had no effect on liver TC (SMD=-0.30, 95% CI: -0.90 to 0.29, P=0.31 and heterogeneity χ^2=0.32, P=0.85, I^2=0%) (Figure 6), only Kim et al. [43] and Ni et al. [46], recorded that ASX reduced significantly liver TC and TG in treated groups. Sensitivity analysis did not modify the effect observed on TC. However, Jia et al. [45] reported that ASX reduced protein kinase B (Akt) activity, glycogen synthase kinase 3 (GSK-3), sterol regulatory element-binding protein 1 (SREBP1) and ribosomal protein S6 kinase beta-1 (S6K1) phosphorylation. Furthermore, ASX reduced TNF-α and IL6 level and increased insulin induced gene 2 (Insig-2a), microtubule-associated proteins 1A/1B light chain 3B I/II (LC3I/II), lysosomal-associated membrane protein 1/2 (LAMP1/2) and beclin-1 protein level in liver, inducing protein expressions [45]. Ni et al. [46], at the contrary, reported that ASX enhanced Akt phosphorylation and reduced lipid peroxidation, c-Jun N-terminal kinases (JNKs), p38 mitogen-activated protein kinases (p38MAPK) and p56 nuclear factor kappa-light-chain-enhancer of activated B cells phosphorylation.

NON-ALCOHOLIC FATTY LIVER DISEASES

Final body weight

Study or Subgroup	HFD + ASX Mean	SD	Total	HFD Mean	SD	Total	Weight	Std. Mean Difference IV, Random, 95% CI
Kim et al. 2017	52.1	1.2	9	54.7	1	9	24.1%	-2.24 [-3.48, -1.00]
Ni et al. 2015 (20W)	33.1	0.6	8	34	1	8	26.3%	-1.03 [-2.09, 0.03]
Ni et al. 2015 (32W)	38.9	1.1	8	40	1.1	8	26.4%	-0.95 [-2.00, 0.10]
Yang et al. 2014	41	1.67	5	39.3	2.43	5	23.3%	0.74 [-0.57, 2.04]
Total (95% CI)			**30**			**30**	**100.0%**	**-0.89 [-1.98, 0.20]**

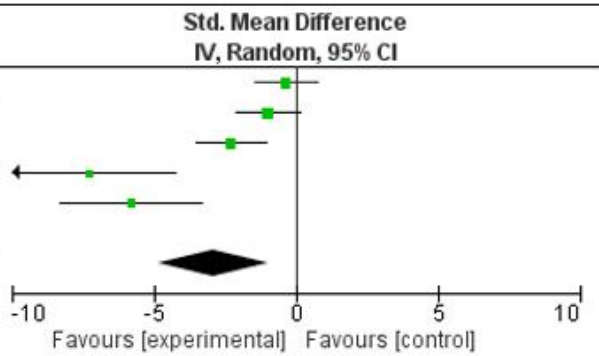
Heterogeneity: Tau² = 0.88; Chi² = 10.57, df = 3 (P = 0.01); I² = 72%
Test for overall effect: Z = 1.60 (P = 0.11)



Serum cholesterol

Study or Subgroup	HFD + ASX Mean	SD	Total	HFD Mean	SD	Total	Weight	Std. Mean Difference IV, Random, 95% CI
Jia et al. 2016 (HiASX)	216	16	10	222.5	22	5	22.8%	-0.34 [-1.42, 0.74]
Jia et al. 2016 (LowASX)	208.1	8.1	10	222.5	22	5	22.6%	-0.97 [-2.12, 0.18]
Kim et al. 2017	216.1	21.3	9	262.8	17.5	9	22.2%	-2.28 [-3.53, -1.03]
Ni et al. 2015 (20W)	122.2	4.5	8	154.2	3.8	8	15.1%	-7.26 [-10.32, -4.20]
Ni et al. 2015 (32W)	149	3.4	8	173.4	4.5	8	17.3%	-5.78 [-8.29, -3.28]
Total (95% CI)			**45**			**35**	**100.0%**	**-2.90 [-4.82, -0.98]**

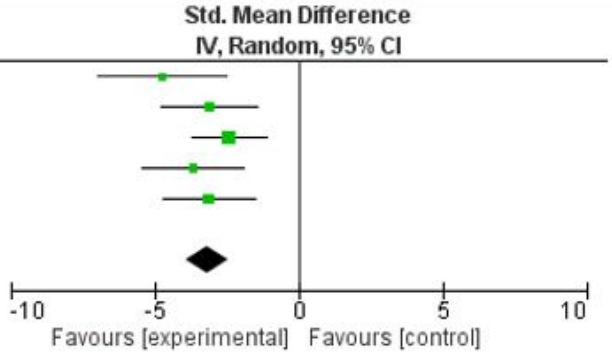
Heterogeneity: Tau² = 3.90; Chi² = 31.32, df = 4 (P < 0.00001); I² = 87%
Test for overall effect: Z = 2.96 (P = 0.003)



Serum triglycerides

Study or Subgroup	HFD + ASX Mean	SD	Total	HFD Mean	SD	Total	Weight	Std. Mean Difference IV, Random, 95% CI
Jia et al. 2016 (HiASX)	58	8	10	97.7	7.7	5	10.5%	-4.72 [-6.97, -2.48]
Jia et al. 2016 (LowASX)	69.1	9.1	10	97.7	7.7	5	18.8%	-3.10 [-4.77, -1.42]
Kim et al. 2017	35.6	2	9	41.9	2.9	9	32.2%	-2.41 [-3.69, -1.13]
Ni et al. 2015 (20W)	32	2.8	8	43.4	3.1	8	17.2%	-3.65 [-5.40, -1.89]
Ni et al. 2015 (32W)	43.1	4.4	8	57.4	4.3	8	21.2%	-3.11 [-4.69, -1.53]
Total (95% CI)			**45**			**35**	**100.0%**	**-3.14 [-3.87, -2.42]**

Heterogeneity: Tau² = 0.00; Chi² = 3.49, df = 4 (P = 0.48); I² = 0%
Test for overall effect: Z = 8.46 (P < 0.00001)



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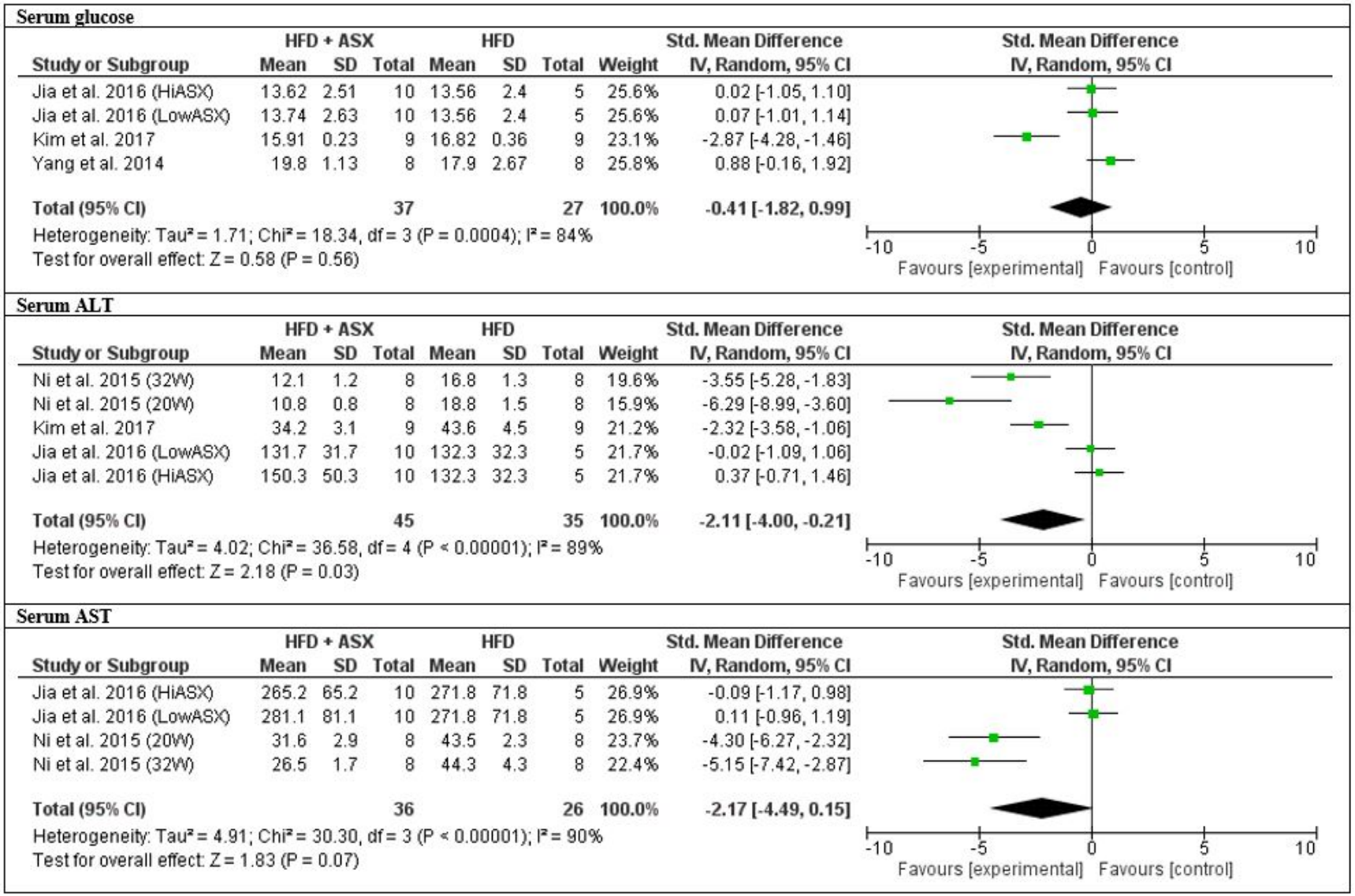
Serum glucose
HFD + ASX
HFD
Std. Mean Difference
Std. Mean Difference
Study or Subgroup Mean SD Total Mean SD Total Weight IV, Random, 95% CI
IV, Random, 95% CI
Jia et al. 2016 (HiASX) 13.62 2.51 10 13.56 2.4 5 25.6% 0.02 [-1.05, 1.10]
Jia et al. 2016 (LowASX) 13.74 2.63 10 13.56 2.4 5 25.6% 0.07 [-1.01, 1.14]
Kim et al. 2017 15.91 0.23 9 16.82 0.36 9 23.1% -2.87 [-4.28, -1.46]
Yang et al. 2014 19.8 1.13 8 17.9 2.67 8 25.8% 0.88 [-0.16, 1.92]
Total (95% CI) 37 27 100.0% -0.41 [-1.82, 0.99]
Heterogeneity: Tau² = 1.71; Chi² = 18.34, df = 3 (P = 0.0004); I² = 84%
Test for overall effect: Z = 0.58 (P = 0.56)
-10 -5 0 5 10
Favours [experimental] Favours [control]
Serum ALT
HFD + ASX
HFD
Std. Mean Difference
Std. Mean Difference
Study or Subgroup Mean SD Total Mean SD Total Weight IV, Random, 95% CI
IV, Random, 95% CI
Ni et al. 2015 (32W) 12.1 1.2 8 16.8 1.3 8 19.6% -3.55 [-5.28, -1.83]
Ni et al. 2015 (20W) 10.8 0.8 8 18.8 1.5 8 15.9% -6.29 [-8.99, -3.60]
Kim et al. 2017 34.2 3.1 9 43.6 4.5 9 21.2% -2.32 [-3.58, -1.06]
Jia et al. 2016 (LowASX) 131.7 31.7 10 132.3 32.3 5 21.7% -0.02 [-1.09, 1.06]
Jia et al. 2016 (HiASX) 150.3 50.3 10 132.3 32.3 5 21.7% 0.37 [-0.71, 1.46]
Total (95% CI) 45 35 100.0% -2.11 [-4.00, -0.21]
Heterogeneity: Tau² = 4.02; Chi² = 36.58, df = 4 (P < 0.00001); I² = 89%
Test for overall effect: Z = 2.18 (P = 0.03)
-10 -5 0 5 10
Favours [experimental] Favours [control]
Serum AST
HFD + ASX
HFD
Std. Mean Difference
Std. Mean Difference
Study or Subgroup Mean SD Total Mean SD Total Weight IV, Random, 95% CI
IV, Random, 95% CI
Jia et al. 2016 (HiASX) 265.2 65.2 10 271.8 71.8 5 26.9% -0.09 [-1.17, 0.98]
Jia et al. 2016 (LowASX) 281.1 81.1 10 271.8 71.8 5 26.9% 0.11 [-0.96, 1.19]
Ni et al. 2015 (20W) 31.6 2.9 8 43.5 2.3 8 23.7% -4.30 [-6.27, -2.32]
Ni et al. 2015 (32W) 26.5 1.7 8 44.3 4.3 8 22.4% -5.15 [-7.42, -2.87]
Total (95% CI) 36 26 100.0% -2.17 [-4.49, 0.15]
Heterogeneity: Tau² = 4.91; Chi² = 30.30, df = 3 (P < 0.00001); I² = 90%
Test for overall effect: Z = 1.83 (P = 0.07)
-10 -5 0 5 10
Favours [experimental] Favours [control]

359

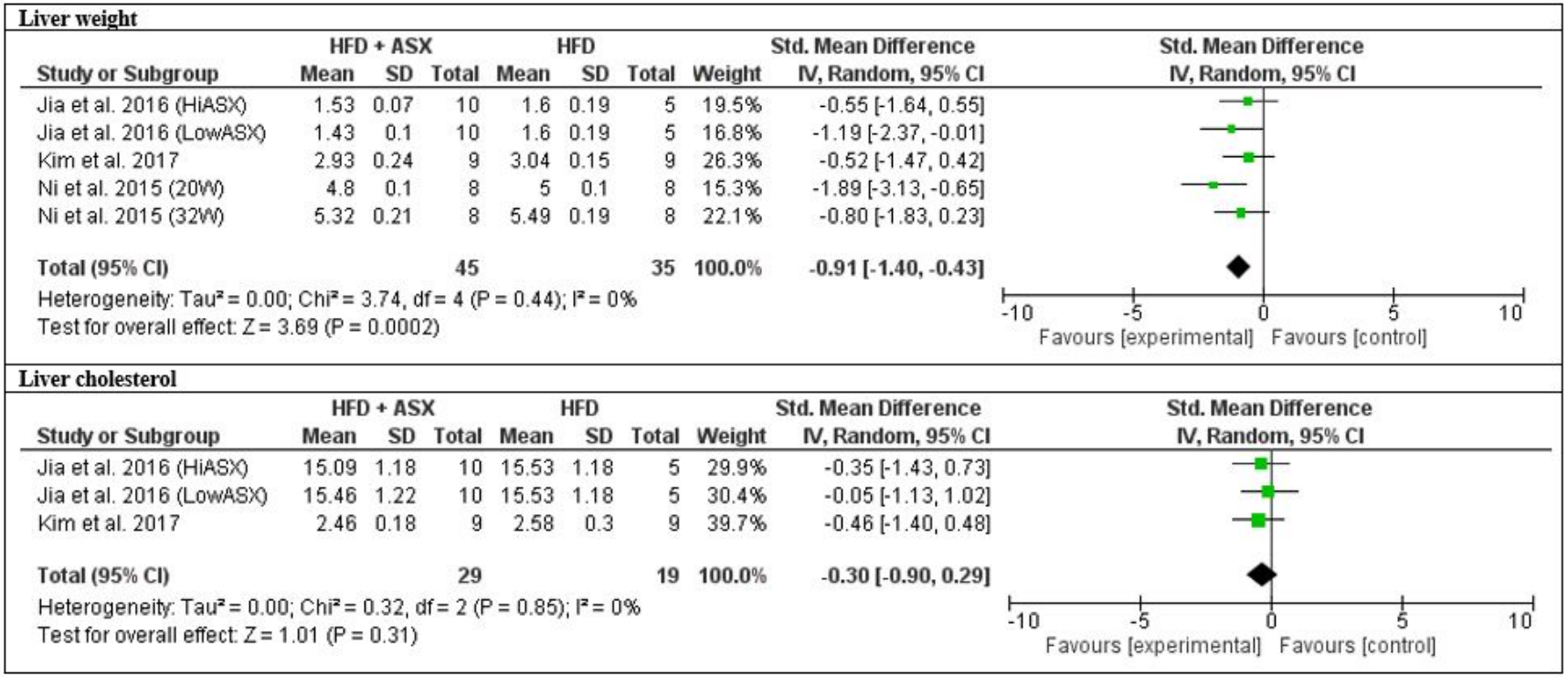

Figure 6: Forest plot comparing different parameters between treatment and control groups in animal models of non-alcoholic fatty liver disease. High astaxanthin concentration (HiASX), low astaxanthin concentration (LowASX). For Ni et al. 2015: 20 weeks of treatment (20W) and 32 weeks of treatment (32W)

365 **4. DISCUSSION**

366 This review aimed to systematically review the effect that ASX had on pathological conditions, such
367 as MetS, T2D and NAFLD, caused by an unbalanced diet (e.g. HFD, HFFD, HF/HS diet) in different
368 animal models. It also analysed how different ASX concentrations influenced different biomarkers
369 of disease in control animals or with disease phenotype.

370 In relation to biomarkers of metabolic syndrome, ASX, at different concentrations and administered
371 for different length of time, induced a significant reduction in adipose tissue weight (P=0.05) and
372 systolic blood pressure (P<0.0001) in control animals. However, it induced a significant increase in
373 few blood biomarkers (e.g. cholesterol, ALT and AST, P<0.10). On the contrary, ASX had positive
374 effects in animal models of T2D and NAFLD. In diabetic mice/rats, ASX significantly reduced serum
375 glucose levels (P=0.04) when administered for different length of time and concentrations. In animal
376 models of NAFLD, ASX significantly improved several disease biomarkers in the blood (e.g.
377 cholesterol, triglycerides, ALT and AST, P<0.10), while reducing liver (P=0.0002) and body weight
378 (P=0.11).

379 Results from this meta-analysis suggest that ASX ameliorates some of the parameters associated with
380 T2D and NAFLD and negatively affected by the pathology. In contrast, in healthy animals (control
381 animals from MetS studies), ASX affected liver function (ALT, AST) and blood lipid (TC) while
382 improving blood pressure and reducing adipose tissue weight. Moreover, the significant
383 heterogeneity measured within studies determining the effect of ASX, in T2D or NAFLD animals,
384 on BW (I^2=88% p<0.0001 and I^2=72% p=0.01, respectively) and glucose levels (I^2=76% p=0.006
385 and I^2=84% p<0.00001, respectively) was lost (P=0.15, P=0.35, respectively) when disease groups
386 were compared by meta-regression analysis (Table S1), suggesting that ASX effect on BW or glucose
387 is independent of disease status.

388 **Table S1**: Summary of SMD on effect of ASX on body weight and glucose levels in disease groups

	No of study	SMD	95% CI	I^2	[1]p value	[2]p value
Body weight						
T2D	4	0.67	-1.16-2.50	88%	P<0.0001	P=0.15

NAFLD	4	-0.89	-1.98-0.20	72%	P=0.01	
Glucose level						
T2D	4	-1.31	-2.58--0.04	76%	P=0.006	P=0.35
NAFLD	4	-0.41	-1.82-0.99	84%	P<0.00001	

[1]p value for heterogeneity within each disease group; [2]p values for heterogeneity between disease groups with meta-regression analysis

In order to better understand and explain some of the changes induced by ASX on different biomarkers of disease, it is important to consider some of the molecular mechanisms by which ASX may affect such parameters.

MetS, as previously mentioned, is a multifactorial pathological condition that affects different organs such as liver, pancreas, adipose tissue, skeletal muscle and intestine, and which can lead to the onset of various diseases, including T2D and NAFLD. A link between the different pathologies analysed in this systematic review - meta-analysis is present and worth of investigation. A diet high in calories and rich in fat and sugars leads to a significant accumulation of visceral fat that makes individuals overweight and, in most cases, obese [1]. Fat accumulation, however, is not the only consequence: by consuming high quantities of fats and carbohydrates, metabolic dysfunction occurs at tissue/cellular level that leads to cells becoming resistant to insulin and to develop glucose intolerance that, if left untreated, induces onset of T2D [48]. Moreover, excessive macronutrient intake in the diet also affects liver function which is impaired by excessive accumulation of fatty acids (FA) in liver cells (NAFLD). All of these physiological dysfunctions are combined with a high degree of chronic inflammation and oxidative stress [8]. Several mechanisms are responsible for the release of ROS: Wright et al. [49] have shown that, in diabetes, ROS release is closely linked to the high fluctuation of glucose in the blood, which subsequently stimulates mitochondrial dysfunction and subsequent production of ROS. Excessive ROS production also affects nitric oxide (NO) bioavailability and induces its sharp decrease. This leads to the formation of superoxide anions activating NF-kβ that is responsible for inducible nitric oxide synthase (iNOS) increase in expression. The whole process ends with the formation of peroxynitrite that is toxic to the vascular endothelium and thus compromises its function [50]. In NAFLD, although mitochondria are involved in the production of ROS [51], there are other causes responsible for oxidative stress. The β-oxidation of fatty acids contributes to

mitochondrial dysfunction, and alteration of the endoplasmic reticulum (ER) as well as NADPH oxidase (Nox) resulting in ROS production and dysregulation of lipid metabolism. Various molecules are subsequently affected by ROS accumulation, including the sterol regulatory element-binding protein 1c (SREBP1c) and patatin-like phospholipase domain-containing 3 (PNPLA3,) which then lead to insulin resistance [52]. MetS, as previously described by Vona et al [6], is characterized by higher levels of oxidative stress observed in obese patients than in lean. Maslov et al [53], in their review, described how the oxidative process in MetS, both generated by the increase in blood glucose (as for T2D) and by the increase in fatty acids ingested with HFD (as for NAFLD), leads to an accumulation of malondialdehyde (MDA) in the adipose tissue. In fact, in mice fed with HFD, Talior et al [54] showed high levels of hydrogen peroxide (H_2O_2) in the plasma of mice affected by MetS. As with the other two diseases, also in the MetS, it appears that Nox is responsible for the production of ROS. The high presence of ROS induces the production of protein kinase C-δ (PKC-δ) responsible for the activation of Nox which, in loop, produces new ROS [55].

Most of the studies, considered in this systematic review, suggest a role for ASX in modulating different pathophysiological parameters such as body and liver weight gain, hyperglycaemia, hyperinsulinemia, insulin resistance, lipid deposition, increase in inflammatory cytokines, and oxidative damage. Animals fed with HFD/HFFD had elevated HOMA-IR index, elevated thiobarbituric acid reactive substances (TBARS) level and reduced insulin sensitivity (quantitative insulin sensitivity check index (QUICKI)), as well as elevated serum levels of ALT, AST, TG, TC, and free fatty acids (FFA) [36].

One of the mechanisms by which ASX affects the different pathophysiological conditions may involve contrasting lipid accumulation. Ikeuchi et al. [38] demonstrated how ASX increased energy expenditure without affecting food intake, while Nishida et al. [32] showed that ASX activated 5' AMP-activated protein kinase (AMPK) in skeletal muscle, and upregulated expression of transcription factors, thus inducing mitochondrial remodelling that subsequently increased oxidative phosphorylation and β-oxidation of fatty acids [46]. These results are supported by Yang et al. [47]

that highlighted how ASX has a lipid-lowering effect, inducing the transcription of acyl-CoA oxidase 1 (ACOX-1), which is also responsible for the oxidation of fatty acids. Yang et al. [47] also argued that ASX induced expression of the peroxisome proliferator-activated receptors (PPAR), a subfamily of nuclear receptors that control many different target genes involved in both lipid metabolism and glucose homeostasis [56], as PPAR increases the expression of ACOX-1. PPAR has a very important role in the lipid-lowering action of ASX, in fact, PPAR, according to Kim et al. [43], induced the expression of mitochondrial proteins such as carnitine palmitoyl-transferase 1 (CPT1) [43]. Kobori et al. [44] also claimed that ASX, by increasing the transcription of PPARα, improved transport, metabolism, and oxidation of FAs, and therefore, reducing their accumulation in adipocytes. This led to an increase in the levels of FA in the blood, especially HDL as confirmed by Kimura et al. [41]. Jia et al. [45] added that the rise in HDL levels was due to the action of liver X receptor alpha (LXRα), which was increased by treatment with ASX whereas hepatic lipogenesis was blocked. ASX inhibited the phosphorylation of Akt, inducing the expression of Insig-2α and consequently reducing SREBP1 and GSK3. In NAFLD, lipid accumulation is an important aspect of the pathology and ASX may reduce not only hepatic steatosis but may interfere with transforming growth factor beta 1 (TGF-β1) activity, a strong profibrogenic factor [47].

Shifting the metabolism towards the use of fatty acids may cause an accumulation of free radicals and ROS at the cellular level. ASX is a powerful antioxidant thanks to the hydroxyl and ketone fractions present on the ionic ring and thanks to its ability to remove singlet oxygen [32]. Yang et al. [47] highlighted how ASX reduced ROS generated by FA β-oxidation, through activation of the Nrf-2 (nuclear factor erythroid 2-related factor 2) pathway. Chen et al. [26], who carried out a study on gestational diabetes (GTD), found that ASX restored the Nrf2/HO-1 (heme oxygenase 1) signaling pathway in the liver. Nrf2, as a transcription factor, plays a key role in the regulation of oxidative stress within cells [57] while HO-1, being a target of Nrf2, helps to reduce oxidative stress [58]. In addition to Nrf2/HO-1, Chen et al. [26] demonstrated that antioxidant enzymes such as SOD, Cat, and GPX were activated in the liver of pregnant animals treated with ASX, and these results were

also supported by Kumar et al. [40] findings in male animals. Obesity may affect endoplasmic reticulum (ER) correct folding of proteins and, when homeostasis is perturbed, accumulation of misfolded proteins occurs that triggers a response in the ER and activation of BiP (binding immunoglobulin protein), responsible for the correct folding of proteins [59]. BiP increases its activity by stimulating mitochondrial oxidative phosphorylation that produces ROS [60]. The administration of ASX acts on the activity of BiP and, consequently, reducing ROS production [33]. ASX antioxidant activity is also responsible for inhibition of cytochrome P4502E1 (CYP2E1) activity in the liver, thus preventing liver damage caused by oxidative stress [36], and for reduction of TBARS (thiobarbituric acid reactive substances, a measure of lipid peroxidation) in adipose tissue [32].

Some studies conducted on human cells line by Chou et al. [61] have shown that ASX also has a direct effect on the production of ROS itself, in fact, following the use of ultraviolet B (UVB) rays, ASX scavenges ROS production in skin cells. Hormozi et al. [62] showed that ASX increases in a dose-dependent manner the activity of superoxide dismutase and catalase in LS-180 tumour cell lines. All these studies, therefore, show how ASX not only acts on the production of ROS when a pro-oxidative mechanism is in place but mostly regulates the endogenous mechanisms of the cells responsible for the elimination of ROS itself as described above.

Some of the beneficial effects of ASX may also be due to its effect on inflammation and the immune system. Gao et al. [31] have shown that ASX reduced production of proinflammatory cytokines such as TNF-α, IL-1β, and interferons-γ (IFNγ). While Bhuvaneswari et al. [33], Kumar et al.[40] and Ni et al. [46] demonstrated that ASX reduced the phosphorylation of IKKβ, NFkβ p56, and MAPK. Nishida et al. [32] reported instead the very important role of ASX in reducing macrophage's infiltration within the adipose tissue avoiding the apoptotic death of the adipocytes. Moreover, Ni et al. [46] reported that mice, affected by NAFLD, had an imbalance ratio between macrophages of type M1 (promoters of apoptosis) and macrophages of type M2 (antagonists of M1). ASX stimulated M2 macrophages and reduced M1. This result was also confirmed by Kim et al. [43] who showed, in animals treated with ASX, a reduction in the expression of F4/80, a macrophage marker. Finally,

492 studies conducted on diabetic animal models have shown that ASX reduced blood glucose levels by
493 improving its metabolism and incorporation into peripheral tissues [32]. Arunkumar et al. [34]
494 reported that ASX increased auto-phosphorylation of the insulin receptor (IR-β), improved
495 translocation of GLUT-4 into the skeletal muscle where it also restored the IRS-PI3K-Akt (insulin
496 receptor substrate-phosphatidylinositol 3-kinase-protein kinase B) metabolic pathway. Uchiyama et
497 al. [42] reported the protective action of ASX against β-pancreatic cells, very sensitive to the attack
498 of ROS, by increasing the level of insulin in the blood. As high blood sugar levels cause blood
499 pressure to rise, ASX may also have a positive effect on blood pressure by reducing glucose levels
500 and improving insulin resistance as shown by this meta-analysis and studied by Preuss et al. [35] that
501 reported how ASX interacted with the renin-angiotensin system (RAS) in a dose-dependent manner:
502 by increasing the dose of ASX, a decrease in blood pressure occurred.
503 This meta-analysis has some limitations mainly related to the big difference that exists between the
504 studies analysed; different animal models, diverse species, and different concentrations of ASX and
505 length of treatment were compared. However, such limitation could be also interpreted as strength of
506 the study: the results obtained are significant and consistent with those present in the literature,
507 validating the effect of ASX despite the wide heterogeneity of the studies included.
508 To our knowledge this is the first systematic review and meta-analysis on the effect of ASX in animal
509 models of obesity-associated diseases. We have shown that ASX has lipid lowering and
510 hypoglycaemic effect, reduced body, liver and adipose tissue weight while improving liver function
511 and blood pressure. We have also provided an explanation for its activity by considering
512 molecular/cellular mechanisms potentially involved. Among such mechanisms, activation of
513 transcription factors and signalling pathways linked to lipid metabolism, insulin secretion and
514 sensitivity and redox homeostasis play an important and differential role at tissue levels (Figure 7).
515 By showing that ASX supplementation in the diet had positive effects on symptoms associated with
516 obesity related diseases in animals, and considering that ASX concentrations used in some of the
517 articles included in this review were based on those used in humans [35], this systematic review and

meta-analysis provides a good starting point to inform future human intervention/supplementation studies.

As present-day sedentary life style and imbalance diet are conducive to people having a high body mass index (BMI), which triggers a series of pathophysiological dysfunctions within the human body with serious consequences, the use of antioxidant supplements may be beneficial. Antioxidants such as vitamin E can reduce and improve some of these aspects, but there is no evidence that many of these are effective in humans [63]. Similarly, ASX has lipid-lowering, hypo-insulin and hypoglycaemic capacity, protects organs from oxidative stress and mitigates the immune system, in animals as suggested in this review. Despite dietary research findings have suggested that consuming greater amounts of antioxidant-rich foods might help to protect against obesity related diseases and several studies in preclinical model of diet induced obesity-associated diseases have shown beneficial effects of antioxidants, rigorous trials of antioxidant supplements in large numbers of people have not found that high doses of antioxidant supplements prevent disease. Several reasons for the lack of substantial benefit of antioxidant supplements in clinical studies can include: i) differences in the chemical composition or doses of antioxidants in foods versus those in supplements may influence their effects; ii) the antioxidant supplements may not have been given for a long enough time to reverse the results of several decades of oxidative stress; iii) specific antioxidants might be more effective than the ones that have been tested; iv) the relationship between free radicals and health may be more complex than has previously shown in *in vitro* and *in vivo* studies; and v) participants included in clinical trials, even if at high risk for particular diseases, were not necessarily under increased oxidative stress. Future research should, therefore, consider some of these factors and explore, in well organised randomised clinical trials, the use of ASX as dietary supplement or nutraceutical to counteract and reduce the negative effects of obesity and associated diseases in humans, considering that toxicity tests have been conducted on healthy volunteers to ensure ASX safety [64].

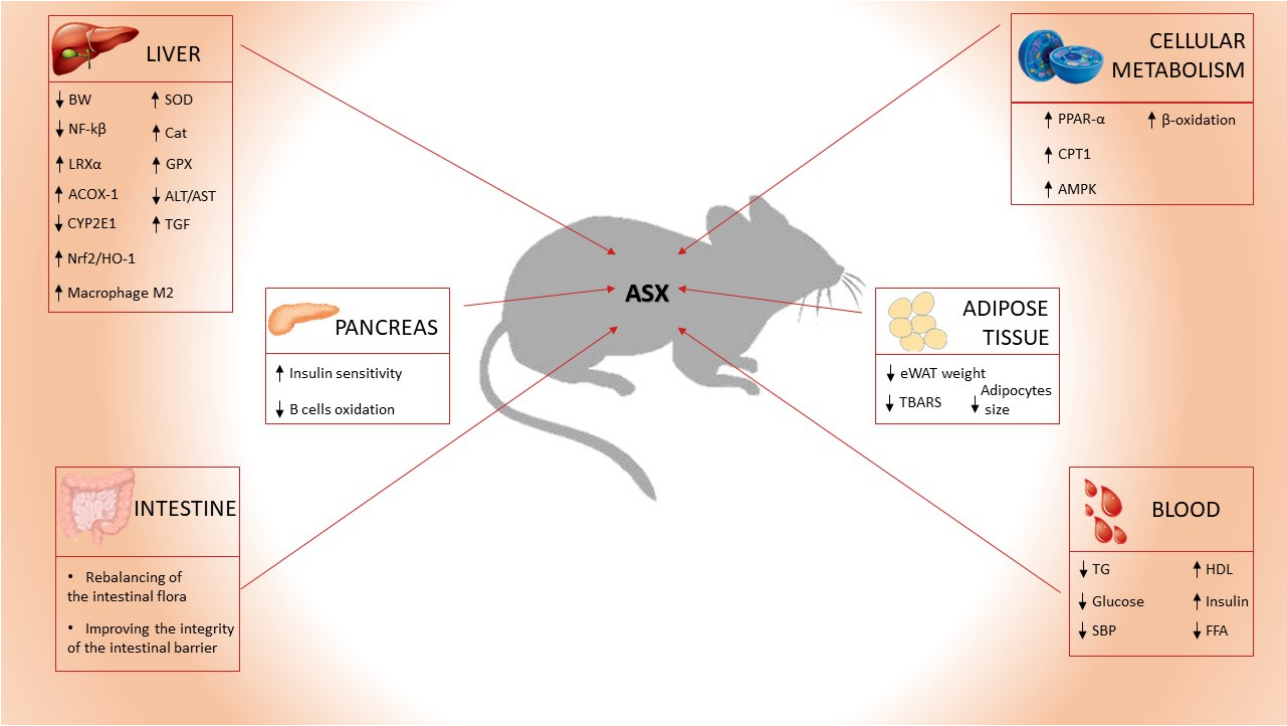

Figure 7: ASX mechanism(s) of action in animal models of obesity-associated diseases**.**

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Authors' contribution

RPR and GB conceptualized the study, developed the protocol, selected articles for full-text review. RPR extracted data from the included studies, and RPR and GB performed all statistical analyses. RPR, ARL, EV, MCP, GM and GB wrote and reviewed the manuscript. .

Conflict of interest

None

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Table S1: Summary of SMD on effect of ASX on body weight and glucose levels in disease groups

	No of study	SMD	95% CI	I^2	[1]p value	[2]p value
Body weight						
T2D	4	0.67	-1.16-2.50	88%	P<0.0001	P=0.15
NAFLD	4	-0.89	-1.98-0.20	72%	P=0.01	
Glucose level						
T2D	4	-1.31	-2.58--0.04	76%	P=0.006	P=0.35
NAFLD	4	-0.41	-1.82-0.99	84%	P<0.00001	

[1]p value for heterogeneity within each disease group; [2]p values for heterogeneity between disease groups with meta-regression analysis