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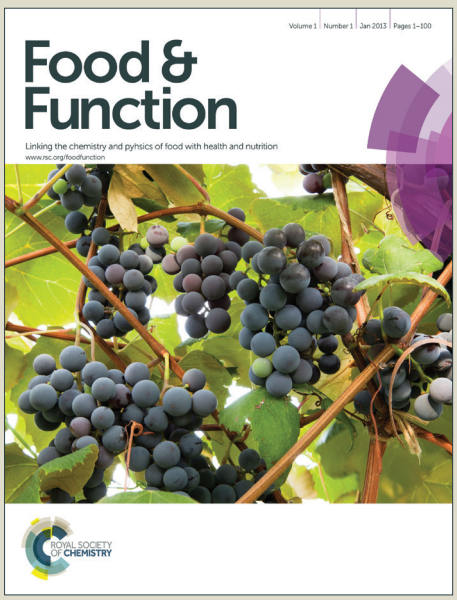

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Effect of chronic consumption of blackberry extract on high-fat induced obesity in rats and its correlation with metabolic and brain outcomes

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Abstract

Flavonoids have been presented as potential protectors against metabolic and cognitive dysfunction. However, mechanisms underlying these 'claims' are not sufficiently explored. To analyse the effect of long-term supplementation with blackberry extract (BE) in a context of high-fat or standard diet, Wistar rats were divided in 4 groups (n=6) fed with standard or high-fat diet, with or without BE supplementation of 25 mg/kg body weight per day. High-fat diet significantly impaired glucose tolerance and increased: body weight, caloric ingestion, very-low-density lipoprotein, triglycerides and cholesterol. Furthermore, it was observed that high-fat diet increased dopamine content in prefrontal cortex and decreased BDNF levels both in prefrontal cortex and plasma. BE supplementation only affected part of those effects. BE slightly improved glucose metabolism and significantly decreased levels of lactate, independently of diet. BE decreased levels of BDNF and also interacted with dopaminergic system, increasing dopamine turnover in striatum, and reverting dopamine content induced by high-fat diet in prefrontal cortex. This study shows that although some particular benefits of anthocyanins supplementation some long-term effects may not be desirable and further studies are needed to optimize ingestion conditions.

Keywords: anthocyanins, dopaminergic system, flavonoids, high-fat diet, neuroprotection, obesity

1-Introduction

Obesity is a health problem that has reached epidemic proportions. In some European countries more than one fourth of population are already obese, presenting body mass index (BMI) higher than 30 kg/m^2 [1]. A phenomenon which is assuming same or larger proportions in several other industrialized countries, i.e. in USA the rate of obesity reach 35% in 2011-2012 [2]. On overall more than 60% of the US and European adult population are obese or overweight BMI>25 kg/m^2 [3]. This epidemic represents an economic and social burden since several comorbidities are associated with obesity [4]. People with obesity have among other features an increased risk for suffering from diabetes, hypertension cardiovascular diseases, cancer and neurogenerative diseases [5]. In the past decade, much attention has been paid to the potential negative effect of obesity on brain function and general risk of dementia [3, 6]. Like obesity also dementia is becoming a worldwide problem and an international priority, with millions of new cases every year [7]. As obesity and its co-morbidities are associated with brain dysfunction, the development of therapeutic strategies to treat one condition can have worthy improvements in the other one.

Diet is an undeniable modifiable factor which may influence both risk of obesity and impaired brain function. It is well documented the exacerbation of both conditions by westernized diets, typically rich in fat and sugar. In vivo studies have also shown that high-fat diet fed animals have several brain disorders among them, dysfunction of dopaminergic system and of neurogenesis impairment [8-10].

Fruits and vegetables, by opposition to high-fat and high-sugar diets have being proved to reduce risk of obesity and associated comorbidities [11, 12]. Also intake of fruits and vegetables have been associated with a decrease risk of cognitive decline or Parkinson disease, in part due to their high composition on natural polyphenols, including flavonoids [13, 14]. In the past years, berries attracted interest as a nutritional approach to improve health, metabolic and cognitive outcomes [15-17]. Blackberries, in particular, are naturally rich in flavonoids, specifically in anthocyanins and although its extracts had already shown positive effects in improving cognitive functions and

78 antioxidant status in animal studies [18-21] there remains a need to further explore and clarify the
79 anthocyanins full potential.

80 This study aimed to explore the beneficial potential of long-term supplementation with blackberry
81 anthocyanins extract in a context of standard or high-fat diet. For this purpose male Wistar rats fed
82 with standard or high-fat diet for 17 weeks were supplemented with blackberry anthocyanin extract
83 (25 mg/kg b.w.) and metabolic evolution as well as biomarkers of brain function were analysed.

2- Methods

2.1-Animal care and study design

Twenty-four male Wistar rats (200-250 g body weight; 7 weeks) were acquired from Harlan Laboratories (Santiga, Spain) and after two weeks of acclimatization divided into four groups (n=6 rats per group): C)- standard diet; BE)- standard diet + blackberry extract; HF)- high-fat diet; HFBE)- high-fat diet + blackberry extract. Animals were fed *ad libitum* with commercial diets: standard chow (4% fat) from Harlan (2014S, Teklad Diets, Harlan Laboratories, Santiga, Spain) and high-fat chow (45% fat) from Research Diet D12451 (New Brunswick, NJ, USA) for 17 weeks. Blackberry extract dose was weekly weight adjusted, (25 mg kg^{-1} body weight), dissolved daily in sterile water and embedded in food pellets that animals had daily access. Animal were maintained at 23–25°C and 12/12 h light–dark cycle, and housed two per cage. Food ingestion was measured twice a week. Animal handling and housing protocols followed European Union guidelines (86/609/EEC) and Portuguese Act (129/92) for the use of experimental animals. The study had ethical approve from Ethical Committee of Faculty of Medicine University of Porto and São João Hospital Center.

2.2-Preparation of blackberry extract

Preparation of anthocyanin blackberry extract was achieved using previously described methods [22, 23]. Briefly, blackberries (*Rubus fruticosus*) were extracted with 50% aqueous ethanol (pH 1.5, acidified with HCl) for 24 hours at 22˚C. The solution obtained was filtered (50 µm nylon membrane) and concentrated using a rotary evaporator under at 30˚C. The concentrated extract was added to a polyamide gel column (Mesh 100–120) to remove sugars. The sugar-free anthocyanin extract was freeze-dried and stored at -20˚C. The anthocyanin extract was analyzed by HPLC at 520 nm, as previously described [24]. Following preparative HPLC, the purified extract was characterised for anthocyanin content by analytical HPLC coupled to UV-Vis, DAD-ESI/MS and NMR techniques.

2.3-Insulin tolerance test and glucose tolerance test

Insulin and glucose tolerance tests were performed at week 3, 8 and 15 of treatment period with one interval day between tests. For insulin tolerance test, after 5 hours of food privation, a solution of insulin (0.5 U/kg weight) was administered by intraperitoneal injection [25]. Glucose levels were measured from tail blood with a *Precision Xceed®* glucometer before (0) and 30, 60, 90 and 120 minutes after insulin injection. Glucose tolerant test was performed after 5 hours of food deprivation, a solution of glucose (2 g/kg weight) was administered by oral gavage as previously described [26]. Glucose levels were measured before (0) and 15, 30, 60, 90 and 120 minutes after glucose administration.

2.4-Blood pressure measurements

Systolic blood pressure was measured with a tail cuff method[27]. To minimize stress-induced variations in blood pressure all measurements were taken in a peaceful environment and after two trials performed in the week before to allow animals to accustom procedure. Values were registered when consistent between three consecutive measures, and pulse stable. This procedure took place at weeks 9 and 14 of treatment period.

2.5-Tissue collection and body composition measurements

At the end of 17 weeks animals were anesthetized with ketamine+xylazine (50 mg kg^{-1}+1 mg kg^{-1}), nasoanal lengths and abdominal circumference were obtained using a measuring tape and body composition of each rat was determined by bioelectrical impedance (Quantum /S bioelectrical impedance analyser, RJL Systems, Akern SRL, Florence, Italy), according to the described in the literature [28] . Animals were kept with isoflurane during blood collection from left ventricle. Blood was collected with heparinised needles and PMSF (phenylmethylsulfonyl fluoride), a protease inhibitor, was added at final concentration 100 µM. After centrifugation at 2000 *g* for 15 min, plasma was collected and stored at -80˚C. Rats were decapitated for brain removal. Pre-frontal cortex, striatum, the remaining brain, liver, subcutaneous adipose tissue-scAT and mesenteric adipose

tissue-mAT were collected, immediately frozen with liquid nitrogen and kept at -80ºC until use. A small portion of scAT and mAT from each animal was also fixed in buffered formaldehyde 10%.

136 **2.6-Biochemical analysis**

137 For urine collection, rats were placed in metabolic cages, after being acclimated during the
138 previous week. Routine plasma and urine biochemical analysis were performed in a certified Clinical
139 Analysis Laboratory. In addition, adiponectin, leptin and brain derived neurotrophic factor (BDNF)
140 were quantified using the following enzyme linked immunoabsorbent assay (ELISA) commercial kits:
141 adiponectin (Life Technologies Ltd, Paisley, UK), leptin (Merck Milipore, Madrid, Spain) and
142 chemiKine brain derived neurotrophic factor (Merck Millipore, Madrid, Spain). For BDNF
143 determination a small portion of whole brain was homogenized using 100 mM Tris/HCl, pH 7,
144 containing 2% bovine serum albumin (BSA), 1 M NaCl, 4 mM EDTA.Na_2, 2% Triton X-100, 0.1%
145 sodium azide and a protease inhibitor (cOmplete mini, Roche Diagnostics, USA). Homogenates were
146 prepared using 4 µL of the described buffer for mg of tissue weight. All following procedures in
147 plasma and brain homogenate were performed according to manufacturer's instructions.

148 **2.7-Morphometric analysis of adipose tissue**

149 Following at least 48 h (4˚C) of formaldehyde fixation, the adipose tissues were dehydrated
150 and finally embedded in paraffin. All samples were coded for a blind analysis and 3 µm-thick sections
151 were obtained with a Leica® Microtome (RM2125RT, Lisbon, Portugal), and stained with
152 haematoxylin and eosin to assess morphology. Digital images were acquired with a fluorescence
153 microscope (Nikon Eclipse 50i®, Melville, USA), at a magnification of x200 from randomly-selected
154 different optical fields. Adipocyte area measurement was performed in 100 random adipocytes,
155 using ImajeJ software® (National Institute of Health, Bethesda, USA).

156 **2.9- Fatty acids analysis**

157 For the analysis of the total fatty acid (FA) composition, 200 mg of liver and 30 mg of adipose
158 tissue, were accurately weighed and prepared as previously described [29]. For quantification
159 purposes, samples were added with 100 µL of tritridecanoin (1.34 mg/ml), used as internal standard
160 prior to derivatization. Fatty acid methyl esters (FAME) were analyzed in a gas chromatograph
161 HP6890A (Hewlett-Packard, Avondale, PA, USA), equipped with a flame-ionization detector (GLC-

FID) and a BPX70 capillary column (50 m x 0.32 mm x 0.25 µm; SGE Europe Ltd, Courtaboeuf, France). Analysis conditions were as follows: injector (split 10:1; injection volume 1 µl) and detector temperatures were 250ºC and 270ºC respectively, carrier gas was Hydrogen (11 psi) and the oven temperature program started at 60ºC (hold 2 min) raised 10ºC/min to 135ºC (hold 2 min), them 10˚C/min to 165ºC (hold 2 min) and finally 10ºC/min to 230ºC (hold 7 min). Supelco 37 and CRM-164 were used for identification of fatty acids. GLC-Nestlé36 was assayed for calculation of response factors and detection and quantification limits (LOD: 0.079 µg FA/ml; LOQ: 0.264 µg FA/ ml).

2.10- Catecholamine determination

Dopamine (DA) and its metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) quantification in striatum was performed by high pressure liquid chromatography with electrochemical detection (HPLC-ED), as previously described [30]. In brief, aliquots of 1.5 ml of 0.2 M perchloric acid in which tissues were kept were placed in 5 ml conical-based glass vials with 50 mg alumina and samples pH was immediately adjusted to pH 8.6 with Tris buffer. The adsorbed catecholamines were then eluted from the alumina with 200 µL of 0.2 M perchloric acid and centrifuge at 1200 *g* Spin-X tubes with 0.22 µm pore membrane (Costar®, Tewksbury MA, EUA); 50 µL of the eluted was injected into HPLC (Gilson Medical Electronics, Villiers, le Bel, France) and 3,4-dihydroxybenzylamine was used as an internal standard. The results were adjusted for tissue weight.

2.10- Statistical analysis

All the groups were tested for effects of diet (D), treatment (BE) and their interaction (D x BE) by two-way ANOVA using GrahPad Prism® 6.0 Software. When interaction between diet and treatment were significantly different, means were compared using Fisher's LSD multiple-comparison post-test. Correlations were tested using Pearson correlation test. Statistical significance was considered when $p<0.05$.

3-Results

3.1- Body weight, weight gain and caloric ingestion

All animals started the treatment with similar weight (246.5 ± 1.9 g) and at end of treatment animals maintained similar weights between groups C and BE (429 ± 28 g and 433 ± 29 g, respectively). Animals from HF and HFBE had 551 ± 68 g and 581 ± 63 g body weight respectively. Increase in body weight over time was more pronounced in groups fed HF diet (Figure 1A). Weight gain was significantly increased by diet (Figure 1B). Effect of supplementation with BE on body weight gain was not significant.

Food ingestion was converted from g to kCal, knowing that standard and high-fat chow have 2.9 kCal and 4.73 kCal per gram, respectively. As show in Figure 1C, caloric ingestion was affected by both diet and by BE supplementation (Figure 1C). Curiously, there was a significant interaction between effects of type of diet and BE supplementation on caloric ingestion. Caloric ingestion of animals supplemented with BE on HF diet was slightly higher (82 kCal/animal/day) than those not supplemented (75 kCal/animal/day), however it was not reflected in weight gain.

3.2- Metabolic parameters

Glucose and insulin tolerance tests were performed at weeks 3, 8 and 15 of the treatment. HF diet impaired overall animals' glucose sensibility seen as an increase of total area under the curve (AUC) (Figure 2A). This effect was seen as soon as after three weeks of study and it was maintained until the end of treatment (Figure 2B, 2C and 2D). Animals from BE group presented a tendency to a lower AUC, significant at the end of test on week 8.

After 15 weeks of treatment, both animals' basal blood glucose and glucose tolerance response were significantly affected by diet but not by BE supplementation (Figure 2D).

Glycaemic levels in insulin tolerance test was affected by diet after only three weeks of treatment (Figure 3A). BE supplementation significantly increase insulin response at 60 min. Curiously, at week 8 (Figure 3B) animals fed with HF diet had a higher response to insulin, presenting a lower peak at min 30; however the animals fed with standard diet were the ones that sustained

the decrease in glycaemia as seen at 120 min. As it can be seen in Figure 3C after 15 weeks, animals from HFBE group had a better response to insulin than HF group.

Systolic blood pressure seemed to be affected by diet but not by BE supplementation after 8 weeks of treatment. No effect was seen after 14 weeks of treatment (Table 1).

3.3- Biochemical parameters

At the end of treatment plasmatic biochemical analyses were performed. As described in Table 2, HF diet affected lipid metabolism, increasing very low dense lipoprotein (VLDL), triglycerides (TG) and cholesterol of the animals. Regarding hepatic enzymes, only alkaline phosphatase (ALP) activity was strongly increased by HF diet. Also, levels of urea were increased by HF diet. BE supplementation decreased aspartate aminotransferase activity (ASAT) and decreased creatinine kinase activity (CK). Plasmatic lactate levels were strongly affected by BE supplementation but not with high-fat diet. Interaction between diet and BE supplementation affected albumin and creatinine levels. While in C animals albumin seemed to decrease and creatinine increase, the opposite effect was seen in HF fed animals.

Renal function was affected by HF diet: there was an increase in urine excretion of urea, sodium and albumin and a decrease in glycosuria. BE also contributed for an increase in renal excretion of urea.

Plasmatic levels of brain derived neurotropic factor (BDNF) were significantly decreased in HF fed animals. BE group showed decreased levels but the supplementation had no effect regarding HF fed groups (Table 3). BDNF levels in brain frontal cortex were measured to correlate with plasmatic ones. Cortical BDNF levels were decreased both by diet and by BE supplementation. There was not a correlation between plasmatic and central BDNF ($r=0.361$ $p=1.129$) considering all tested animals. However when considering each group isolated, control groups (C) had a strong association between both localizations ($r=-0.967$ $p=0.033$) but both HF diet and BE supplementation disrupted this correlation.

3.4- Adipose tissue distribution, adipokine levels, and fatty acid composition

HF diet was the main contributing factor to variance in nasoanal length, waist circumference, free fatty mass, fatty mass weight and percentage, leading to increase in all these parameters (Table 4). The plasmatic levels of adipokines adiponectin and leptin (Figure 4A and 4B), were both increased by HF diet. BE supplementation also contributed to an increase in animals' leptin levels but not in adiponectin levels. The ratio between the pro-inflammatory leptin and the anti-inflammatory adiponectin showed that there is still an increase induced by HF diet but no effect by BE (Figure 4C).

Morphology of adipose tissue was only affected by type of diet (Figure 5A). Surprisingly, HF diet increased adipocyte area only in subcutaneous depots (Figure 5A). Fatty acid composition was analysed on liver and on two distinct deposits of adipose tissue, known to have different metabolic implications: the subcutaneous and mesenteric adipose tissue. The concentration of each fatty acid is described in Tables 5-7B. Compared to C animals, HF diet animals had more accumulation of total monounsatured fatty acids (MUFA) in mesenteric adipose tissue, less polyunsatured fatty acids (PUFA) (Table 5A) and no differences in total saturated fatty acids (SFA) (Table 5B). This effect was mainly due to more accumulation of fatty acids as the monounsatured oleic acid (C18:1 c9) and less as polyunsatured linoleic acid (C18:2 c9c12). In subcutaneous adipose tissue (Tables 6A and 6B), HF diet increased SFA and MUFA with no effect on PUFA. BE supplementation did not had effect on the global SFA, MUFA or PUFA. Nevertheless, some differences were observed in particular fatty acids within these classes, specifically in C17i and C15:1 c10, both increased in mAT of BE group; also C17i was increased in scat of these animals. There were no significant effects of BE on fatty acid composition in liver (Table 7).

3.5- Dopamine content

Dopamine (DA) levels were quantified in pre-frontal cortex and striatum. DA levels were affected by type of diet in pre-frontal cortex and there was an interaction of BE extract with this effect. As it can be seen in Figure 6A, although not having effect on C animals, supplementation with BE reverted the increase in DA content seen in HF animals. In striatum, type of diet did not affect DA levels while BE supplementation significantly decreased DA content (Figure 6B). Levels of the

dopamine metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) were not affected by any factor (Figure 6C). This reflected on an increase in DOPAC/DA ratio, indicating a higher DA turnover with BE supplementation (Figure 6D).

4- Discussion

This study aimed to analyse the impact of long-term BE anthocyanins consumption on a global metabolic approach in rats with or without diet-induced obesity. Several metabolic parameters were analysed: resistance to insulin, glucose tolerance, blood pressure, biochemical parameters and adipose tissue morphology, function and fatty acid storage.

Wistar rats were supplemented with an anthocyanin BE in a normal and in an obesity context for 17 weeks. The HF diet insult resulted, as expected, in overweight and some metabolic dysfunctions as impairment in glucose tolerance and insulinemic response, increase in percentage of fat body weight, increase in scAT adipocyte size and increase in the adipokines production. Although after 9 weeks of treatment, systolic blood pressure seemed altered, hypertension was not installed at the end.

As diet seems to be a key factor in controlling and counteracting the installation of obesity and associated comorbidities, an extract from blackberry, rich in anthocyanins was simultaneous given to a group of animals. This supplementation seemed to induce a better glycaemic response in both BE and HFBE animals. Some studies have shown a decrease in glycaemic response after anthocyanin consumption. The mechanisms behind this effect are not completely clear, but it is possible to involve an effect on glucose transport [31]. Cyanidin-3-glucoside and delphinidin-3-glucoside have been associated with anti-diabetic properties in different in vitro and in vivo models [32]. Obesity has associated a low-grade chronic inflammation component, which is still unclear whether it is a cause or a consequence of associated co-morbidities. Increase in adipocyte size for instance, prompts adipose tissue inflammation, which is also dependent on the type of fatty acids stored [33]. Adipocytes size has been shown to be an indirect measurement of inflammation as it correlates with several rick factor of metabolic dysfunction [33]. The propensity to lower adipocytes size in mesenteric tissue may be predictive of a decreased risk of inflammation by BE supplementation. Localization of adipose

292 tissue can predict the risk of metabolic consequences, more associated with visceral fat, while
293 subcutaneous fat can have a protector effect [34]. Our results show that type of diet significantly
294 increase cholesterol levels, but curiously this effect was only visible on HFBE group and not on HF
295 group as would be expected from previous literature [35, 36]. This effect may be, in part, due to the
296 decrease in HDL seen in HF groups and prevented in HFBE group.

297 A very unusual but relevant result was the reduction of lactate release to bloodstream after
298 BE consumption. Hiperlactatemia have been found in obese humans [37, 38] and previous studies have
299 shown that, besides muscle, lactate can also be produced in other tissues including adipose tissue [39].
300 This lower lactate levels in groups with anthocyanins BE extract consumption may be seen as
301 positive since lactate levels are co-related with cardiovascular diseases and overall mortality [40].

302 BE intake decreased BDNF content in both plasma and brain of standard-fed animals which is
303 in agreement with Klein et al. that have shown that blood BDNF concentrations correlate positively
304 with BDNF levels in the hippocampus of rats and pigs [41]. It is well documented that BDNF is involved
305 in synaptic plasticity, neuronal differentiation and survival of neurons and thus its increase is usually
306 associated with beneficial outcomes. Nevertheless, Miyazaki et al. [42] observed significant increases
307 in serum BDNF levels in patients with neurodevelopmental disorders, such as autism or mental
308 retardation, compared to normal controls. Also, Si-Hoon demonstrated plasma BDNF levels has
309 significant positive correlations with the severity of inattention symptoms in children [43]. While El-
310 Gharbawy et al, 2006 [44] showed that plasmatic BDNF concentrations were decreased in obese
311 individuals, a recent study have also shown that obesity does not affect BDNF levels [45]. Low levels of
312 BDNF have been associated with changes in dopamine receptors [46] and by opposite increasing levels
313 of BDNF showed to increase dopamine turnover [47, 48]. BDNF gene expression in the frontal cortex of
314 the DAT knockout mice was shown to be reduced [49]. Both HF diet and BE supplementation
315 decreased BDNF levels but the mechanisms behind this same effect may be different.

316 In previous animal studies supplementation with blueberries, also rich in anthocyanins, or
317 with the flavonoids alone, for six weeks resulted in increased hippocampal levels of BDNF [50, 51].
318 Supplementation with BE, although decreasing BDNF levels, showed increased dopamine turnover in

striatum. Previous studies have suggested an association between glucose and dopamine levels [52], with low levels of glucose in the brain inhibiting the dopamine release [52, 53]. Interference of flavonoids with glucose transporters which was already shown in previous reports [31, 54] and include modulation of glucose access to the brain, could have a role in the dopamine changes observed in this study.

The ratio leptin/adiponectin has been correlated with metabolic parameters and predictor of cardiovascular risk [55]. Diet increased this ratio with no effects by BE supplementation. These animals have also increased production in adiponectin and leptin, produced by adipose tissue which is known to be involved in metabolic regulation.

Anthocyanins has been suggested as potentially increasing plasma long chain fatty acids levels, inclusive the eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), the main very long-chain (n-3) PUFA [56], although this issue remains controversial [57]. In the present study, animals fed with anthocyanins rich extract did not shown any difference in long-chain fatty acids composition. Although the biological significance was not clear BE significantly increased methylhexadecanoic acid (C17i) in standard fed animals in both adipose tissues deposits. Branched-chain FAs are ubiquitous in nature and present in particularly large quantities in bacteria but rarely found in other organisms [58]. This fact joined to lack of detection of this fatty acid on standard diet (data not shown) suggested that somehow BE could be changing either the animal metabolism or the production of fatty acids by intestinal bacteria [59].

Flavonoids' effect, similarly with many other xenobiotics, varies with the supplemented dose, many times without a dose-dependency, especially regarding in vivo effects [60, 61]. The dose given to the animals - 25 mg/body weight – would correspond to approximately 243 mg blackberry extract in a human adult with 60 kg, using the formula to human equivalent dose (HED) based on body surface area as described by Reagan-Swow [62]. This dose could be easily achieved by diet eating as much as 100 g blackberries a day [63] or could also be introduced as a food supplement if its risk-benefit so justifies. Future studies should test different doses to maximize the potential of blackberry supplementation minimizing undesirable effects.

This study confirmed that high-fat high-carbohydrate diet-induced obesity can prompt several features of metabolic dysfunction on Wistar rats, being some of them partially reverted with low doses of blackberry extract supplementation for a long period. Decrease of plasma lactate levels appeared as the strongest effect of blackberry supplementation independently of the fat content of diet. Blackberry supplementation was also able to modulate levels of dopamine and its clearance while reducing BDNF levels, which biological relevance is still a matter of debate. Further interventional studies should clarify these outcomes. The interest in effective intervention strategies to prevent/treat obesity and related pathologies have been increasing, along with a recent interest regarding the close associations between obesity and brain dysfunction. These results advanced the knowledge about the therapeutic potential of berries and may empower the achievement of specific recommendations for berry intake or purified blackberry extract in the future.

Author's contributions

MM contributed for the experimental design, data acquisition and analysis and drafted the manuscript. CM, SN, JF and LA contributed to data acquisition. IF and NM were responsible for the preparation of blackberry extract. AF and CC were responsible for study conception, conduction of experiments, data interpretation, preparation and critical revision of the manuscript.

Author Disclosure Statement:

The authors declare no competing financial interests.

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374 are gratefully acknowledged.

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Table 1 – Systolic blood pressure (SBP) at a middle and end stage of the study design

	C	BE	HF	HFBE	D	BE	D x BE
SBP- 9 weeks	137.5 (11.2)	129.6 (5.7)	145.2 (9.8)	139.7 (14.5)	0.058	0.15	0.79
SBP-15 weeks	129.9 (14.3)	133.3 (9.0)	137.3 (13.4)	135.9 (20.5)	0.42	0.86	0.69

SBP-Systolic blood pressure. Standard diet (C); standard diet + blackberry anthocyanins extract (BE); high-fat diet (HF) or high-fat diet + blackberry anthocyanins extract (HFBE). Values are expressed as mean (SD). The significance of diet (D), blackberry extract (BE) or interaction between both factors (D x BE) were tested by two-way anova and expressed as p values.

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Table 2- Biochemical parameters in plasma and urine samples

	C	BE	HF	HFBE	D	BE	D x BE
Plasma analyses							
Cholesterol (mg/dL)	103.8 (16.1)	100.0 (19.7)	105.6 (17.2)	133.2 (13.6)	**<0.05**	0.14	0.05
HDL (mg/dL)	80.8 (14.9)	88.3 (16.7)	75.0 (12.8)	89.2 (13.8)	0.72	0.14	0.64
VLDL (mg/dL)	30.0 (9.5)	26.3 (2.5)	37.2 (5.9)	36.3 (5.3)	**<0.01**	0.45	0.64
TG (mg/dL)	150.2 (47.6)	131.5 (11.8)	185.0 (29.8)	181.3 (27.2)	**<0.01**	0.45	0.62
ASAT (U/L at 37ºC)	163.6 (67.1)	101.0 (31.0)	168.6 (41.6)	117.3 (25.0)	0.61	**<0.05**	0.78
ALAT (U/L at 37ºC)	47.2 (9.2)	51.5 (11.9)	41.4 (8.2)	41.2 (4.5)	0.07	0.60	0.63
ALP (U/L at 37ºC)	79.7 (11.6)	92.8 (5.8)	132.0 (29.8)	135.2 (17.0)	**<0.001**	0.33	0.55
Proteins (g/dL)	6.08 (0.35)	6.00 (0.22)	6.16 (0.19)	6.47 (0.33)	0.05	0.41	0.15
Albumin (g/dL)	3.67 (0.2)^{a,b}	3.37 (0.2)^{a}	3.52 (0.2)^{a,b}	3.77 (0.3)^{b}	0.26	0.81	**<0.05**
Creatinine (mg/dL)	0.40 (0.10)^{a}	0.53 (0.02)^{b}	0.52 (0.05)^{b}	0.44 (0.11)^{a,b}	0.62	0.54	**<0.01**
CK (U/L at 37ºC)	1087 (358)	443 (189)	783 (236)	485 (155)	0.27	**<0.001**	0.15
Urea (mg/dL)	30.2 (1.7)	32.2 (2.6)	36.6 (4.5)	40.2 (5.8)	**<0.001**	0.14	0.67
Uric acid (mg/dL)	0.83 (0.17)	0.74 (0.14)	0.92 (0.23)	0.93 (0.31)	0.20	0.71	0.62
Iron (µg/dL)	291.0 (35.3)	271.8 (37.6)	259.4 (32.2)	268.2 (14.8)	0.29	0.75	0.40
Sodium (mmol/L)	140.8 (4.6)	142.5 (3.3)	136.0 (1.6)	141.5 (4.7)	0.14	0.06	0.32
Potassium (mmol/L)	5.50 (0.75)	5.55 (0.53)	5.78 (0.61)	6.00 (0.82)	0.29	0.69	0.80
Chlorides (mmol/L)	103.2 (3.7)^{a,b}	100.8 (1.6)^{a}	100.2 (3.3)^{a}	107.8 (8.0)^{b}	0.40	0.27	**<0.05**
Calcium (mg/dL)	9.9 (0.97)	10.7 (0.30)	10.7 (0.45)	11.1 (0.59)	0.07	0.07	0.60
Phosphorus (mg/dL)	10.3 (0.71)	10.2 (1.25)	10.3 (0.37)	10.8 (0.66)	0.40	0.67	0.46
Magnesium (mg/dL)	2.66 (0.16)	2.66 (0.16)	2.32 (0.23)	2.20 (0.17)	**<0.001**	0.40	0.36
Lactate (mg/dL)	7.85 (1.94)	5.58 (1.00)	7.90 (1.85)	3.80 (1.82)	0.25	**<0.001**	0.23
Urine analyses							
Total urine (24h)	17.3 (6.0)^{a,b}	12.7 (5.5)^{a}	12.5 (6.5)^{a}	22.5 (7.1)^{b}	0.38	0.35	**<0.05**
Glucose (mg/dL)	14.8 (1.3)	19.0 (3.7)	10.2 (1.5)	11.2 (0.7)	**<0.001**	0.08	0.09
Urea 24h (g/day)	0.17 (0.03)	0.19 (0.05)	0.24 (0.03)	0.35 (0.11)	**<0.01**	**<0.05**	0.14
Sodium (mmol/day)	0.53 (0.26)	0.62 (0.31)	0.70 (0.14)	0.93 (0.25)	**<0.05**	0.17	0.51
Potassium (mmol/day)	1.78 (0.36)	2.02 (0.71)	1.95 (0.35)	2.58 (0.62)	0.14	0.08	0.41
Microalbuminuria (mg/day)	0.04 (0.04)	0.05 (0.02)	0.16 (0.13)	0.12 (0.07)	**<0.05**	0.50	0.82

Standard diet (C); standard diet + blackberry anthocyanins extract (BE); high-fat diet (HF) or high-fat diet + blackberry anthocyanins extract (HFBE); HDL-High dense lipoprotein; VLDL-Very low dense lipoprotein; TG-triglycerides; ASAT-aspartate aminotransferase; ALAT- alanine aminotransferase; ALP-alkaline phosphatase; CK-creatinine kinase. Values are expressed as mean (SD). The significance of diet (D), blackberry extract (BE) or interaction between both factor (D x BE) were tested by two-way anova and expressed as p values. Pos-hoc Fishers's LSD test was performed when interaction between factors was present. Mean values with different superscript letters are significantly different (p<0.05).

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Table 3- Brain derived neurotropic factor (BDNF) levels on plasma and brain homogenates

	C	BE	HF	HFBE	D	BE	D x BE
BDNF plasmatic (pg/mL)	18.84 (4.0)[a]	12.16 (3.7)[b]	8.07 (2.1)[b]	8.289 (3.7)[b]	<0.001	0.06	<0.05
BDNF brain (pg/mg tissue)	0.88 (0.15)[a]	0.57 (0.10)[b]	0.68 (0.10)[b]	0.57 (0.04)[b]	<0.05	<0.001	<0.05

Standard diet (C); standard diet + blackberry anthocyanins extract (BE); high-fat diet (HF) or high-fat diet + blackberry anthocyanins extract (HFBE); Values are expressed as mean (SD). The significance of diet (D), blackberry extract (BE) or interaction between both factor (D x BE) were tested by two-way anova, expressed as p values, and followed by Fishers's LSD test. Mean values with different superscript letters are significantly different (p<0.05).

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Table 4- Body dimension and composition

	C	BE	HF	HFBE	D	BE	D x BE
Nasoanal lenght (cm)	25.2 (0.18)	25.0 (0.58)	26.7 (1.14)	27.1 (0.82)	< 0,0001	0.752	0.348
Waist circunference (cm)	18.3 (0.87)	18.6 (0.83)	21.1 (1.10)	21.7 (1.25)	< 0,0001	0.296	0.724
Free fatty mass (g)	246.6 (11.5)	248.3 (11.0)	295.1 (27.5)	307.3 (26.1)	< 0,0001	0.417	0.539
Fatty mass (g)	181.2 (16.6)	183.9 (16.9)	259.2 (41.2)	278.3 (40.0)	< 0,0001	0.400	0.525

Standard diet (C); standard diet + blackberry anthocyanins extract (BE); high-fat diet (HF) or high-fat diet + blackberry anthocyanins extract (HFBE). Values are expressed as mean (SD). The significance of diet (D), blackberry extract (BE) or interaction between both factors (D x BE) were tested by two-way anova and expressed as p values.

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391 **Table 5a.** Saturated fatty acid composition (µg FA/ mg tissue) in mesenteric adipose tissue (MAD)
392 samples.

	C	BE	HF	HFBE
SFA	205.56 (33.31)	208.37 (29.45)	212.44 (35.87)	233.19 (27.53)
C12	0.32 (0.08)	0.29 (0.06)	0.29 (0.03)	0.35 (0.06)
C14	6.39^{a} (1.34)	5.97^{a} (1.23)	4.63^{b} (0.36)	5.61^{a,b} (0.53)
C15 ai	0.07^{a} (0.03)	0.07^{a} (0.02)	0.03^{b} (0.00)	0.04^{b} (0.00)
C15	2.02^{a} (0.37)	1.99^{a} (0.21)	0.89^{b} (0.11)	0.99^{b} (0.06)
C16	170.20^{a} (27.52)	170.35^{a} (26.99)	142.96^{b} (21.97)	161.19^{a,b} (15.16)
C17i	0.52^{a} (0.14)	0.75^{b} (0.13)	0.35^{b} (0.07)	0.40^{b} (0.05)
C17 ai	0.54^{a} (0.13)	0.55^{a} (0.14)	0.42^{b} (0.07)	0.48^{b} (0.06)
C17	1.70^{a} (0.37)	1.79^{a} (0.22)	2.52^{b} (0.47)	2.62^{b} (0.41)
C18 i	1.13^{a} (0.29)	1.47^{a} (0.23)	0.72^{b} (0.20)	0.74^{b} (0.19)
C18	20.44^{a} (4.53)	22.59^{a} (3.16)	57.96^{b} (13.41)	59.09^{b} (12.53)
C20	0.70 (0.24)	0.80 (0.17)	0.81 (0.23)	0.75 (0.27)
C21	0.07^{a} (0.02)	0.09^{a} (0.02)	0.05^{b} (0.02)	0.05^{b} (0.01)
C22	0.23^{a} (0.09)	0.27^{a} (0.07)	0.18^{b} (0.06)	0.17^{b} (0.06)
C23	0.05^{a,b} (0.01)	0.06^{b} (0.01)	0.04^{a} (0.01)	0.04^{a} (0.00)
C24	1.18^{a} (0.30)	1.33^{a} (0.15)	0.57^{b} (0.13)	0.68^{b} (0.12)

393 ai: branched chain fatty acid, anteiso; i: branched chain fatty acid, iso. Standard diet (C); standard diet + blackberry
394 anthocyanins extract (BE); high-fat diet (HF) or high-fat diet + blackberry anthocyanins extract (HFBE). Values are expressed
395 as mean (SD). ^{A,B,C,D} superscript letters in a row for significant differences among groups ($p<0.05$) after one-way anova followed
396 by bonferroni.
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401 **Table 5b.** Unsaturated fatty acid composition (µg FA/ mg tissue) in mesenteric adipose tissue (MAD)
402 samples

	C	BE	HF	HFBE
MUFA	235.25^{a} (35.89)	242.47^{a} (30.67)	331.81^{b} (50.51)	353.60^{B} (36.72)
C14:1	0.39^{a} (0.15)	0.37^{a} (0.12)	0.10^{b} (0.01)	0.13^{B} (0.04)
C15:1 c10	0.62^{a} (0.15)	0.76^{a} (0.13)	0.34^{b} (0.10)	0.38^{B} (0.07)
C16:1 t9	0.13^{a} (0.04)	0.13^{a} (0.02)	0.22^{b} (0.03)	0.25^{B} (0.02)
C16:1 c7	2.78^{a} (0.38)	2.65^{a} (0.38)	3.56^{b} (0.52)	3.79^{B} (0.33)
C16:1 c9	24.14^{a} (7.77)	22.47^{a} (8.71)	7.29^{b} (1.00)	10.00^{B} (2.47)
C16:1 c11	0.33^{a} (0.09)	0.28^{a} (0.08)	0.12^{b} (0.02)	0.16^{B} (0.02)
C17:1 c9	0.20^{a} (0.03)	0.19^{a} (0.03)	0.15^{b} (0.02)	0.17^{AB} (0.02)
C17:1 c10	1.08^{a} (0.24)	1.04^{a} (0.21)	1.26^{b} (0.15)	1.48^{B} (0.12)
C18:1 t	0.87^{a} (0.33)	0.95^{a} (0.24)	2.48^{b} (0.40)	2.47^{B} (0.30)
C18:1 c9	167.98^{a} (25.95)	174.29^{a} (18.91)	290.91^{b} (45.08)	307.25^{B} (34.37)
C18:1 c11	30.29^{a} (4.53)	32.11^{a} (4.34)	20.01^{b} (3.08)	21.89^{B} (2.19)
C18:1 c12	0.13^{a} (0.05)	0.14^{a} (0.05)	0.38^{b} (0.03)	0.41^{B} (0.03)
C18:1 c13	0.57^{a} (0.11)	0.56^{a} (0.09)	0.44^{b} (0.06)	0.54^{AB} (0.06)
C18:1 t16	0.48 (0.13)	0.55 (0.13)	0.51 (0.09)	0.53 (0.09)
C20:1 c9	2.02^{a} (0.44)	2.32^{a} (0.34)	3.19^{b} (0.78)	3.21^{B} (0.63)
C20:1 c11	3.20^{a} (0.76)	3.57^{a} (0.59)	0.88^{b} (0.24)	0.99^{B} (0.21)
C22:1 c9	0.11^{a} (0.05)	0.15^{a,b} (0.03)	0.08^{b} (0.03)	0.08^{B} (0.02)
C24:1	0.10^{a} (0.05)	0.11^{a} (0.03)	0.06^{b} (0.01)	0.05^{B} (0.02)
PUFA	245.70^{a} (30.41)	249.96^{a} (30.35)	181.19^{b} (15.12)	194.72^{b} (12.22)
C18:2 t9t12	0.17 (0.04)	0.17 (0.04)	0.15 (0.02)	0.18 (0.02)
C18:2 c9t12	0.06^{a} (0.02)	0.05^{A} (0.01)	0.09^{b} (0.02)	0.12^{b} (0.02)
C18:2 t9c12	0.57^{a} (0.11)	0.53^{A} (0.11)	0.27^{b} (0.04)	0.30^{b} (0.06)
C18:2 c9c12	228.73^{a} (28.13)	233.44^{A} (28.34)	168.69^{b} (14.20)	179.88^{b} (11.73)
C18:3 t9t12c15	0.34 (0.06)	0.37 (0.04)	0.36 (0.06)	0.37 (0.05)
C18:3 c6c9c12	0.83^{a} (0.16)	0.75^{AB} (0.11)	0.64^{b} (0.08)	0.76^{b} (0.05)
C18:3 c9t12t15	0.37 (0.09)	0.45 (0.07)	0.43 (0.07)	0.45 (0.08)
C18:3 c9c12c15	7.64^{a} (1.77)	7.37^{A} (1.64)	4.80^{b} (0.53)	5.83^{b} (0.99)
C18:2 c9t11	0.22^{a} (0.09)	0.24^{A} (0.08)	0.63^{b} (0.06)	0.70^{b} (0.06)
C20:2 c11c14	1.16^{a} (0.28)	1.27^{A} (0.16)	2.08^{b} (0.45)	2.37^{b} (0.36)
C20:3 c8c11c14	0.65 (0.21)	0.64 (0.09	0.52 (0.08)	0.66 (0.09)
C20:4 AA	3.19^{a} (1.08)	2.98^{A} (0.37)	1.65^{b} (0.23)	2.05^{b} (0.35)
C20:3 c11c14c17	0.11^{a} (0.04)	0.10^{A} (0.01)	0.19^{b} (0.03)	0.23^{b} (0.05)
C20:5 n3	0.18^{a} (0.04)	0.16^{A} (0.03)	0.08^{b} (0.01)	0.09^{b} (0.02)
C22:2 c13c16	0.14^{a} (0.04)	0.13^{A} (0.02)	0.09^{b} (0.02)	0.08^{b} (0.02)
C22:5 n6	0.39^{a} (0.16)	0.41^{A} (0.08)	0.16^{b} (0.03)	0.18^{b} (0.04)
C22:5 n3	0.42^{a} (0.16)	0.44^{A} (0.13)	0.20^{b} (0.04)	0.23^{b} (0.06)
C22:6 DHA	0.54^{a} (0.29)	0.46^{A} (0.08)	0.18^{b} (0.04)	0.25^{b} (0.07)
ug/mg	686.51 (92.98)	700.80 (81.61)	725.44 (98.09)	781.51 (70.09)

403 c:cis double bond; t: trans double bond; AA: Arachidonic acid; n3: omega 3 fatty acid; n6: omega 6 fatty acid; DHA:
404 Docosahexanoic fatty acid. Standard diet (C); standard diet + blackberry anthocyanins extract (BE); high-fat diet (HF) or high-
405 fat diet + blackberry anthocyanins extract (HFBE). Values are expressed as mean (SD). ^{A,B,C,D} superscript letters in a row for
406 significant differences among groups ($p<0.05$) after one-way anova followed by bonferroni.
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409 **Table 6a.** Saturated fatty acid composition (µg FA/ mg tissue) in subcutaneous adipose tissue (SAD)
410 samples

	C	BE	HF	HFBE
SFA	212.75^{a,b} (29.98)	249.04^{a,b} (22.50)	263.69^{b} (41.84)	285.47^{b} (46.28)
C12	0.49 (0.14)	0.45 (0.11)	0.55 (0.12)	0.51 (0.10)
C14	7.44 (0.91)	7.75 (1.82)	7.69 (1.33)	7.86 (1.14)
C15 ai	0.08^{a} (0.03)	0.08^{a} (0.02)	0.06^{b} (0.01)	0.05^{b} (0.01)
C15	2.25^{a} (0.45)	2.57^{a} (0.34)	1.32^{b} (0.21)	1.36^{b} (0.18)
C16	176.36 (24.19)	204.10 (18.35)	185.34 (29.67)	201.54 (30.12)
C17i	0.47^{a} (0.14)	0.82^{b} (0.14)	0.47^{a} (0.05)	0.52^{a} (0.09)
C17 ai	0.54 (0.10)	0.61 (0.10)	0.60 (0.09)	0.65 (0.09)
C17	1.73^{a} (0.45)	2.20^{a} (0.42)	2.92^{b} (0.47)	3.20^{b} (0.45)
C18i	1.03^{a} (0.15)	1.48^{a} (0.20)	0.80^{b} (0.07)	0.91^{b} (0.23)
C18	20.10^{a} (5.20)	26.15^{a} (5.26)	61.63^{b} (10.49)	66.43^{b} (15.68)
C20	0.49^{a} (0.13)	0.64^{a,b} (0.15)	0.63^{a,b} (0.13)	0.74^{b} (0.20)
C21	0.07 (0.02)	0.10 (0.01)	0.05 (0.02)	0.12 (0.21)
C22	0.16 (0.04)	0.22 (0.04)	0.14 (0.02)	0.16 (0.03)
C23	0.06 (0.01)	0.08 (0.03)	0.07 (0.01)	0.07 (0.02)
C24	1.48^{a,b} (0.30)	1.80^{a} (0.21)	1.41^{a,b} (0.31)	1.37^{b} (0.19)

411 ai: branched chain fatty acid, anteiso; i: branched chain fatty acid, iso. Standard diet (C); standard diet + blackberry
412 anthocyanins extract (BE); high-fat diet (HF) or high-fat diet + blackberry anthocyanins extract (HFBE). Values are expressed
413 as mean (SD). ^{A,B,C,D} superscript letters in a row for significant differences among groups ($p<0.05$)) after one-way anova
414 followed by bonferroni.
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418 **Table 6b.** Unsaturated fatty acid composition (µg FA/ mg tissue) in subcutaneous adipose tissue
419 (SAD) samples

	C	BE	HF	HFBE
MUFA	249.77[a] (32.29)	281.16[a] (29.12)	407.67[b] (60.69)	449.86[b] (61.37)
C14:1	0.44[a] (0.06)	0.45[a] (0.12)	0.21[b] (0.04)	0.22[b] (0.05)
C15:1 c10	0.67[a] (0.13)	0.98[b] (0.16)	0.50[c] (0.05)	0.52[c] (0.11)
C16:1 t9	0.17[a] (0.03)	0.16[a] (0.03)	0.33[b] (0.06)	0.35[b] (0.04)
C16:1 c7	3.20[a] (0.47)	3.35[a] (0.41)	4.65[b] (0.82)	4.80[b] (0.59)
C16:1 c9	28.26[a] (7.49)	27.48[a] (6.84)	15.68[b] (2.53)	18.55[b] (5.64)
C16:1 c11	0.39[a] (0.07)	0.38[a] (0.08)	0.21[b] (0.05)	0.21[b] (0.03)
C17:1 c9	0.21 (0.03)	0.24 (0.04)	0.21 (0.04)	0.21 (0.03)
C17:1 c10	1.26[a] (0.23)	1.34[a] (0.17)	1.88[b] (0.27)	2.10[b] (0.29)
C18:1 t	1.10[a] (0.27)	1.19[a] (0.34)	3.30[b] (0.51)	3.33[b] (0.46)
C18:1 c9	176.73[a] (22.18)	201.44[a] (22.99)	348.64[b] (52.56)	383.85[b] (53.61)
C18:1 c11	31.49[a,b] (4.43)	37.04[a] (4.08)	25.13[c] (3.82)	28.02[c] (3.76)
C18:1 c12	0.15[a] (0.05)	0.15[a] (0.03)	0.51[b] (0.07)	0.54[b] (0.07)
C18:1 c13	0.60 (0.11)	0.68 (0.07)	0.68 (0.09)	0.75 (0.11)
C18:1 t16	0.42[a] (0.08)	0.48[a] (0.08)	0.61[b] (0.07)	0.66[b] (0.09)
C20:1 c9	1.92[a] (0.37)	2.35[a] (0.44)	3.94[b] (0.60)	4.37[b] (0.80)
C20:1 c11	2.79[a] (0.79)	3.43[a] (0.63)	1.21[b] (0.20)	1.41[b] (0.30)
C22:1 c9	0.10 (0.03)	0.14 (0.02)	0.10 (0.02)	0.11 (0.02)
C24:1	0.08 (0.01)	0.10 (0.03)	0.07 (0.01)	0.09 (0.05)
PUFA	275.73[a,b] (49.14)	324.67[b] (54.96)	255.31[b] (38.89)	261.10[b] (34.10)
C16:2 c9t12	0.02 (0.01)	0.03 (0.01)	0.02 (0.01)	0.02 (0.01)
C16:2 c9c12	0.07[a] (0.01)	0.08[a] (0.03)	0.05[b] (0.01)	0.04[b] (0.01)
C18:2 t9t12	0.21 (0.04)	0.24 (0.04)	0.22 (0.03)	0.25 (0.03)
C18:2 c9t12	0.07[a] (0.02)	0.08[a] (0.03)	0.13[b] (0.03)	0.15[b] (0.02)
C18:2 t9c12	0.68[a] (0.11)	0.72[a] (0.12)	0.41[b] (0.08)	0.43[b] (0.06)
C18:2 c9c12	253.66[a,b] (44.95)	299.16[b] (51.39)	230.87[a] (35.64)	236.42[a] (31.56)
C18:2 c9c15	n.d[a]	n.d[a]	0.26[b] (0.07)	0.27[b] (0.04)
C18:3 t9t12c15	0.36 (0.06)	0.40 (0.06)	0.44 (0.06)	0.49 (0.07)
C18:3 c6c9c12	0.96 (0.15)	1.02 (0.19)	0.93 (0.14)	1.04 (0.13)
C18:3 c9t12t15	0.31[a] (0.06)	0.43[a] (0.08)	0.51[b] (0.07)	0.55[b] (0.09)
C18:3 c9c12c15	9.55[a,b] (2.16)	11.22[b] (2.20)	8.61[a] (1.55)	8.53[a] (1.32)
C18:2 c9t11	0.29[a] (0.13)	0.28[a] (0.10)	0.89[b] (0.15)	0.96[b] (0.14)
C20:2 c11c14	1.58[a] (0.40)	1.81[a] (0.18)	4.38[b] (0.58)	4.42[b] (0.68)
C20:3 c8c11c14	0.82[a] (0.22)	0.93[a,b] (0.14)	1.09[b,c] (0.16)	1.20[c] (0.20)
C20:4 AA	4.79 (0.98)	5.46 (1.06)	4.24 (0.80)	4.16 (0.55)
C20:3 c11c14c17	0.16[a] (0.04)	0.19[a] (0.06)	0.46[b] (0.07)	0.44[b] (0.06
C20:5 n3	0.21[a,b] (0.06)	0.24[b] (0.08)	0.17[a] (0.04)	0.16[a] (0.03)
C22:2 c13c16	0.12[a,b] (0.02)	0.14[b] (0.05)	0.10[a] (0.02)	0.11[a] (0.04)
C22:5 n6	0.53[a] (0.12)	0.64[a] (0.16)	0.33[b] (0.06)	0.36[b] (0.07)
C22:5 n3	0.61 (0.21)	0.76 (0.27)	0.62 (0.16)	0.56 (0.12)
C22:6 DHA	0.73[a] (0.21)	0.82[a] (0.19)	0.58[b] (0.13)	0.56[b] (0.12)
ug/mg	738.25[a] (101.11)	854.86[a] (102.26)	926.67[b] (137.22)	996.42[b] (133.87)

420 c:cis double bond; t: trans double bond; AA: Arachidonic acid; n3: omega 3 fatty acid; n6: omega 6 fatty acid; DHA:
421 Docosahexanoic fatty acid. [A,B,C,D] superscript letters in a row for significant differences among groups ($p<0.05$). Standard diet
422 (C); standard diet + blackberry anthocyanins extract (BE); high-fat diet (HF) or high-fat diet + blackberry anthocyanins extract
423 (HFBE). Values are expressed as mean (SD). [A,B,C,D] superscript letters in a row for significant differences among groups
424 ($p<0.05$) after one-way anova followed by bonferroni.

425

426 **Table 7.** Fatty acid composition (µg FA/ mg tissue) in liver samples

	C	BE	HF	HFBE
SFA	11.05 (1.54)	10.47 (1.14)	12.87 (2.54)	12.30 (2.34)
C14	0.09[a] (0.02)	0.09[a] (0.02)	0.13[b] (0.06)	0.14[b] (0.04)
C15	0.05 (0.00)	0.05 (0.01)	0.05 (0.01)	0.04 (0.01)
C16	5.70 (0.82)	5.45 (0.71)	6.46 (1.54)	6.10 (1.49)
C17	0.11 (0.01)	0.11 (0.02)	0.11 (0.02)	0.10 (0.02)
C18	4.91[a,b] (0.75)	4.58[b] (0.59)	5.90[a] (1.25)	5.70[a] (0.95)
C24	0.14 (0.03)	0.14 (0.03)	0.19 (0.05)	0.18 (0.06)
MUFA	3.47 (0.64)	3.36 (0.76)	6.79 (2.61)	6.60 (2.01)
C81:1 t	0.05 (0.01)	0.04 (0.01)	0.10 (0.03)	0.10 (0.03)
C16:1 c7	0.05[a] (0.01)	0.05[a] (0.01)	0.12[b] (0.06)	0.12[b] (0.06)
C16:1 c9	0.44[a] (0.04)	0.49[a] (0.10)	0.29[b] (0.08)	0.36[a,b] (0.14)
C18:1 c9	1.75[a] (0.50)	1.53[a] (0.49)	5.42[b] (2.23)	5.17[b] (1.62)
C18:1 c11	1.08[a] (0.13)	1.16[a] (0.17)	0.75[b] (0.21)	0.74[b] (0.19)
C18:1 t16	0.05[a] (0.01)	0.05[a] (0.01)	0.03[b] (0.01)	0.03[b] (0.00)
C20:1 c9	0.03[a] (0.02)	0.04[a] (0.01)	0.09[b] (0.03)	0.08[b] (0.03)
C20:1 c11	0.07[a] (0.01)	0.06[a] (0.01)	0.03[b] (0.01)	0.03[b] (0.01)
PUFA	11.63 (1.51)	11.26 (1.54)	12.97 (2.67)	12.24 (1.91)
C18:2 c9c12	4.42 (0.44)	4.15 (0.51)	5.70 (1.48)	5.06 (1.14)
C18:3 c6c9c12	0.08[a,b] (0.02)	0.06[b] (0.01)	0.11[a] (0.03)	0.11[a] (0.03)
C18:3 c9c12c15	0.07[a] (0.02)	0.05[a] (0.02)	0.13[b] (0.05)	0.12[b] (0.03)
C20:2 c11c14	0.09[a] (0.01)	0.10[a] (0.02)	0.14[b] (0.03)	0.14[b] (0.03)
C20:3 c8c11c14	0.20 (0.03)	0.22 (0.04)	0.19 (0.04)	0.21 (0.04)
C20:4 AA	5.64 (1.19)	5.54 (1.14)	5.35 (1.50)	5.28 (1.11)
C20:3 c11c14c17	0.05 (0.01)	0.05 (0.01)	0.05 (0.01)	0.05 (0.01)
C20:5 n3	0.05 (0.01)	0.05 (0.01)	0.04 (0.01)	0.04 (0.01)
C22:5 n6	0.11 (0.02)	0.11 (0.04)	0.10 (0.02)	0.12 (0.02)
C22:5 n3	0.17 (0.03)	0.19 (0.04)	0.17 (0.03)	0.16 (0.04)
C22:6 DHA	0.75[a] (0.12)	0.74[a] (0.17)	1.00[b] (0.25)	0.97[b] (0.18)
µg/mg	26.15[a,b] (2.97)	25.09[b] (2.57)	32.63[a] (6.91)	31.14[a] (5.78)

427 c:cis double bond; t: trans double bond; AA: Arachidonic acid; n3: omega 3 fatty acid; n6: omega 6 fatty acid; DHA:
428 Docosahexanoic fatty acid. Standard diet (C); standard diet + blackberry anthocyanins extract (BE); high-fat diet (HF) or high-
429 fat diet + blackberry anthocyanins extract (HFBE). Values are expressed as mean (SD). [A,B,C,D] superscript letters in a row for
430 significant differences among groups ($p<0.05$) after one-way anova followed by bonferroni.
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437 Figure 1- Effects of blackberry anthocyanin extract (BE) on weight of Wistar rats fed a standard or
438 high-fat diet during 17 weeks, expressed as weight evolution during the treatment time (A) or
439 weight gain at the end of 17 weeks (B) and effects on food ingestion (C). C-standard diet fed group;
440 BE- standard diet supplemented with blackberry anthocyanin extract fed group; HF- high-fat diet fed
441 group; HFBE-High-fat diet supplemented with blackberry anthocyanin extract group (n=6). Results
442 are expressed in mean ± SEM. Statistical significance was considered when $p<0.05$.

443

444 Figure 2- Effects of blackberry anthocyanin extract (BE) on glycaemic response to oral glucose test in
445 Wistar rats fed a standard or high-fat diet. A) Area under curve of total glycaemic response during
446 120 min after oral gavage of a solution of glucose (2 g/kg weight). B) C) and D) represent glycaemic
447 response each time point during the 120 min, measured at 3, 8 and 15 weeks of treatment. C-
448 standard diet fed group; BE- standard diet supplemented with blackberry anthocyanin extract fed
449 group; HF- high-fat diet fed group; HFBE-High-fat diet supplemented with blackberry anthocyanin
450 extract group (n=6). Letters were presented at a specific point every time the following differences
451 were seen *a*)-C vs BE; *b*)-C vs HF; *c*)-C vs HFBE; *d*)-BE vs HF; *e*)-BE vs HFBE; *f*)-HF vs HFBE. Statistical
452 significance was tested by two-way ANOVA considering time and diet as factors, followed by Fisher's
453 LSD test. Different superscript letters represent statistical differences between means ($p<0.05$).

454

455 Figure 3- Effects of blackberry anthocyanin extract (BE) on glycaemic response to insulin tolerance
456 test in Wistar rats fed a standard or high-fat diet during A) 3 weeks; B) 8 weeks and C) 15 weeks. C-
457 standard diet fed group; BE- standard diet supplemented with blackberry anthocyanin extract fed
458 group; HF- high-fat diet fed group; HFBE- high-fat diet supplemented with blackberry anthocyanin
459 extract group (n=6). Letters were presented at a specific point every time the following differences
460 were seen *a*)-C vs BE; *b*)-C vs HF; *c*)-C vs HFBE; *d*)-BE vs HF; *e*)-BE vs HFBE; *f*)-HF vs HFBE. Statistical
461 significance was tested by two-way ANOVA considering time and diet as factors, followed by Fisher's
462 LSD test. Different superscript letters represent statistical differences between means ($p<0.05$).

463

464 Figure 4- Effects of blackberry anthocyanin extract (BE) on the release of adypokines on Wistar rats
465 fed a standard or high-fat diet during 17 weeks (n=6). Results are expressed in mean ± SEM.
466 Statistical significance was tested by two-way ANOVA considering diet and blackberry
467 supplementation as factors, followed by Fisher's LSD test. Statistical significance was considered
468 when $p<0.05$.

469

470 Figure 5- Effects of blackberry anthocyanin extract (BE) on adipocytes area of mesenteric adipose
471 tissue (mAT) (A) and subcutaneous adipose tissue (scAT) (B) of Wistar rats fed a standard or high-fat

472 diet during 17 weeks (n=6). Results are expressed in mean ± SEM. Statistical significance was
473 considered when $p<0.05$.
474 Figure 6- Effects of blackberry anthocyanin extract (BE) on the release of dopamine and 3,4-
475 dihydroxyphenylacetic acid (DOPAC) on Wistar rats fed a standard or high-fat diet during 17 weeks
476 (n=6). Results are expressed in mean ± SEM. Statistical significance was tested by two-way ANOVA
477 considering diet and blackberry supplementation as factors, followed by Fisher's LSD test in
478 prefrontal cortex. Statistical significance was considered when $p<0.05$.
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480

1. A. Berghofer, T. Pischon, T. Reinhold, C. M. Apovian, A. M. Sharma and S. N. Willich, *BMC public health*, 2008, **8**, 200.
2. C. L. Ogden, M. D. Carroll, B. K. Kit and K. M. Flegal, *Jama*, 2014, **311**, 806-814.
3. A. K. Dahl and L. B. Hassing, *Epidemiologic reviews*, 2012, DOI: 10.1093/epirev/mxs002.
4. F. Muller-Riemenschneider, T. Reinhold, A. Berghofer and S. N. Willich, *European journal of epidemiology*, 2008, **23**, 499-509.
5. G. A. Bray, *The Journal of clinical endocrinology and metabolism*, 2004, **89**, 2583-2589.
6. M. A. Beydoun, H. A. Beydoun and Y. Wang, *Obesity Reviews*, 2008, **9**, 204-218.
7. M. Prince, R. Bryce, E. Albanese, A. Wimo, W. Ribeiro and C. P. Ferri, *Alzheimers Dement*, 2013, **9**, 63-75.
8. S. E. Kanoski and T. L. Davidson, *Physiology & behavior*, 2011, **103**, 59-68.
9. H. R. Park, M. Park, J. Choi, K. Y. Park, H. Y. Chung and J. Lee, *Neurosci Lett*, 2010, **482**, 235-239.
10. J. K. Morris, G. L. Bomhoff, B. K. Gorres, V. A. Davis, J. Kim, P. P. Lee, W. M. Brooks, G. A. Gerhardt, P. C. Geiger and J. A. Stanford, *Experimental neurology*, 2011, **231**, 171-180.
11. B. Buijsse, E. J. Feskens, M. B. Schulze, N. G. Forouhi, N. J. Wareham, S. Sharp, D. Palli, G. Tognon, J. Halkjaer, A. Tjonneland, M. U. Jakobsen, K. Overvad, A. D. van der, H. Du, T. I. Sorensen and H. Boeing, *The American journal of clinical nutrition*, 2009, **90**, 202-209.
12. M. González-Castejón and A. Rodriguez-Casado, *Pharmacological Research*, 2011, **64**, 438-455.
13. L. Letenneur, C. Proust-Lima, A. Le Gouge, J. F. Dartigues and P. Barberger-Gateau, *American journal of epidemiology*, 2007, **165**, 1364-1371.
14. X. Gao, A. Cassidy, M. Schwarzschild, E. Rimm and A. Ascherio, *Neurology*, 2012, **78**, 1138-1145.
15. S. Norberto, S. Silva, M. Meireles, A. Faria, M. Pintado and C. Calhau, *J Funct Foods*, 2013, **5**, 1518-1528.
16. A. Basu, M. Rhone and T. J. Lyons, *Nutrition reviews*, 2010, **68**, 168-177.
17. C. Rendeiro, J. D. Guerreiro, C. M. Williams and J. P. Spencer, *The Proceedings of the Nutrition Society*, 2012, **71**, 246-262.
18. B. O. Cho, H. W. Ryu, C. H. Jin, D. S. Choi, S. Y. Kang, D. S. Kim, M. W. Byun and I. Y. Jeong, *Journal of agricultural and food chemistry*, 2011, **59**, 11442-11448.
19. B. Shukitt-Hale, V. Cheng and J. A. Joseph, *Nutritional neuroscience*, 2009, **12**, 135-140.
20. N. M. Hassimotto and F. M. Lajolo, *Journal of the science of food and agriculture*, 2011, **91**, 523-531.
21. I. Serraino, L. Dugo, P. Dugo, L. Mondello, E. Mazzon, G. Dugo, A. P. Caputi and S. Cuzzocrea, *Life Sciences*, 2003, **73**, 1097-1114.
22. J. Azevedo, I. Fernandes, A. Faria, J. Oliveira, A. Fernandes, V. de Freitas and N. Mateus, *Food chemistry*, 2010, **119**, 518-523.
23. J. Oliveira, V. Fernandes, C. Miranda, C. Santos-Buelga, A. Silva, V. de Freitas and N. Mateus, *Journal of agricultural and food chemistry*, 2006, **54**, 6894-6903.
24. I. Fernandes, J. Azevedo, A. Faria, C. Calhau, V. de Freitas and N. Mateus, *Journal of agricultural and food chemistry*, 2009, **57**, 735-745.
25. C. Barrientos, R. Racotta and L. Quevedo, *Nutrition Research*, 2010, **30**, 791-800.
26. C. van den Brom, C. Bulte, B. Kloeze, S. Loer, C. Boer and R. Bouwman, *Cardiovascular Diabetology*, 2012, **11**, 74.
27. J. Ibrahim, B. C. Berk and A. D. Hughes, *Clinical and experimental hypertension*, 2006, **28**, 57-72.
28. K. Rutter, L. Hennoste, L. C. Ward, B. H. Cornish and B. J. Thomas, *Laboratory animals*, 1998, **32**, 65-71.
29. P. Castro-Gómez, J. Fontecha and L. M. Rodríguez-Alcalá, *Talanta*, 2014, **128**, 518-523.
30. P. Soares-Da-Silva and M. H. Fernandes, *Acta physiologica Scandinavica*, 1991, **143**, 287-293.
31. A. Faria, D. Pestana, J. Azevedo, F. Martel, V. de Freitas, I. Azevedo, N. Mateus and C. Calhau, *Molecular nutrition & food research*, 2009, **53**, 1430-1437.

32. P. V. A. Babu, D. Liu and E. R. Gilbert, *The Journal of nutritional biochemistry*, 2013, **24**, 10.1016/j.jnutbio.2013.1006.1003.
33. M. Itoh, T. Suganami, R. Hachiya and Y. Ogawa, *International journal of inflammation*, 2011, **2011**, 720926.
34. I. J. Neeland, C. R. Ayers, A. K. Rohatgi, A. T. Turer, J. D. Berry, S. R. Das, G. L. Vega, A. Khera, D. K. McGuire, S. M. Grundy and J. A. de Lemos, *Obesity*, 2013, **21**, 19.
35. S. K. Panchal, L. Ward and L. Brown, *Eur J Nutr*, 2013, **52**, 559-568.
36. X.-Y. Li, Z.-X. Zhao, M. Huang, R. Feng, C.-Y. He, C. Ma, S.-H. Luo, J. Fu, B.-Y. Wen, L. Ren, J.-W. Shou, F. Guo, Y. Chen, X. Gao, Y. Wang and J.-D. Jiang, *Journal of Translational Medicine*, 2015, **13**, 278.
37. J. Lovejoy, F. D. Newby, S. S. Gebhart and M. DiGirolamo, *Metabolism*, 1992, **41**, 22-27.
38. M. DiGirolamo, F. D. Newby and J. Lovejoy, *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, 1992, **6**, 2405-2412.
39. G. van Hall, *Acta Physiol*, 2010, **199**, 499-508.
40. K. Matsushita, E. K. Williams, M. L. Mongraw-Chaffin, J. Coresh, M. I. Schmidt, F. L. Brancati, R. C. Hoogeveen, C. M. Ballantyne and J. H. Young, *Am J Epidemiol*, 2013, **178**, 401-409.
41. A. B. Klein, R. Williamson, M. A. Santini, C. Clemmensen, A. Ettrup, M. Rios, G. M. Knudsen and S. Aznar, *Blood BDNF concentrations reflect brain-tissue BDNF levels across species*, 2011.
42. K. Miyazaki, N. Narita, R. Sakuta, T. Miyahara, H. Naruse, N. Okado and M. Narita, *Brain Dev*, 2004, **26**, 292-295.
43. S.-H. Shim, Y. Hwangbo, Y.-J. Kwon, H.-Y. Jeong, B.-H. Lee, H.-J. Lee and Y.-K. Kim, *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, 2008, **32**, 1824-1828.
44. A. H. El-Gharbawy, D. C. Adler-Wailes, M. C. Mirch, K. R. Theim, L. Ranzenhofer, M. Tanofsky-Kraff and J. A. Yanovski, *The Journal of clinical endocrinology and metabolism*, 2006, **91**, 3548-3552.
45. E. Gajewska, M. Sobieska, D. Lojko, K. Wieczorowska-Tobis and A. Suwalska, *European review for medical and pharmacological sciences*, 2014, **18**, 3246-3250.
46. K. Sakata and S. M. Duke, *Neuroscience*, 2014, **260**, 265-275.
47. J. A. Siuciak, C. Boylan, M. Fritsche, C. A. Altar and R. M. Lindsay, *Brain research*, 1996, **710**, 11-20.
48. C. A. Altar, C. B. Boylan, C. Jackson, S. Hershenson, J. Miller, S. J. Wiegand, R. M. Lindsay and C. Hyman, *Proceedings of the National Academy of Sciences of the United States of America*, 1992, **89**, 11347-11351.
49. F. Fumagalli, G. Racagni, E. Colombo and M. A. Riva, *Molecular psychiatry*, 2003, **8**, 898-899.
50. C. Rendeiro, D. Vauzour, M. Rattray, P. Waffo-Teguo, J. M. Merillon, L. T. Butler, C. M. Williams and J. P. Spencer, *PloS one*, 2013, **8**, e63535.
51. C. Rendeiro, D. Vauzour, R. J. Kean, L. T. Butler, M. Rattray, J. P. Spencer and C. M. Williams, *Psychopharmacology*, 2012, **223**, 319-330.
52. K. Blum, P. K. Thanos and M. S. Gold, *Frontiers in psychology*, 2014, **5**, 919.
53. C. M. Adler, I. Elman, N. Weisenfeld, L. Kestler, D. Pickar and A. Breier, *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology*, 2000, **22**, 545-550.
54. M. Meireles, F. Martel, J. Araujo, C. Santos-Buelga, S. Gonzalez-Manzano, M. Duenas, V. de Freitas, N. Mateus, C. Calhau and A. Faria, *The Journal of membrane biology*, 2013, **246**, 669-677.
55. G. D. Norata, S. Raselli, L. Grigore, K. Garlaschelli, E. Dozio, P. Magni and A. L. Catapano, *Stroke*, 2007, **38**, 2844-2846.
56. M. C. Toufektsian, P. Salen, F. Laporte, C. Tonelli and M. de Lorgeril, *The Journal of nutrition*, 2011, **141**, 37-41.
57. D. Vauzour, N. Tejera-Hernandez, C. O'Neill, V. Booz, B. Jude, I. Ernst, N. Rigby, J. M. Silvan, P. Curtis, A. Cassidy, S. de Pascual-Teresa, G. Rimbach and A. M. Minihane, *The Journal of Nutritional Biochemistry*, DOI: http://dx.doi.org/10.1016/j.jnutbio.2014.09.005.
58. M. Kniazeva, Q. T. Crawford, M. Seiber, C. Y. Wang and M. Han, *PLoS biology*, 2004, **2**, E257.

589 59. A. Faria, I. Fernandes, S. Norberto, N. Mateus and C. Calhau, *Journal of agricultural and food*
590 *chemistry*, 2014, **62**, 6898-6902.
591 60. E. J. Calabrese and R. B. Blain, *Regulatory Toxicology and Pharmacology*, 2011, **61**, 73-81.
592 61. A. Murakami, *Archives of biochemistry and biophysics*, 2014, **557**, 3-10.
593 62. S. Reagan-Shaw, M. Nihal and N. Ahmad, *FASEB journal : official publication of the*
594 *Federation of American Societies for Experimental Biology*, 2008, **22**, 659-661.
595 63. X. Wu, G. R. Beecher, J. M. Holden, D. B. Haytowitz, S. E. Gebhardt and R. L. Prior, *Journal of*
596 *agricultural and food chemistry*, 2006, **54**, 4069-4075.

597

Figure 1

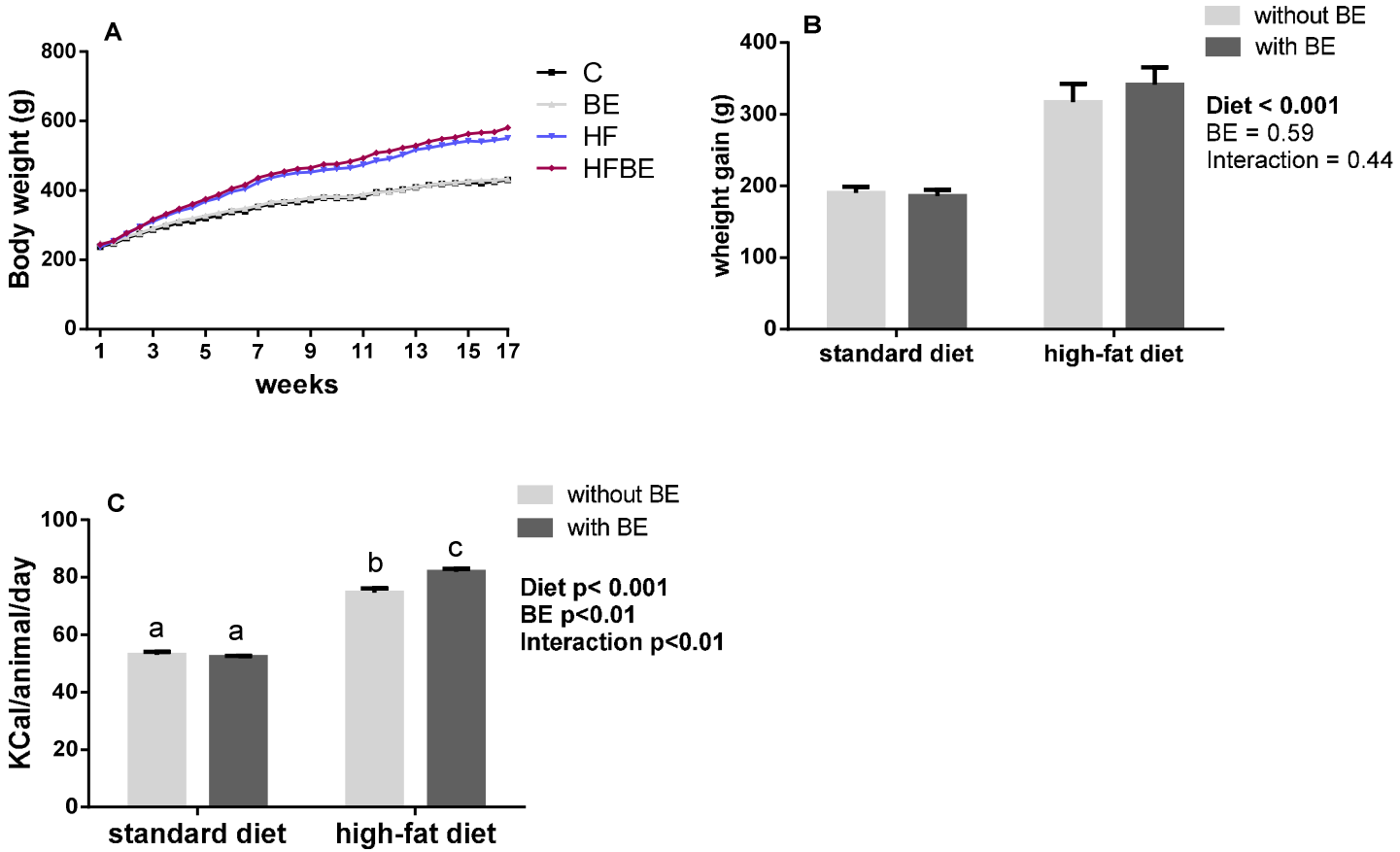

Figure 2

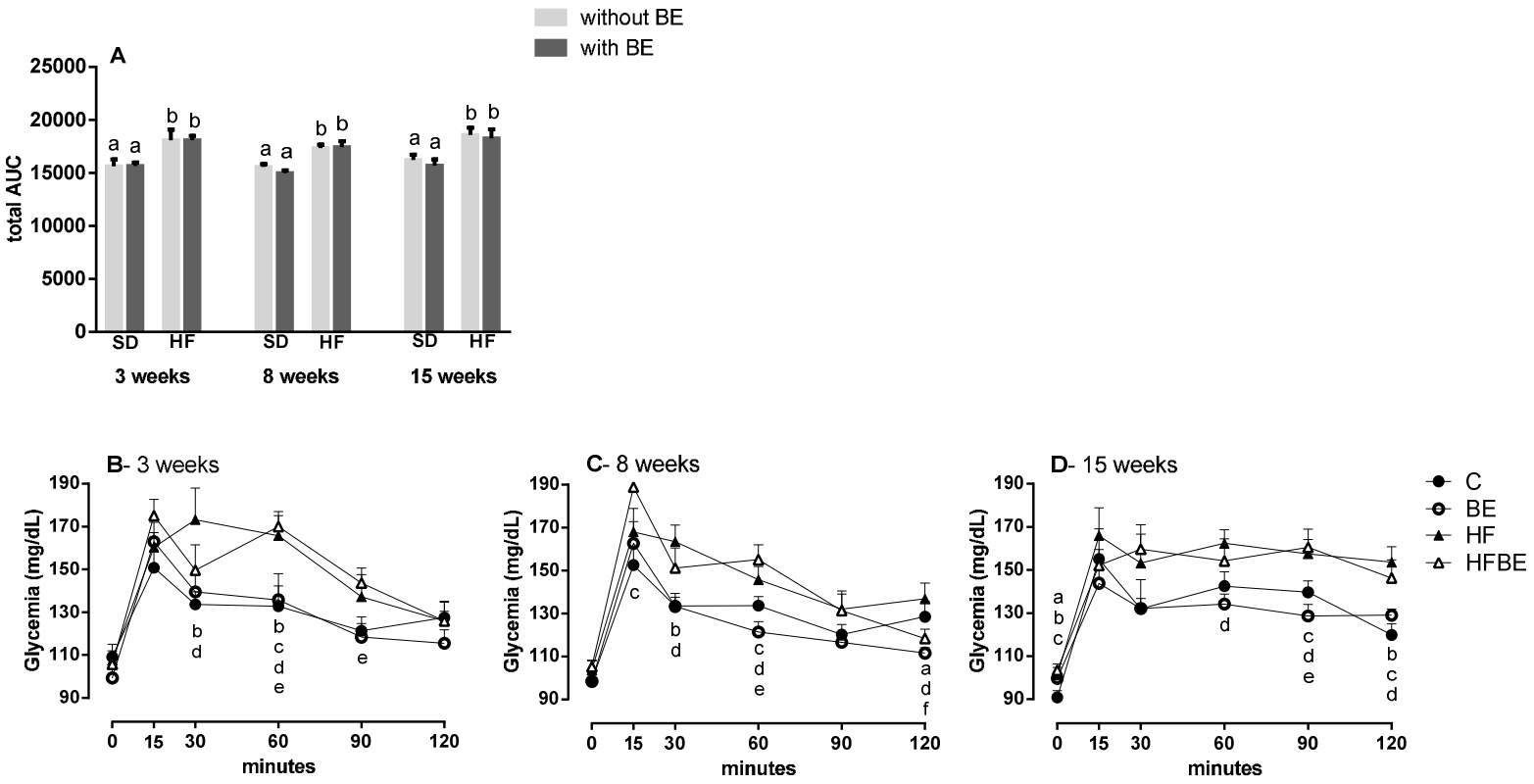

Figure 3

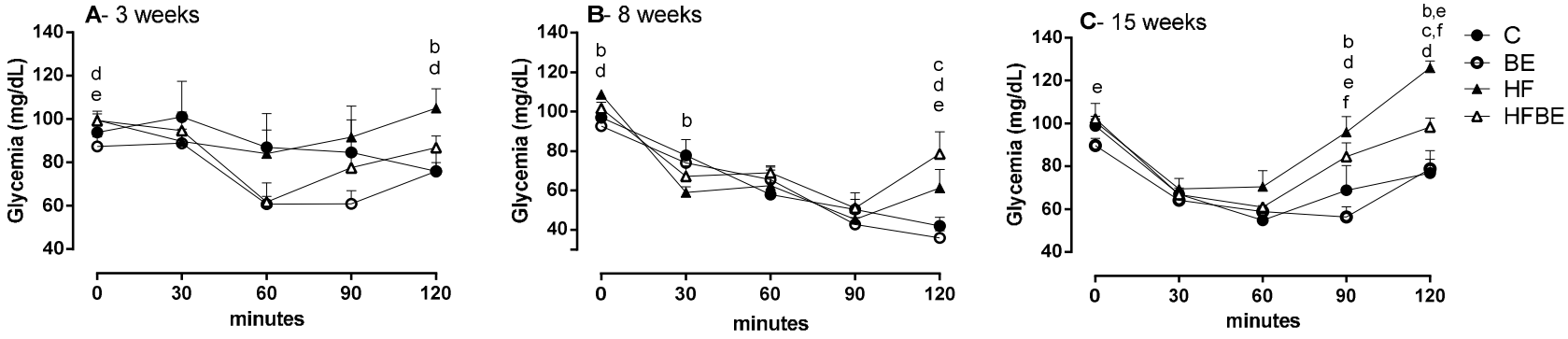

Figure 4

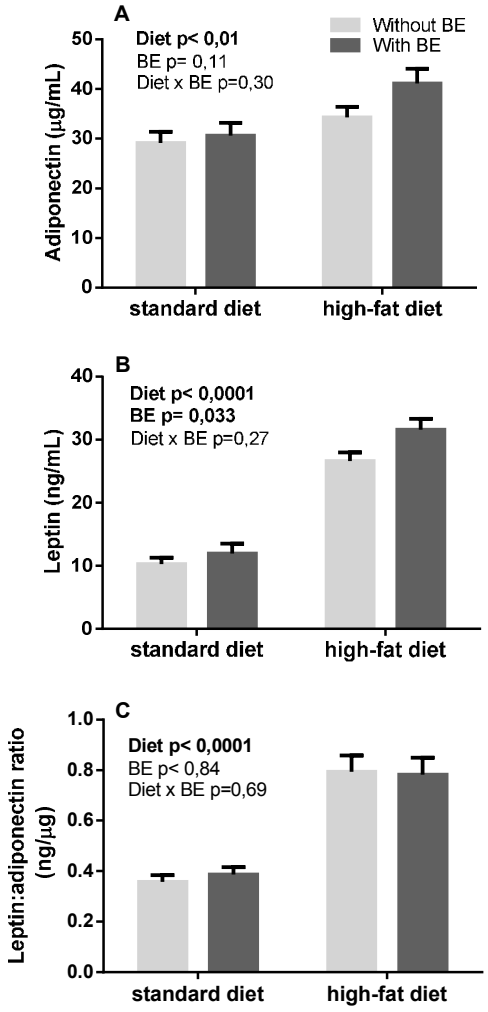

Figure 5

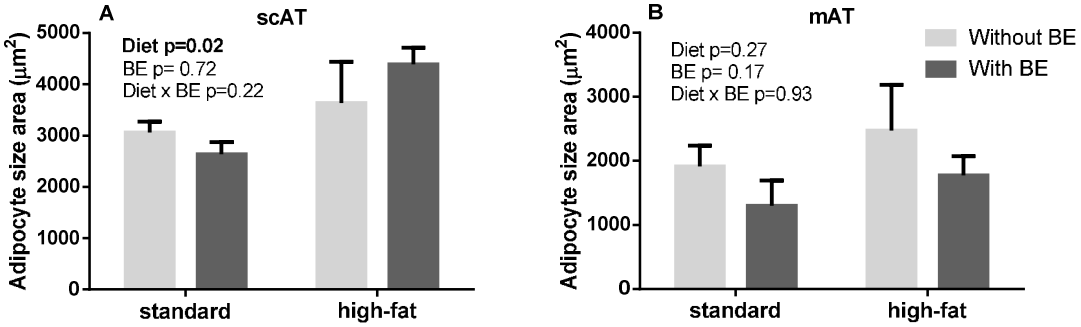

Figure 6