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Metabolic effects of a cafeteria diet and its withdrawal and responses to a blackberry supplement in rats

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This work has been completed within the Chemistry and Biochemistry Department, in the area of Biochemistry and Molecular Biology of Pharmacy Faculty in CEU-San Pablo University, under the supervision of Dr. Emilio Herrera Castillón, Emeritus Professor of Biochemistry and Molecular Biology, and Dra. Encarnación Amusquivar Arias, Assistant Professor of Biochemistry and Molecular Biology.

Dr. Emilio Herrera Castillón and Dra. Encarnación Amusquivar Arias, as supervisors of the present work, agree that this work have been met the necessary requirements for submission and constitute an original contribution to the subject matter.

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Index

Figures

Tables

Abbreviations

AA	arachidonic acid
ACOD	acyl-CoA-oxidase
ACS	acyl-CoA-synthetase
AgRP	agouti-related protein
ALA	α-linolenic acid
AMP	adenosine-5-phosphate
ANOVA	analysis of variance
ATGL	adipose triglyceride lipase
ATP	adenosine 3'-phosphate
AUC	Area Under the Curve
CAT	catalase
CART	cocaine- and amphetamine-related transcript
CD	Cafeteria group
ChE	cholesterol esterase
CHOD	cholesterol oxidase
DHA	docosahexaenoic
DHAP	dihydroxyacetone phosphate
ELOVL6	elongase 6
EPA	eicosapentaenoic
FAS	fatty acid synthase
GK	glycerol kinase
GLUT 4	glucose transporter type 4
GOD	glucose oxidase enzyme
GP1	to glycerol-1-phosphate
GSK3	glycogen synthase kinase 3
HOMA	Homeostasis Model Assessment of Insulin Resistance
HSL	hormone-sensitive lipase
IL-6	interleukin 6
IL-11	interleukin 11
IL-12	interleukin 12
IR	insulin receptor
IRS-1	insulin receptor substrate 1
ISI	Insulin Sensitivity Index
LepRb	leptin receptor
LPL	lipoprotein lipase
MGL	monoglyceride lipase
NAFLD	non-alcoholic fatty liver disease
NEFA	non-esterified fatty acids
NPY	neuropeptide Y
OA	oleic acid
OGTT	oral glucose tolerance test
PA	palmitic acid
PKC	protein kinase C
POA	palmitoleic acid
POD	peroxidase enzyme
NPY	neuropeptide Y
OA	oleic acid
OGTT	oral glucose tolerance test
PA	palmitic acid
PKC	protein kinase C
POA	palmitoleic acid
POD	peroxidase enzyme
POMC	pro-opiomelanocortin
PPAR	peroxisome proliferator-activated receptor
PPi	pyrophosphoric acid
QUICKI	Quantitative Insulin Sensitivity Check Index
ROS	reactive oxygen species
SA	stearic acid
SD	standard group
SOD	superoxide dismutase
TAG	triacylglycerol
TG	triglyceride
TMB	3,3',5,5'-tetramethylbenzidine
TNF-alpha	tumor necrosis factor alpha
VLDL	very-low density lipoprotein
WHO	World and Health Organization

RESUMEN

Está claramente demostrada la estrecha relación entre la dieta y la salud tanto a largo como a corto plazo. De hecho, varios estudios han descrito que los hábitos alimentarios tradicionales pueden tanto incrementar como reducir la prevalencia de determinadas enfermedades. Dietas ricas en grasa y reducidas en vegetales y frutas se asocian frecuentemente con el desarrollo de sobrepeso y obesidad que a su vez se relacionan con un incremento en el riesgo de desarrollar enfermedades cardiovasculares, diabetes y otras. Sin embargo, los mecanismos por los cuales esto ocurre y cómo estos efectos podrían ser invertidos necesitan aún ser aclarados.

Por otro lado, se ha descrito que algunos vegetales y frutas, además de su valor nutricional convencional, tienen compuestos funcionales capaces de ofrecer beneficios para la salud. Se conoce que estos compuestos activos presentes en productos naturales tienen actividad anti-cáncer, anti-obesidad, anti-diabetes y otras. Entre los compuestos descritos en la literatura por su efecto como reguladores del metabolismo de la glucosa están las antocianinas

encontradas en los frutos del bosque. A pesar de algunos estudios prometedores describiendo los efectos beneficiosos de estos frutos, todavía no se conoce el mecanismo por el que afectan al metabolismo. En esta Tesis, hemos evaluado los efectos de la ingesta de una dieta rica en grasa, la denominada “dieta de cafetería”, en ratas hembra y macho, y tras un periodo de ingesta de dieta control. Adicionalmente, estudiamos los efectos de las moras (*Rubus fruticosus*), un fruto del bosque rico en antocianinas, en ratas hembra y macho alimentadas con dieta de cafetería.

Para el desarrollo de esta Tesis se llevaron a cabo dos protocolos experimentales. En el experimento I, ratas Sprague-Dawley hembra y macho de 21 días de edad fueron aleatoriamente asignadas a dos grupos experimentales: grupo estándar alimentadas con dieta control y grupo cafetería alimentadas con dieta de cafetería, rica en grasa, durante 90 días. Tras este periodo, los animales fueron sometidos al test de tolerancia oral a la glucosa (OGTT) y la mitad de los animales fueron sacrificados para recogida de plasma, hígado y tejido adiposo. La otra mitad de los animales de ambos grupos comenzaron a recibir dieta control hasta el día 200 de experimento, cuando fueron sometidos a otro OGTT y sacrificados para recogida de plasma, hígado y tejido adiposo.

La ingesta de alimentos y el peso corporal fueron medidos semanalmente. Tras el sacrificio, el peso de los tejidos adiposos e hígado también fueron registrados. En plasma se evaluó el perfil de ácidos grasos, niveles de glucosa, insulina, triglicéridos, glicerol, ácidos grasos no esterificados, colesterol, leptina, adiponectina y resistina. En los tejidos (hígado y tejidos adiposos) se valoró el perfil de ácidos grasos y el contenido de triglicéridos y colesterol. La actividad de las enzimas lipoproteína lipasa y glicerol quinasa fue evaluada en los tejidos adiposos recogidos.

Para el experimento II, ratas Sprague Dawley hembras y machos de 70 días de edad fueron aleatoriamente asignadas a cuatro grupos experimentales:

grupo estándar alimentadas con dieta control, grupo estándar con *Rubus* alimentadas con dieta estándar suplementada con extracto de *Rubus*, grupo cafetería alimentadas con dieta de cafetería y grupo cafetería con *Rubus* alimentadas con dieta de cafetería suplementada con extracto de *Rubus* durante 80 días. Tras ese período todos los animales fueron sacrificados para recogida de plasma, hígado y tejidos adiposos. Los pesos de los tejidos adiposos e hígado fueron registrados. En plasma, se evaluó el perfil de ácidos grasos, los niveles de glucosa, insulina, triglicéridos, glicerol, ácidos grasos no esterificados, colesterol y β-hidroxibutirato. En los tejidos (hígado y tejido adiposo) se determinaron el perfil de ácidos grasos, el contenido de triglicéridos y colesterol.

En el experimento I, los pesos de los diferentes tejidos adiposos y de hígados resultaron más altos en las ratas hembra alimentadas con dieta de cafetería que con dieta control a los 90 días de experimento. Este efecto desapareció cuando tras esa alimentación las ratas hembra fueron alimentadas con dieta control durante 110 días. Sin embargo, en los machos alimentados con dieta de cafetería no se observó ese incremento de los pesos de tejido adiposo o de hígado. La respuesta de los parámetros metabólicos a la alimentación con dieta de cafetería resultó ser mayor en machos que en hembras. Las actividades de la lipoproteína lipasa y de la glicerol quinasa fueron mayores en algunos tejidos adiposos de ratas hembra alimentadas con cafetería que en los machos. La dieta de cafetería cambió el perfil de ácidos grasos en plasma, hígado y tejido adiposo, tanto en hembras como en machos, con incrementos en la concentración de ácidos grasos saturados y monoinsaturados. Los tests de tolerancia oral a la glucosa mostraron un disminuido índice de sensibilidad insulínica en las ratas hembra que fueron alimentadas con dieta de cafetería, mientras que no se observaron cambios en los machos.

En el experimento II, la administración de una dieta de cafetería a machos y a hembras durante 80 días produjo un aumento de la adiposidad. El suplemento de la dieta con extracto de *Rubus* a las ratas hembra y macho

alimentadas con dieta control incrementó la concentración de β -hidroxibutirato en el plasma de estos animales además de incrementar su sensibilidad insulínica. Por otro lado, la respuesta al suplemento de extracto de *Rubus* en las ratas alimentadas con dieta de cafetería no fue tan evidente como en las ratas alimentadas con dieta control. En cuanto a los perfiles de ácidos grasos, el suplemento de la dieta control con extracto de *Rubus* también produjo un incremento de ácidos grasos poliinsaturados en el hígado de las hembras y de los machos a los 80 días de tratamiento con dieta control pero no en los grupos alimentados con dieta de cafetería.

Globalmente, los efectos de la dieta de cafetería fueron más manifiestos en hembras que en machos, y desaparecieron tras la ingesta de dieta control durante 110 días. El suplemento de la dieta control con extracto de *Rubus* produjo en las ratas un estado catabólico a pesar de tener una ingesta calórica normal. Sin embargo, los efectos del extracto de *Rubus* desaparecieron cuando eran administrados en la dieta de cafetería, y esta falta de respuesta la interpretamos en función de la intensa resistencia insulínica producida por esta dieta.

INTRODUCTION

1. Diet and health

The close relationship between diet and health is clearly substantiated. The influence of the diet in human health was first identified by epidemiological observations. The results of these epidemiological studies showed that environmental factors, especially food compounds, exert an important role in hormones that act on the prevention and development of disease (1).

This research field is known as nutritional epidemiology and predominantly focus on studying diseases and their relationship with a food or nutrient. These studies are fundamentally limited by the methodology used since people are not fed exclusively with a single nutrient or food. Thus, in the latest years, complementary methods to analyse dietary patterns have been developed to minimize these limitations (2, 3). Also, due to this fact a variety of studies using

more controlled methods are necessary to broaden the knowledge about food and health especially those using animal models.

Multiple factors are able to affect the energy balance which could lead to an increase of energy accumulation (4). The number of people with excess of weight in the world is increasing in the last 40 years (5). Modifications on the food industry producing energy-dense foods together with the expanding urbanization, sedentary work positions and changes in the modes of transport contribute to the increase of excess body weight worldwide (5, 6).

The consequences of overweight and obesity are also documented. An excess of weight increases the risk of developing cardiovascular diseases, which were the leading cause of death worldwide in 2021 (5). Moreover, the risk of developing musculoskeletal disorders and certain types of cancers such as breast, colon, kidney, gallbladder, liver, prostate, endometrial and ovarian are increased in individuals with overweight and obesity. Apart from all these situations, it is well known that diabetes and insulin resistance are also conditions related to the excess of body weight (5, 7).

Furthermore, nutritional epidemiological studies not only found a relationship between dietary patterns and disease development but also showed that specific foods play an important role inducing or preventing certain diseases. In fact, a low intake of fruits and vegetables is associated to a higher mortality caused by cardiovascular diseases (8). Additionally, these studies have described that beyond their traditional nutritional value, fruits and vegetables could also collaborate improving specific metabolic conditions such as an excessive inflammatory molecules production (9). Moreover, there are several natural products identified such as flavones, xanthones and anthocyanins, that are described to be able to regulate glucose metabolism and consequently improve insulin resistance (10).

The forest fruits (raspberries, blueberries, blackberries and others) are some of those natural products due to their high phytochemical levels that are associated with numerous health benefits (11). When they are in high amounts, the carotenes and anthocyanins present in these fruits show a relevant antioxidant activity (12). Thus, in addition to being rich in vitamins and minerals, forest fruits contribute to a high antioxidant activity due to the presence of extra antioxidant compounds (13).

In fact, in the past, plants and fruits were often used to treat diseases rather than preventing diseases. However, the biological activity of those fruits is not always completely understood, because most of their active molecules are not yet isolated and characterized. In particular, the blackberries are one of the fruits that are known to be used as medicine from prehistory period, nevertheless there is still a lack of knowledge about their benefits for health (14).

2. Metabolism in obesity and/or overweight

Overweight and obesity are defined by the World and Health Organization (WHO) as an excess of a body fat accumulation (15). Fundamentally, the excess of fat accumulation is associated to a higher energy intake rather than to a reduced energy expenditure which results in a positive energy balance and consequently weight gain (16). Apart from the energy imbalance, obesity development has been described as a complex process involving genetic and environmental factors that are able to modify the energy homeostasis system and lead to overweight and obesity (17).

An important role is given to genetic factors for the individual susceptibility to excess weight accumulation. Several studies have identified genes and mutations that are responsible for some obesity cases (18). Most of these genes are involved in the central signalling for the control of food intake and energy

expenditure in the hypothalamus (19). In the archeus nucleus of the hypothalamus, orexigenic and anorexigenic neurons receive the peripheral signs to control food intake. There are two populations of neurons that control food intake. One of them expresses neuropeptide Y (NPY) and agouti-related protein (AgRP) that when activated increase food intake (20). The second neuron population expresses pro-opiomelanocortin (POMC) (21) and cocaine- and amphetamine-related transcript (CART) that when activated inhibit food intake (22). These neurons express receptors for peripheral molecules such as insulin, leptin, ghrelin and others that are released accordingly to the fed state and body adiposity. It is described that mutations of genes that codify these peripheral molecules and their receptors as well as in POMC and its receptor (MC4R) cause severe obesity that often starts from childhood (23).

An essential peripheral molecule that crosses the blood-brain barrier described in 1994 is leptin (24). Leptin is an adipokine released to plasma by adipose tissue proportionally to the size of this tissue, and it its related with a reduction of food intake. Once in the blood leptin has as main target the neurons from hypothalamus, midbrain and brainstem playing an important role on the energy homeostasis. Leptin initiate its cellular effects binding leptin receptor (LepRb), that is expressed in higher amounts in the brain, especially hypothalamus. To produce its effects in the hypothalamus leptin demands an intact melanocortin system (POMC neurons) through which leptin leads to a reduction of food intake. Additionally, leptin acts through adrenergic system stimulating an increase on the energy expenditure (25). All these effects taken together made leptin be considered one of the most promising molecules for the cure of obesity in the latest decades. However, it has been shown lately that a small percentage of obese people could be cured by simply taking leptin as medicine daily. In fact, mutations in leptin gene or its receptor (LepRb) have been identified as a rare cause of obesity in humans (26, 27). However, an impaired leptin anorexigenic effect is described even though no genetic mutation in genes

involved in leptin signaling occurs, and this effect is known as leptin resistance. Although obese individuals show a high leptin plasma levels due to their high adipose tissue size, it is described that the anorexigenic leptin effect is impaired. The hyperleptinemia found in obese individuals could be related to modifications in leptin receptor sensitivity, an impaired leptin cellular cascade activation and/or a reduced leptin transportation throughout the blood-brain barrier. These alterations may be caused mainly by elevated cellular inflammation and increased plasma non-esterified fatty acids levels that occur in obese individuals. It is important to point out that leptin resistance is a more common condition in humans than mutations in leptin gene and leptin receptor (28, 29).

Despite that, it is known that monogenic severe obesity is rare in humans. The most accepted hypothesis for genetic obesity is that an interaction of multiple alleles with modest alterations on food intake and energy expenditure contribute to increase the fat mass in some individuals. In fact, it is described that multiple alleles obesity occurs more often in human population (30).

However, only genetic factors are not able to explain the high prevalence of overweight and obesity worldwide. One of the environmental factors involved in overweight and obesity development is the diet. Modifications in modern western society through industrialization created novel food technologies that increased the access to food significantly. Though, this available industrialized food products are often highly caloric and rich in fat and refined carbohydrates that are added in order to increase palatability which contribute to an excessive food intake. On the other hand, modern society has also reduced physical activity levels, which together with a high energy dense food intake, contribute to increase overweight and obesity worldwide (31).

Indeed, most of the obese individuals show a high caloric intake and reduced energy expenditure. Part of this excess of energy intake is due to an increased fat intake which is known to increase the risk of obesity, coronary heart

disease and certain types of cancer (32, 33). Additionally, an elevated consumption of saturated fatty acids by itself is considered a risk for cardiovascular disease. Saturated fatty acids are also able to modify adipokine release, such as adiponectin and leptin, by the adipose tissue favouring weight gain (34). Also, it is described that *trans* fatty acids intake is able to inhibit the central anorexigenic effect of insulin in rats leading to hyperphagia, that could promote an energy imbalance and foment weight gain (35). Moreover, it is described that the effects of specific diets on insulin secretion could contribute to overweight and obesity development. Carbohydrate intake, especially refined sugars, stimulate insulin release in order to reduce glucose plasma levels. The high insulin plasma levels lead to a high glucose and fatty acid uptake in the adipocyte contributing to increase the adipose tissue size and body weight (36, 37). On the other hand, n-3 fatty acids are known for their action reducing body weight and improving plasma and liver lipid profile. Also, it is described that n-3 fatty acid intake is able to increase adiponectin (anorexigenic adipokine) plasma levels and reduce leptin and insulin plasma levels which contribute for a reduction on fat accumulation in adipose tissue (34).

3. Animal models to study the effects of fat rich diets

The use of animals for scientific purposes is considered relevant for research investigations, even though the results may be not necessarily equal to what occurs in humans (38). Thus, it is important to use a valid animal model that would be able to mimic a specific human disease accordingly to the research objectives of the study. Different animal models have been employed on the study of overweight and obesity. They are fundamentally divided into two groups: genetically modified models and obesity models induced by diet (39, 40). In the present study we were interested on animal obesity model induced by diet.

For obesity and metabolic syndrome studies rodents are the animals mostly used. There are a variety of dietary protocols described to produce overweight and obesity by a similar process as it occurs in humans. However, the results are controversial since the dietary composition and experiment length varies remarkably among the studies (41). Thus, the profile of obesity and/or diabetes produced by each protocol could be completely different among the studies which makes comparison particularly difficult. For example, diets rich in a specific carbohydrate such as fructose usually affect differently insulin sensitivity when compared to sucrose rich diets (42, 43).

Another type of diet often used to produce overweight/obesity are the high fat diets. As occurs with the carbohydrate rich diets, the source of lipids in different high fat diets and the length of experiments vary among studies, and also make the comparison of the results quite difficult. The majority of studies use diets containing 45% to 60% of the dietary energy from fat (44-47). It is described that male mice fed with control diet supplemented with lard for 12 weeks showed higher body weight than control group from week 5 to week 12 of treatment (34). In male rats fed with a high fat diet containing lard, an increased body weight was described when compared to the control group after 4 weeks of experiment (48).

Beyond the effects on body weight, high fat diets are able to induce several metabolic alterations. It is described a reduction on insulin sensitivity of rats fed a high fat diet (20% of energy from fat) for five weeks (49). Modifications in cardiovascular system such as arteriosclerosis, hypertension, vascular endothelial tissue and others are described as an effect of high fat diet intake by rats (50). Additionally, high fat diets could lead to non-alcoholic fatty liver disease as well as modify lipid metabolism in liver and adipose tissue (51, 52). Central effects are also known as a result of a high fat diet intake affecting spatial learning and memory acquisition in rats and mice (53, 54). Studies using high fat diets also found diverse results accordingly to the type of the lipid source used to prepare the diet. Diets rich in saturated fatty acids such as the ones with high proportion of lard are described to produce more deleterious

effects than high fat diets rich in mono/polyunsaturated fatty acids (55-57). In fact, when a high fat diet made with fish oil containing n-3 polyunsaturated fatty acids is offered to rats, these animals show an improvement of metabolic parameters when compared to control group (58-62).

Although high fat diets are prone to produce overweight/obesity and its metabolic effects, a single fat source in the diet is not compatible with the variety of fat sources humans ingest on a daily basis. Thus, animal models that use a "cafeteria diet" are able to reflect more accurately the human habits that lead to overweight/obesity. This model consists in offering to rats or mice a diet containing a variety of typical human food components that have been associated to the development of overweight/obesity and a higher risk of developing type 2 diabetes (63, 64). This type of diet has been applied in different types of experimental studies aiming to increase knowledge of metabolic characteristics during overweight/obesity development and also to investigate treatment possibilities (64).

Apart from the effects upon obesity, other metabolic effects have been described after cafeteria diet consumption, including insulin resistance, liver and adipose tissue inflammation (65). Also, some authors have described that after a short-term cafeteria diet consumption there is a different gender response such as an overall lower lipolytic activity in female than in male rats, although such response didn't have relationship with a different energy intake between both sex groups (66). Thus, the understanding of the mechanisms by which the cafeteria diet affects metabolism could contribute to new targets to understand overweight/obesity development and its potential treatment.

4. Diabetes and insulin resistance

Diabetes is a chronic disease characterized by permanent increased glucose plasma levels which can lead to damages in arteries and veins, retina,

nerve cells and kidney cells (67). The prevalence is increasing worldwide especially in middle- and low-income countries. It is estimated that from 2019 to 2030, the number of people diagnosed with diabetes would rise about 10%, being higher in urban areas than in rural areas (68). Diabetes is classified into two types of disorders with different characteristics. Type 1 diabetes, also known as insulin-dependent diabetes, occurs when β-pancreatic cells do not produce insulin or produce a reduced amount of insulin which is not enough for reducing plasma glucose levels. Then there is type 2 diabetes which is the most common type of diabetes that occurs when individuals become resistant to insulin and/or when β-pancreatic cells produce lower amounts of insulin than the ones that are required to maintain normoglycemia (69). In the present study we focused on type 2 diabetes that is mainly developed in adults having high body weight.

There are several factors involved in development of type 2 diabetes such as diet, physical activity level, microbiota, obesity and genetic factors (70-72). However as mentioned above, the main characteristic of type 2 diabetes is the presence of insulin resistance. Insulin resistance is defined by a reduced insulin ability to stimulate glucose uptake by different tissues causing hyperglycemia. At the same time, these permanent increased glucose levels lead to increased insulin secretion by β-pancreatic cells that caused also hyperinsulinemia (73).

The exact mechanisms involved on insulin resistance are still unclear. Nevertheless, there are some pathways that are known to contribute to a reduced insulin effect. To start its cellular effects insulin binds insulin receptor (IR). When insulin resistance occurs, it is observed a reduction in IR expression in skeletal muscle, adipose tissue and liver and an IR reduced tyrosine kinase activity. Also, changes in insulin receptor substrate 1 (IRS-1) seem to be a crucial part of the mechanisms involved in insulin resistance (74). Modifications on the first steps of insulin pathway in the cell culminates on a reduction of GLUT4 translocation to plasma membrane and consequently a reduction on glucose uptake by the cell (75). Moreover, development of insulin resistance is known to be related to

several conditions as hypertension, dyslipidemia, vascular alterations and obesity. Some of these conditions are consistent with most of the definitions for metabolic syndrome (76, 77). The criteria for the metabolic syndrome are proposed for several different health organizations. The International Diabetes Federation defined metabolic syndrome as central obesity plus two of the following parameters: elevated triacylglycerols, reduced HDL cholesterol, elevated blood pressure, and elevated fast plasma glucose (78-81).

Under normal conditions adipocytes are highly sensitive to insulin. When prolonged hyperinsulinemia occurs adipose tissue continue to accumulate triacylglycerols inside the adipocyte (82). In obese individuals with hyperinsulinemia, the adipose tissue rich in triacylglycerols release protein factors that attract macrophages which infiltrate in the adipose tissue. The presence of macrophages triggers an inflammatory response that impairs fatty acid deposition in adipocytes which then facilitates the fatty acid release to the blood torrent in the form of non-esterified fatty acids (NEFA) (83-85). The NEFA in plasma are then able to reach muscle and liver cells in increased amounts. This excess of fatty acids released from adipose tissue enters muscle and liver cells where they are converted again into triacylglycerols and then deposited as lipid droplet in the cytoplasm of those cells. The triacylglycerol accumulation in those tissues (i.e. liver and muscle) other than adipose tissue leads to a reduced insulin sensitivity in liver and muscle which is one of the characteristics of type 2 diabetes (86). Additionally, it is described that an increased diacylglycerol concentration in the cells (which is an intermediate of triacylglycerol production) is associated with protein kinase C (PKC) activation (87). PKC pathways are involved in several diabetic vascular alterations such as atherosclerosis, cardiomyopathy, retinopathy and nephropathy. In endothelial cells PKC activation lead to increased permeability of albumin (88, 89) as well as other macromolecules and also modify nitric oxide bioavailability, which decrease its vasodilator effect (90). The smooth muscle cells are also affected by PKC

activation since this protein reduce apoptosis in these cells impairing adjustments in the vascular tone. The activated PKC in monocytes contributes to its initial adhesion to the vasculature and also their differentiation in macrophages. Additionally, PKC is involved in an increased production of extracellular matrix in kidneys that contributes to diabetic nephropathy (91).

On the other hand, it is also described that insulin resistance development is associated with diet-induced inflammation. Inflammatory diets such as the Western-diet which is characterized by high intake of high-fat foods, refined grains, high-sugar foods, high red meat intake and are able to contribute to increase plasma and tissue concentrations of inflammatory mediators (36). In normal conditions, these inflammatory mediators are responsible for tissue repairing and immunity against infections. However, when cellular inflammation is excessively increased it modifies IRS-1 phosphorylation which could interrupt insulin signaling in the cell reducing its effects (92). In obese individuals proinflammatory cytokines are expressed in adipose tissue together with the macrophage infiltration as a response to the higher amounts of triacylglycerols storage. These cytokines are released to plasma leading to a systemic inflammation, thus the effects of increased inflammation in the adipose tissue are able to reach other tissues (93, 94).

Another mechanism described to play a role on insulin resistance development involves reactive oxygen species (ROS) (95). ROS are mainly produced by mitochondria as a result of aerobic metabolic processes. Low ROS levels work as a regulator of cellular growth and are also important for the immunologic system. However, high ROS levels are associated with lipid peroxidation, protein oxidation, enzymatic inactivation, excessive activation of pro-inflammatory genes, as well as DNA damages (96). It is important to point out that the amount of ROS present in the cells is not only a result of the internal production but determined by the balance between production and the antioxidant mechanisms such as the antioxidant enzyme activities (catalase and

superoxide dismutase for example) and the presence of antioxidant molecules (ascorbic acid, tocopherols, phenolic compounds and others). Also, it is known that oxidative stress caused by excessive ROS presence could activate serine kinases and transcription factors that are associated with insulin signaling impairment (95).

In fact, diabetic individuals show a higher production of ROS and a reduced plasma antioxidant molecule level. Prolonged hyperglycemia generates ROS that increase overall oxidative stress in diabetes due to an excess of glucose flux through glycolysis and the tricarboxylic acid cycle (97, 98). Elevated glucose levels also react with lipids and proteins through glycation reactions that end up producing advanced glycosylation end products and ROS in each step of these reactions (99). On the other side, antioxidant enzymes are also target for glycation reactions which impair their action and consequently the efficiency of the antioxidant defense system in diabetic individuals (99-101). Additionally, it is known that antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) are poorly expressed in β-cells. Thus, beta-cells are more susceptible to oxidative stress (102). Moreover, it is described that oxidative stress is crucial for apoptosis induction of pancreatic β-cells (103) reducing the number of cells able to produce insulin in pancreas. As a result of this, chronic hyperglycemia (as it occurs in type 2 diabetes) is able to damage pancreatic β-cells reducing pancreatic insulin production. Therefore, ROS and oxidative stress are deeply involved in diabetes development specially in the case of type 2 diabetes (104).

5. White adipose tissue

5.1 White adipose tissue metabolism

The white adipose tissue stores energy for the whole body as lipids (105). Lipoproteins (chylomicrons and VLDL) containing triacylglycerols circulate in the blood torrent and when these large particles reach the adipose tissue, the triacyclglycerols are metabolised by the enzyme known as lipoprotein lipase (LPL). LPL is an acylglycerol hydrolase which was first known as clearing factor due to its action on reducing turbidity of elevated triglyceride plasma after heparin administration (106). In the presence of heparin, LPL is released from tissues without changing its activity so that LPL activity is able to be detected (107). LPL is present in the endothelial cell lining capillaries of the adipose tissue and it is also present in heart, liver and skeletal muscle, but it is more active in the adipose tissue. It is produced in the rough endoplasmic reticulum and it is activated in the Golgi apparatus (108). The active LPL is secreted in secretory vesicles that reach the membrane surface. In the case of adipose tissue, LPL leave the adipocyte to a proteoglycan chain that is attached to the endothelium membrane where LPL is able to act on the triacylglycerols of the chylomicrons and VLDLs present in the circulation. These lipoproteins have in their surface the protein apo C – II which activates LPL catalytic activity to hydrolyse each triacylglycerol into three molecules of fatty acids and one molecule of glycerol. The release of fatty acids are then taken up by the adipose tissue, passing throughout the endothelial membrane to reach the adipocytes. Most of the glycerol released by the action of the LPL remains in the blood torrent and a small part of glycerol molecules is metabolized by glycerol kinase activity in the adipocytes (109). The control of LPL varies extensively from one type of tissue to another even though LPL structure is equal. In heart, LPL activity increase during fasting but decrease in adipose tissue. Also, insulin is able to increase LPL activity in adipose tissue whereas it decreases LPL activity in heart and lungs (110, 111).

It is known that LPL knockout mice die 48 hours after birth due to failure of an effective triacylglycerol clearance (112, 113). Also, heterozygote mice for LPL deficiency show hypertriglyceridemia and the same occurs to humans carrying a mutation on LPL gene (112). These data demonstrate the importance of LPL for the lipid metabolism. As lipid content vary throughout the day due to lipid intake and the body requirements for energy, LPL is sensibly regulated by multiple mechanisms including glucocorticoid and insulin (114, 115).

After a meal, insulin is liberated from β-pancreatic cells as a response to higher glucose levels in plasma. Increased insulin levels up-regulate adipose tissue LPL activity that stimulate fatty acid accumulation in the adipose tissue in post-prandial period as described above. Once inside the adipocyte, fatty acids are re-esterified into triacylglycerol for storage. Conversely, decreased insulin levels inhibit LPL activity and consequently reduces fatty acid accumulation in adipocyte (116, 117).

However, during fasting or strenuous exercise the fatty acids stored in the adipose tissue are released to the plasma to be used as energetic fuel to the whole body in a process called lipolysis. Lipolysis is strictly controlled by insulin, catecholamines and also natriuretic peptides (115, 118, 119). Three enzymes are involved in lipolysis: adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL) and monoglyceride lipase (MGL). The hormones involved in lipolysis activate the ATGL that acts selectively hydrolysing triacylglycerol to generate diacylglycerols and free fatty acids. The free fatty acids generated by ATGL activity leave the adipocyte to plasma and the diacylglycerols are hydrolysed by HSL. The HSL is a multifunctional enzyme that hydrolyse diacylglycerols and other compounds (120). HSL activity is determined by several hormones. Catecholamines and glucagon stimulate HSL activity through the increase of intracellular levels of cAMP. On the other hand, HSL activity is inhibited by prostaglandins, insulin and adenosine that results in reduced lipolysis (119, 121, 122). HSL acts preferentially against positions sn-1 and sn-3 position of

triacylglycerol mobilizing fatty acid to lately be released in the circulation mainly linked to albumin. ATGL and HSL enzymes are a rate-limiting step on lipolysis and are under strict regulation. Monoacylglycerol lipase (MGL) hydrolases only monoacylglycerols to generate free fatty acid and a glycerol molecule that is mainly released to circulation (119).

Although predominantly the glycerol molecules necessary for the triacylglycerol formation in the adipose tissue are obtained from adipocyte glucose metabolism, it is described that a small proportion of glycerol from triacylglycerols breakdown could be used in the fatty acid re-esterification. This process could occur due to the action of the enzyme known as glycerol kinase. Glycerol kinase catalyses the transfer of the phosphate group from ATP to glycerol producing glycerol-3-phosphate which is essentially an inversible reaction. Glycerol-3-phosphate is important for triacylglycerol production and consequently could collaborate on the increase of the adipose tissue size (123).

In 1957, Wieland & Suyter (124) described the lack of glycerol kinase in adipose tissue and therefore it was accepted that adipose tissue was not able to metabolize glycerol for triacylglycerol synthesis. Indeed, in vitro studies from that year using C^{14} labelled-glycerol demonstrated an impaired C^{14}-glycerol uptake by adipose tissue. Ten years later using a more sensible radiochemistry assay, Robinson & Newsholme (125) showed that glycerol kinase is present in the adipose tissue although its activity is very low thus the authors concluded that adipose tissue glycerol kinase activity could not be relevant for glycerol metabolization. However, in 1970, our laboratory group demonstrated a higher glycerol kinase activity in adipose tissue samples from rats (126). The mathematical analysis of the experimental model was able to calculate the real speed of glycerol metabolization in adipose tissue (127) and demonstrated that it is affected by several factors as the nutritional state, albumin plasma levels, presence of antilipolytic or lipolytic drugs and thyroid function of those animals (127, 128).

5.2 White adipose tissue as an endocrine organ

In the past, white adipose tissue was considered only an organ for triacylglycerol storage but in the last decades research advances have revealed that adipose tissue is also an endocrine organ. In fact, adipose tissue is responsible for releasing to the circulation several molecules that are essential for body energy homeostasis (105). Thus, information about the level of lipid storage is transmitted to the central nervous system that controls food intake through several pathways including hormone release to circulation (129).

The molecules released by adipose tissue to the circulation are collectively known as adipokines (130). As commented above, leptin was the first described protein released by the adipose tissue (24). Leptin levels on the blood torrent are not only proportional to the amount of body adipose tissue (131) but also vary throughout the day due to energy intake and follow a circadian cycle (132). Lately, leptin mRNA was identified in other tissues as skeletal muscle, lymphoid tissue and others. Besides of its effects in the control of food intake as previously commented, leptin affects reproductive system, immune function, insulin resistance and others. Leptin is considered a mediator of inflammation in auto-immune diseases and other diseases. Long-chain helical family of cytokines such as interleukin 6 (IL-6), interleukin 11 (IL-11) and interleukin 12 (IL-12) has structural homology with leptin. Moreover, Leptin receptor (LepRb) is similar to the superfamily of class I cytokine receptor. Additionally, immune cells as neutrophils, natural killer cells, macrophages and monocytes express LepRb (133). Thus, leptin acts on regulation of oxidative stress in several immune cells improving phagocytosis and stimulating the release of inflammatory cytokines (134, 135). On the other hand, it is described that secondary inflammation caused by several diseases could lead to leptin resistance impairing leptin control of food intake. Therefore, inflammation and leptin affect each other in a bidirectional manner (136).

A year after leptin discovery, other adipokine was described simultaneously by four different research groups in both mouse and human named adiponectin. Adiponectin is produced only by adipose tissue, both white and brown. The adiponectin biological activity varies accordingly to its molecular weight being greater in high molecular weight adiponectin molecules (137). In human plasma, adiponectin levels represent 0.01% of the total amount of plasma proteins and its concentration is not strongly influenced neither by the circadian rhythms nor postprandial state (132, 138, 139). Additionally, total adiponectin levels in women are higher than in men (140) and a higher proportion of high molecular weight adiponectin is described in woman compared to men (141). This effect is described to be due to an inhibitory effect of androgenic hormones on adiponectin production (142), being also responsible for the differences in fat distribution between genders (143).

To produce its effects plasma adiponectin needs to bind an adiponectin receptor. Adipo R1 and Adipo R2 are the adiponectin receptors expressed in skeletal muscle, liver and adipose tissues (144). The levels of mRNA of both adiponectin receptors are possibly negatively regulated by insulin via activation of PI3-kinase and inactivation of Foxo1 (145). Tsuchida (145) et al. studied insulin-resistant ob/ob mice with hyperglycemia and hyperinsulinemia and found a decreased expression of Adipo R1 and R2 in muscle and adipose tissue in these animals compared to control group. Moreover, in obese animals not only the plasma adiponectin is decreased but also the quantity of adiponectin receptors.

Obesity is the major factor that determine plasma adiponectin levels. The increased lipid accumulation reduces adiponectin production by mature adipocyte (146, 147). It is described that adiponectin shows a protective effect in the cardiovascular system, peripheric insulin resistance, systemic inflammation, fatty liver and also in β-pancreatic cells function (148). However, the mechanisms by which adiponectin exert these effects are not yet completely understood.

Opposite to the positive effects of adiponectin, the adipokine named resistin is described to be involved in the development of insulin resistance and also could lead to proinflammatory effects. Resistin was initially found in mouse (149), being considered as a hormone that connects obesity to diabetes. Human resistin is mainly produced by blood monocyte, macrophages and bone marrow cells (150). Also, resistin seem to be produced by preadipocytes rather than mature adipocytes (151). However, other studies have found resistin mRNA also in mature adipocytes (152). In mouse, resistin is a 11kDa polypeptide with 94 amino acids and it is described that its main production occurs in the adipose tissue although resistin mRNA was also found in skeletal muscle and intestinal epithelium (149, 150, 153). Moreover, mature resistin segment in mouse is 72% identical to mature rat resistin (149). In fact, important differences are described between human and animal resistin concerning gene expression, protein, the main production site and its physiological effects (154).

In obese individuals resistin plasma levels are increased and it is described to be related to type 2 diabetes and metabolic syndrome development. Resistin increases glucose production in liver stimulating glycogen phosphorylase activity and reducing glycogen synthase activity. Also, resistin decreases insulin receptor which reduces glucose uptake in skeletal muscle regardless of insulin-activated pathway (155). Moreover, resistin initiates inflammatory pathways and it is involved in the differentiation of monocytes in macrophages. In animals and humans, resistin produces vascular alterations that increases smooth muscle proliferation (156) and promotes monocyte infiltration and adhesion in endothelial cells. Although plasma resistin is described to be increased in the conditions cited above, other research groups fail on identifying this connexion. Thus, resistin relevance for the development of type 2 diabetes, obesity and metabolic syndrome is still under debate (157).

Beyond these three adipokines, it is described that the adipose tissue releases many other molecules. For example, it is known that tumor necrosis

factor alpha (TNF-alpha) is a multifunctional cytokine involved in inflammation, apoptosis and cytotoxicity that is also expressed by subcutaneous and visceral adipose tissue (158) although TNF-alpha is mainly released by immune cells (159). Likewise, IL-6 is another pro-inflammatory cytokine released by the adipose tissue. The adipose tissue IL-6 production corresponds from 10 to 30% of the total amount of the IL-6 circulating plasma levels (160). There are several other adipokines described by numerous studies such as angiotensin, omentin, plasminogen activator inhibitor 1 and others with varied effects in different tissues (161-163). However, it is outside of our experiment study to enter more deeply in the description of these different adipokines.

5.3 Sex differences in adipose tissue

Body fat distribution is different between females and males in mammal species including humans. These differences are driven mainly by sexual hormones and their receptors. Women have more subcutaneous adipose tissue while men have more visceral adipose tissue (164, 165). Also, women predominantly accumulate fat in lower body (gluteal-femoral) while men predominantly accumulate fat in upper body (central/abdominal) (166). The adipose tissue distribution correlates with a higher or lower risk of developing chronic diseases. Visceral fat (abdominal) is associated with elevated insulin, non-esterified fatty acids (NEFA) and triglyceride (TG) plasma levels (167) whereas subcutaneous fat has been described due to its protection against adverse effects of obesity in both sexes (168, 169).

In laboratory animals such as rats and mice sex differences in adipose tissue has also been reported. One of the potential reasons for expansion of adipose tissue is the number of adipocyte precursor cells. Female C57BL/6J mice fed with a low-fat diet showed more adipocyte precursor cells in inguinal and

gonadal white adipose tissue than males (170). However, other studies have described that male mice on a high fat diet showed increased weight gain and insulin resistance in relation to females fed with high fat diet (171). The possible reason for these effects seems to be linked to estrogen. Female mice submitted to ovariectomy show increased weight gain that is improved by estrogen replacement (172). Rodent models knockout for estrogen receptor α are also used in studies that aim to evaluate sex differences in white adipose tissue metabolism. In fact, it is described that female and male mice knockout for estrogen receptor α in adipose tissue showed elevated visceral adiposity and alterations compatible with metabolic syndrome (173).

Additionally, it is described that some adipokines released by the adipose tissue could interact with sex hormones and modify adipose tissue metabolism. It is known that leptin signalling in the hypothalamus is modulated by estradiol (174). Indeed, high estradiol levels in rodents improve leptin signalling including the reduction of food intake (175). Also, it is described that estradiol prevent leptin resistance in rodents fed with high fat diet (176). On the other hand, the literature reports reveal contradictory results concerning adiponectin and estradiol plasma levels. In adults, females show higher adiponectin concentrations than males which indicates that estradiol may be involved in adiponectin However, ovariectomized adult mice show elevated plasma adiponectin levels and mice at the age of puberty when ovariectomized still show a rise in adiponectin plasma levels (177).

In the case of overweight and obesity caused by a high fat diet exposure, it is described that the increase in adipose tissue inflammation varies accordingly to gender (178). In visceral adipose tissue there are more macrophage infiltration than in subcutaneous adipose tissue in obesity. These macrophages are responsible to produce proinflammatory cytokines that can be involved in insulin resistance and several other vascular dysfunctions that could culminate in

atherosclerosis. Thus, as females predominantly accumulate subcutaneous adipose tissue, they are more protected than males to develop the metabolic disturbances caused by inflammation in obesity caused by high fat diet intake (179-181).

6. Energy metabolism: glucose, lipids and fatty acids

6.1. Glucose metabolism

The carbohydrates ingested through the diet start to be digested in the mouth by the action of salivary amylase. However, the major carbohydrate digestion occurs in the small intestine by enzymatic action of pancreatic amylase (182) and the membrane-bound intestinal brush border enzymes, called glucosidases. Then, monomeric carbohydrates such as glucose resulting from glucosidases action are absorbed by the enterocytes where subsequently are transported to the blood. The glucosidase enzymes play an important role on glucose availability for blood uptake; thus, they are used as a target for antidiabetic drugs that reduce plasma glucose levels by inhibiting carbohydrate digestion (183-185). Also, enterocytes glucose transporters are used as pharmaceutical targets for plasma glucose reduction by blocking the passage of glucose from the small intestine to the blood (186, 187).

Once in the blood, glucose reaches all tissues but to enter in the cells, membrane transporters are necessary. Thus, glucose uptake is mediated by a large family of glucose transporters, GLUT. Liver transporters (GLUT1, GLUT2) and cerebral neurons transporters (GLUT3) are continuously present in the plasma membrane. On the other hand, the main glucose transporter in skeletal muscle, cardiac muscle, cerebral neurons and adipose tissue (GLUT4) are stored in intracellular small vesicles that are released to the membrane upon insulin

stimulation. Therefore, glucose uptake in those tissues is dependant of insulin (188).

Insulin is produced in pancreas as a large precursor protein that need to be metabolized by proteases to release a mature functional insulin to the blood torrent. While exists as a proinsulin peptide, this hormone is stored in β-cells secretory granules. The most important stimulant for insulin release from these secretory granules is the increased glucose levels in plasma. Hence, glucose uptake through GLUT2 in β-pancreatic cell stimulate the conversion of proinsulin to insulin by specific proteases that hydrolyses two peptide bonds of proinsulin producing mature insulin and peptide C that are release to the blood by exocytosis. This insulin release triggers insulin synthesis from transcription to proinsulin production (189). Moreover, it is known that insulin pancreatic release occurs in two phases: a fast phase that lasts about 5 – 10 min and a slow phase that can lasts for hours. The fast phase releases insulin already present in the secretory granules while during the slow phase the secretory granules release insulin recently produced and it can last as long as glucose plasma levels are maintained at high levels (190, 191).

Besides plasma glucose levels, other molecules and hormones also control insulin release by pancreas. Some amino acids (leucine for example); β-adrenergic agonists and intestinal hormones (cholecystokinin and gastrin) are able to stimulate insulin release. On the other hand, catecholamines (through α-adrenergic receptors), cortisol and somatostatin are able to inhibit insulin release form β-pancreatic cells (189, 190, 192).

Insulin starts its cellular effects by binding the insulin receptor (IR) in the plasma membranes present in various organs. Liver and adipose tissue are organs particularly rich in IR. The IR activation triggers a signal pathway that culminate in a variety of biological effects from cellular metabolism to cell survival and growth (193-195). In adipose tissue and muscles insulin lead to GLUT4

translocation to the membrane and glucose entrance into the cells (196, 197). Also, in muscle insulin stimulates glycogen (form of carbohydrate storage) synthesis and amino acid uptake for protein synthesis (198). As mentioned, insulin lead to several effects but in the present study we will focus on its liver and adipose tissue effects.

In liver, glucose is stored as a long carbohydrate molecule known as glycogen and its metabolism is controlled by insulin as well as by glucagon and catecholamines, the counterregulatory hormones (199, 200). When insulin binds IR in liver it triggers a pathway that lead to glycogen synthase kinase 3 (GSK3) inhibition through its phosphorylation. GSK3 inhibit the activity of glycogen synthase, the enzyme responsible for producing glycogen in the liver, but when GSK3 is phosphorylated, it does not inhibit glycogen synthase activity, that culminates in a stimulus for glycogen production (201). The glycogen storage in liver supplies other tissues of glucose during short-term fasting periods. However, during long-term fasting periods when glycogen is depleted, liver cells are able to synthesize glucose from glycerol, pyruvate, lactate and amino acids through a process known as gluconeogenesis that is potentially inhibited by insulin. These substrates for gluconeogenesis are produced by the liver and by other tissues and reach the liver through the blood torrent. However, in a fed state when there is a high plasma glucose level, hepatocytes convert glucose into fatty acids (202).

6.2 Fatty acid metabolism

Several cell types are able to use fatty acids as source of energy. They can be obtained through the diet or synthesized by an organ (mainly liver) and exported to others. Fatty acids from the diets are transported through the blood torrent esterified in form of triacylglycerols in chylomicrons. Triacylglycerols that reach the liver can be hydrolysed to release fatty acids that are oxidized by

hepatocytes to produce energy or used as precursors of ketone bodies synthesis. In this way, part of the acetyl-CoA produced during fatty acid oxidation is converted to acetone, acetoacetate and β-hydroxybutyrate, these compounds are known as ketone bodies. Acetone is produced in small amounts when compared to the other two ketone bodies. Acetoacetate and β-hydroxybutyrate are transported through the blood torrent to extra-hepatic tissues (203, 204). In tissues like skeletal muscle ketone bodies are converted to acetyl-CoA and then oxidized in the citric acid cycle providing energy to these tissues.

However, when there is an excess of fatty acids arriving to liver not all of them are oxidized. They are re-esterified to triacylglycerols and packed together with cholesterol esters and specific apolipoproteins producing very-low density lipoprotein (VLDL) and released to the blood torrent. Therefore these fatty acids return to plasma to be metabolized by other tissues (205, 206). Moreover, different fatty acids are also used as substrate for the synthesis of different types of fatty acids.

Saturated fatty acids can be synthesized *de novo* in different tissues. This process occurs in the cytosol of the cells from surplus citrate released by mitochondria. Citrate is metabolized to acetyl-CoA by the action of citrate lyase. Then acetyl-CoA is converted to malonyl-CoA by the acetyl-CoA carboxylase. Malonyl-CoA is subsequently converted to palmitic acid (PA) (16:0) by the action of fatty acid synthase (FAS) (figure 1). The reaction catalysed by acetyl-CoA carboxylase is a rate-limiting step for fatty acid synthesis. Hence its action is regulated by hormones, being activated by insulin and inhibited by glucagon and adrenaline as well as by surplus palmitoyl-CoA. Fatty acid biosynthesis occurs mainly in the liver and the adipose tissue (207). Also, it is known that a high intake of specific polyunsaturated fatty acids could modify lipogenic enzymes expression in liver throughout a family of nuclear transcription factors known as peroxisome proliferator-activated receptor (PPAR) (208). Moreover, synthetic fibers and thiazolidinediones that are often used in hypertriglyceridemia and

diabetes are PPAR ligands that are able to mediate glucose and lipid metabolism (209).

Long chain saturated fatty acids are synthesized from PA by the action of elongase 6 (ELOVL6) that adds two carbons to PA (16:0) producing stearic acid (SA) (18:0) (figure 1). PA and SA are precursors of two monounsaturated fatty acids found in higher amounts in mammals that are oleic acid (OA, 18:1 n-9) and in a minor amount palmitoleic acid (POA, 16:1 n-7). These two fatty acids are produced by adding one double bound between carbon 9 and carbon 10 in a process known as desaturation. The enzyme involved in the synthesis of OA is Δ^9 desaturase (figure 1) (210).

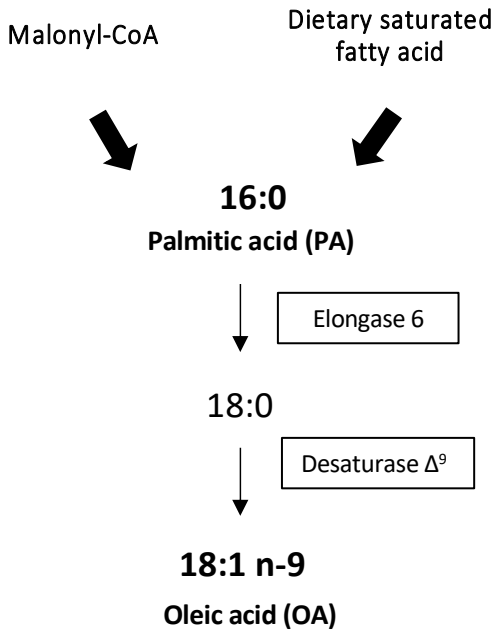

Figure 1: Fatty acid synthesis, elongation and desaturation.

Mammals are capable to introduce a double bound in the position Δ^9 of fatty acids, however, they are incapable of introducing double bounds between carbon number 10 and the methyl end of fatty acids. Thus, mammals cannot synthesize linoleic acid (LA) (18:2 n-6) and α-linolenic acid (ALA) (18:3 n-3). However, vegetables are able to synthesize these two fatty acids since they have desaturases that add carbons in the positions Δ^{12} and Δ^{15}. Therefore, these fatty acids are considered essential fatty acids for mammals and must be acquired through the diet, and are precursors of several other fatty acids (211, 212).

Once LA and ALA are ingested, they can be converted to other polyunsaturated fatty acids as shown in figure 2. Long chain n-6 polyunsaturated fatty acids are produced from LA throughout the action of several elongase and desaturase enzymes as shown in figure 2 (212). Among them, the arachidonic acid (AA, 20:4 n-6) is an essential precursor of regulatory lipids and eicosanoids that plays an important role in inflammation, immunity, cell proliferation and others (213). In the case of n-3 polyunsaturated fatty acid pathway, eicosapentaenoic (EPA, 20:5 n-3) and docosahexaenoic (DHA, 22:6 n-3) acids are produced by the action of the same enzymes that produce long chain n-6 fatty acids, but from ALA as shown in figure 2 (212). N-3 fatty acid family plays an important role lowering plasma lipids, preserving endothelial function and improving insulin sensitivity among others (214).

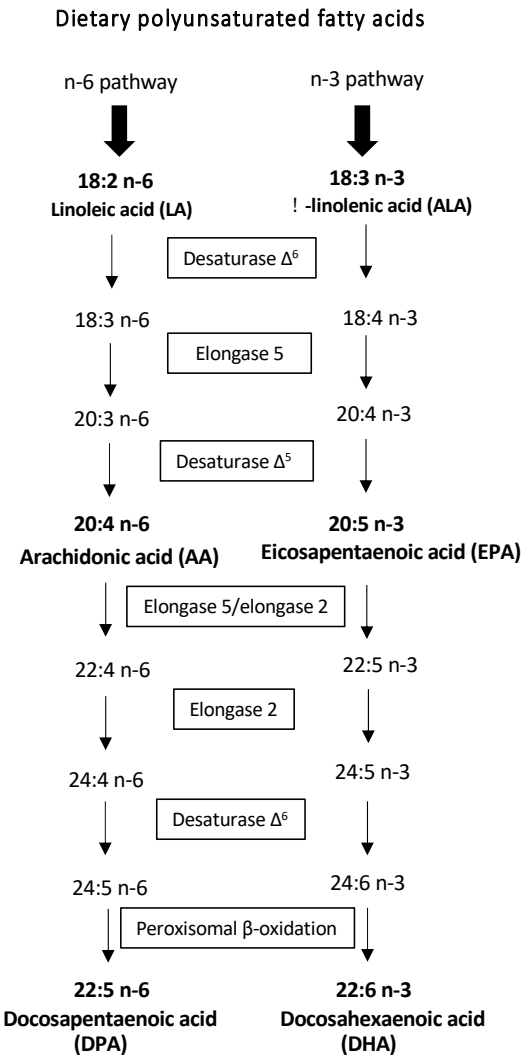

Figure 2: Biosynthesis pathway of long chain n-6 and n-3 polyunsaturated fatty acid

7. *Rubus berries and health*

Rubus berries are known due to their high levels of phenolic compounds that are involved in the health benefits reported in the literature (215). In plants these compounds are responsible for the pigments of the fruits, to attract pollinators, to protect against ultraviolet light and to function as an antioxidants (216). In mammals, the compounds present in blackberries show a variety of benefits (12).

The most important phenolic compounds present in the blackberries are the anthocyanins. Anthocyanins are water-soluble pigments that are responsible for the red, purple, blue and black colour of the fruit (217). Additionally, they show an important antioxidant capacity due to the anthocyanin chemical structure. Phenolic compounds usually contain an aromatic ring (figure 3) that are able to delocalize and stabilize unpaired electrons (13).

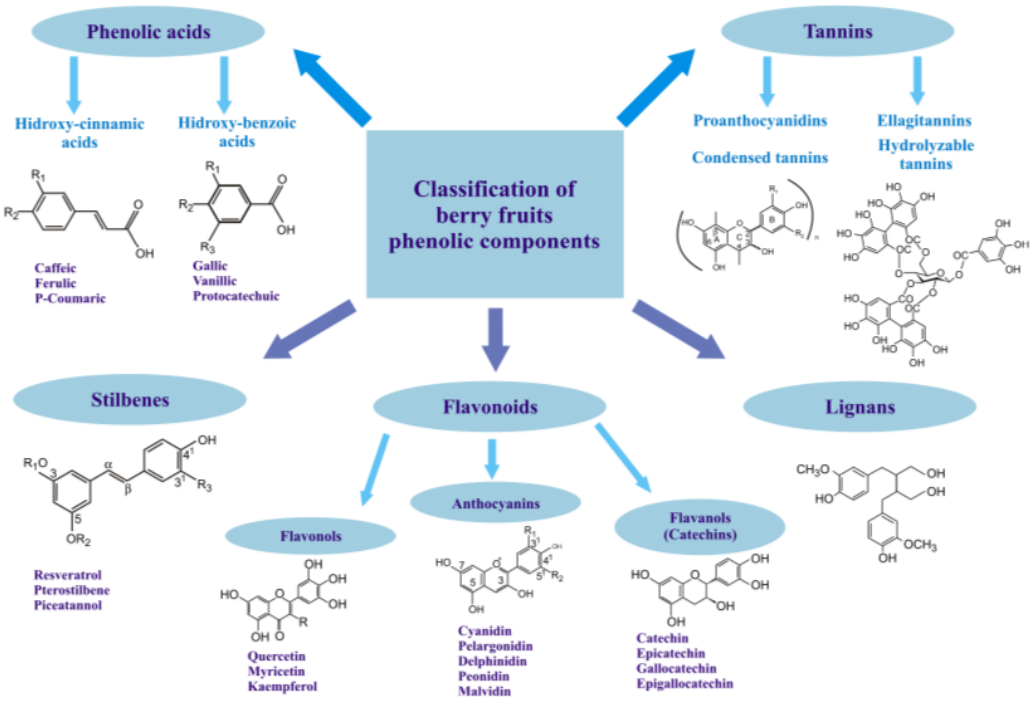

Figure 3: Phenolic components in berry fruits (218).

Antioxidant properties of blackberries due to their high amount of phenolic compounds are relevant in mammals when there is an imbalance between free radical production and antioxidant capacity. A high free radical production leads to damage in a variety of molecules that result in impaired cell function (219). *In vivo* studies analyzing the effects of different *Rubus* berries described attenuation of oxidative stress throughout scavenging activity and improved activity of antioxidant enzymes. On the contrary, high amounts of oxidant molecules are associated with neurodegenerative and cardiovascular diseases and cancer development (220).

Additionally, by reducing oxidative stress, the inflammatory response is also reduced. Although inflammation is an important natural reaction of the immune system, it is described that the excessive production of inflammatory

mediators is associated with chronic inflammatory state. And such state is related to cancer, obesity and type 2 diabetes development. Thus, *Rubus* berries collaborate on reducing the risk of developing several chronical diseases although there are still unknown mechanisms related to the health benefits of *Rubus* berries (12).

8. Blackberry fruit

Botanically blackberries are categorized in the rose family, genus *Rubus* and subgenus *Rubus*. *Rubus* species are native in all continents but most of them are native from the South Pacific Islands. The European blackberries, *Rubus fruticosus*, were originated in the Caucasus though they were spread across the entire European continent and also introduced into America, Asia and Oceania (14).

Blackberries are considered, in botanic, a drupe fruit which is a fruit that contains only one seed. Drupe fruits are originated from one single ovary of the flower as well as cherries and olives. However, in the case of blackberries approximately 50 to 100 ovaries in the same flower produces drupelets that end up forming a blackberry fruit. The aggregate of drupelets occurs due to the epidermal hairs that maintains several drupelets tangled to each other. When bad cohesion among the drupes occurs, the quality of the blackberry is severely reduced since the fruits become crumbly (221).

This fruit is consumed by humans in a diverse range of processed products beyond as fresh fruits. The food products prepared from blackberries vary from wine and beer to desserts and jams (220). Additionally, as mentioned above, blackberries and their products are considered a highly nutritious food due to their high levels of minerals, vitamins and phytochemical compounds (222).

OBJECTIVES

En la Introducción de esta Tesis hemos descrito cómo la dieta puede influir eficazmente en el desarrollo de sobrepeso/obesidad, así como en otras alteraciones metabólicas, como son la diabetes y la resistencia insulínica. En un intento de lograr una dieta semejante o próxima a la utilizada frecuentemente por el hombre, aplicable a la investigación animal, se desarrolló la denominada “dieta de cafetería”. Algunos autores han encontrado que tras el consumo de la dieta de cafetería durante un periodo corto en la rata se observa una respuesta distinta en función del género, con una menor actividad lipolítica en hembras que en machos. Por otra parte, tal como describimos en la Introducción, el suplemento dietético con bayas de *Rubus* puede contribuir a reducir el riesgo de padecer distintas enfermedades crónicas mediante una disminución del estrés oxidativo. A pesar de ello, existen aún mecanismos desconocidos de los beneficios de esta fruta sobre la salud.

En base a lo anterior, los objetivos concretos del presente estudio son:

1. Evaluar los efectos de una dieta rica en grasas, como es la dieta de cafetería, en ratas hembras y en machos, así como la respuesta a un periodo posterior de dieta control o standard.

2. Evaluar los efectos de una dieta rica en grasas, como es la dieta de cafetería, suplementada con un extracto de *Rubus*, en ratas hembras y en machos.

MATERIAL AND METHODS

1. Experimental Design

1.1 Animals

All the experimental protocols for this experiment were supervised and approved by the Animal Research Ethics Committee from the Pharmacy Faculty of the University San Pablo-CEU (reference 101112).

Sprague Dawley rats were obtained by the animal quarters of the University San Pablo-CEU from its usual provider. The animals were housed under a constant 12h light/dark cycle, with continuous ventilation, at 22± 2ºC and 55± 10% of relative humidity.

The rats were separated in cages of 5 animals of the same gender throughout all the experiment receiving experimental diets and water *ad libitum*.

1.2 Experimental groups

Two different experiments were carried out to develop this thesis, they were named I and II.

1.2.1 Experiment I: Gender and long-term effects of cafeteria diet

Figure 4 shows the design of Experiment I. Sprague Dawley rats weaned at 21 days of age weighing approximately 100g were fed with standard pellet diet (Harlan Tekland Global 14% Protein Rodent Maintenance Diet 2014, Madison, WI, USA) for a period of five days for adaptation. The rats were then randomly separated into two groups:

1. Standard group (SD) that was maintained on the pellet diet
2. Cafeteria group (CD) which was given a cafeteria diet

For the whole experiment rats were allowed to eat the corresponding diet *ad libitum.* The composition of the cafeteria diet is shown in table 1. The cafeteria diet was prepared in our laboratory mixing the ingredients shown in table 1. The solid ingredients were crushed mechanically to obtain a homogeneous blend and then the liquid ingredients were added one after the other to the blend. Finally, a homogeneous dough was obtained and pellets were formed with it. The pellets were maintained at -20ºC until use.

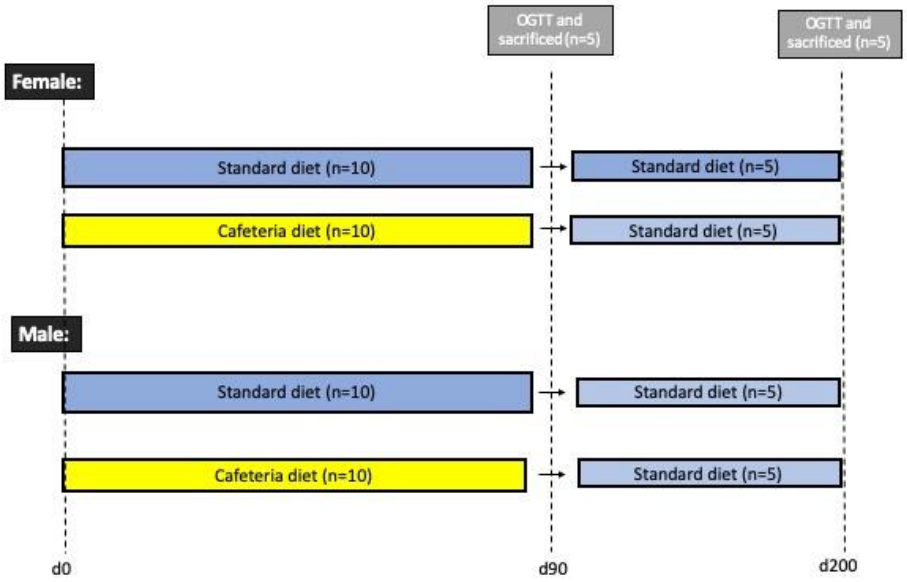

Figure 4: Experimental design of experiment I

"OGTT": Oral glucose tolerance test carried out at days 0 (d0), 90 (d90) and 200 (d200) of the experiment.

After 90 days of dietary treatment, the animals were subjected to an oral glucose tolerance test (OGTT) as detailed in section 3.1. Half of the rats from each experimental group were sacrificed (figure 4) and their plasma, liver and adipose tissues collected for further analysis as detailed in section 2.

From day 90 of experiment the remaining rats from both groups were fed with the pellet standard diet and at day 200 all rats were subjected to another OGTT (figure 4). After the test the animals were sacrificed and their plasma, liver and adipose tissues were collected for further analysis as detailed in section 2.

Table 1: Cafeteria diet composition (g/100g)

Ingredients	Producer	Quantity
Pellets	*Harlan Tekland,* Madison MI	23
Condensed milk	*Nestlé*, Barcelona	35
Sucrose	*Azucarera*, Madrid	7
Muffins	*Panrico SA*, Barcelona	6
Croissants	*Bimbo*, Barcelona	6
Powdered milk	*Central Lechera Asturiana*, Asturias	15
Lard	*El Pozo*, Murcia	8

The fatty acid composition of both diets were analysed as detailed in section 4 and the results are shown in the section Results and Discussion.

1.2.2 Experiment II: Effects of cafeteria diet supplemented with Blackberry extract in female and male rats

Figure 5 shows experimental design of experiment II. Female and male Sprague Dawley rats of 70 days old were fed with standard pellet diet (Harlan Tekland Global 14% Protein Rodent Maintenance Diet 2014, Madison, WI, USA) for a period of five days for adaptation. Animals were randomly divided into four experimental groups:

1. Standard group (SD) that was maintained on the pellet diet

2. Standard plus *Rubus* group (SD+R) that received standard diet supplemented with *Rubus* extract
3. Cafeteria group (CD) which was given a cafeteria diet
4. Cafeteria plus *Rubus* group (CD+R) that received cafeteria diet supplemented with *Rubus* extract

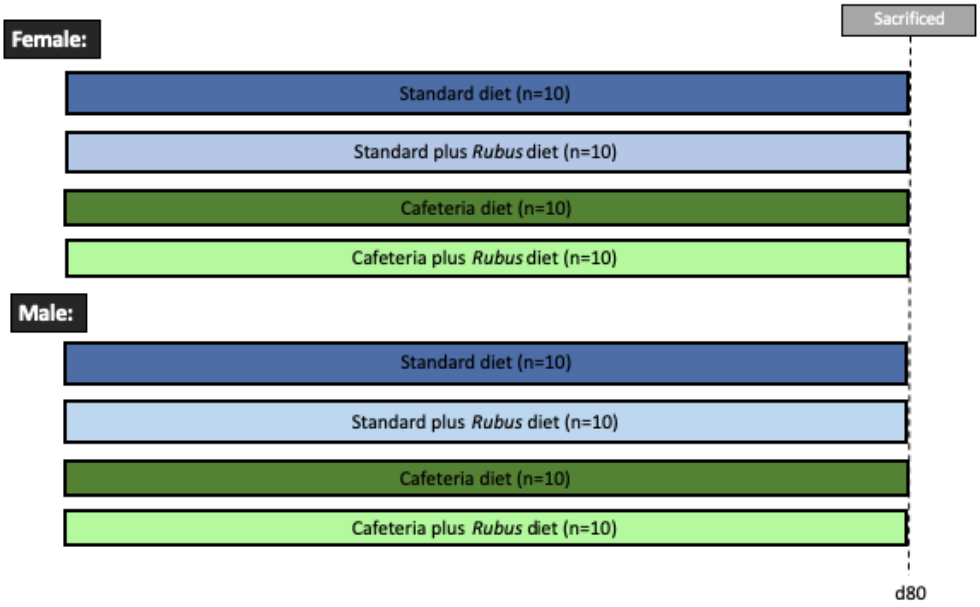

Figure 5: Experimental design of experiment II

"d80": 80 days of experiment

For the whole experiment (80 days) rats received their corresponding diet *ad libitum.* The composition of the diets is shown in table 2. The standard and cafeteria diets were prepared in our laboratory mixing the ingredients shown in table 2. For cafeteria diet, the solid ingredients were crushed mechanically to obtain a homogeneous blend and then the liquid ingredients were added one after the other to the blend. Finally, a homogeneous dough was obtained and pellets were formed with it. The pellets were maintained at -20ºC until use. The pellets of the standard diet were crushed mechanically and mixed with water and

agar or *Rubus* extract, water and agar in order to enable to form the pellets again. The pellets of both standard diets were also maintained at 20ºC until use.

Table 2: Diets composition of experiment II (g/100g)

Ingredients	Producer	Standard	Standard + *Rubus*	Cafeteria	Cafeteria + *Rubus*
Pellets	*Harlan Tekland,* Madison MI	58	58	19	19
Condensed milk	*Nestlé*, Barcelona			35	35
Sucrose	*Azucarera*, Madrid			7	7
Muffins	*Panrico SA*, Barcelona			6	6
Croissants	*Bimbo*, Barcelona			6	6
Powdered milk	*Central Lechera Asturiana*, Asturias			15	15
Lard	*El Pozo*, Murcia			8	8
***Rubus* extract**			4		4
Water		40	36	4	
Agar	*Sigma-Aldrich*, Missouri, USA	2	2		
Caloric content (Kcal)		108	108	403	403

Rubus extract

The blackberries (*Rubus* sp. var.*Loch Ness*) used in the present study were kindly provided by Agrícola El Bosque (Lucena del Puerto, Spain). Blackberries were lyophilized and then the anthocyanins were extracted using 80% methanol in 0.1% HCl as previously described by García-Seco et al. (223). Afterwards, the extract was sonicated for 10 min and then centrifuged at 3000 rpm for 10 min at 4ºC. Methanol was removed by evaporation using a rotavapor at 40ºC. *Rubus* extract was stored at -20ºC until use.

Anthocyanins quantification

Quantification of total anthocyanins was performed by pH differential method as previously described (224) with minor modifications. Diluted 1:9 (v/v) extract sample was used for anthocyanin determination. 0.5 mL of sample was diluted 1:14 (v/v) in a pH 1.0 solution (HCl 0.2M) and in a pH 4.5 acetate solution (1M). The tubes were incubated in the dark for 15 min at room temperature. The absorbances were measured at λ510 and λ700 nm, respectively in ultraviolet-visible spectrophotometer (*Shimadzu 300*, Tokyo, Japan) and calculated using the following equation:

$$A = (A510 - A700)\ pH\ 1{,}0 - (A510 - A700)\ pH\ 4$$

Where A is absorbance of the diluted sample. The concentration was calculated applying the Lambert-Beer´s law using cyanidin-3-glucoside as control. Our *Rubus* extract concentration of anthocyanins was 5.42 g of cyanidin-3-glucoside per 100g of extract.

2. Sample collection

After 3 hours of fasting, the rats were anesthetised with CO_2 for 30 seconds. They were then sacrificed by decapitation and had the trunk blood

collected into ice-chilled receptacles containing Na_2EDTA. Plasma was separated from fresh blood by centrifugation at 3000rpm for 30 min at 4ºC and stored at -80ºC until analysis.

Liver and different fat depots were rapidly dissected and placed into liquid nitrogen before weighing and were kept at -80ºC until analysis.

3. Glucose overload test

3.1 Oral Glucose Tolerance Test

On the day specified in section 1.2.1 (90 days for half of the rats and 200 days for the other half of experiment I), after three hours of fasting the rats were weighed and had their tail blood collected in tubes containing 1g Na_2EDTA/L for plasma separation and analysis of basal insulin and glucose. Subsequently rats received an oral 50% glucose solution at 2g glucose per Kg body weight. Afterwards, tail blood was collected at 7.5, 15, 30 and 60 min for plasma separation and later analysis.

3.2 Insulin Sensitivity Indexes

The glucose and insulin area under the curve (AUC) were calculated by the trapezoid's method. Additionally, the ratio between them (AUC glucose/AUC insulin) was calculated as an index of glucose tolerance.

Other insulin sensitivity indexes were calculated with basal glucose and insulin levels. The Insulin Sensitivity Index (ISI), Homeostasis Model Assessment of Insulin Resistance (HOMA) and the Quantitative Insulin Sensitivity Check Index (QUICKI) were calculated as previously described (225), by using the following equations:

$$ISI = \frac{10000}{\sqrt{FPG \times FPI \times meanG \times meanI}}$$

$$HOMA\text{–}IR = \frac{FPG \times FPI}{2{,}430}$$

$$QUICKI = \frac{1}{logFPI + logFPB}$$

Where FPG is fasting plasma glucose (mg/dL), FPI is fasting plasma insulin (µL/mL), and mean G and mean I are the mean glucose and insulin concentration values along the OGTT.

4. Diet analysis

4.1 Extraction of lipids from the diets

Lipids were extracted and purified according to the method of Folch et al. (226). For this, 200 mg of a diet sample were added to tubes containing 3 mL of chloroform:methanol 2:1 (v:v), butylhydroxytoluene (BHT) (50 mg/L) and 200 µL of the fatty acid 19:1 n-9 (*Sigma*, St. Louis, MO, USA) as internal standard. The tubes were agitated by inversion for 4 hours. Subsequently a piece of cotton wool was added to the tubes to function as a filter when the liquid was aspirated and transferred to another screw cap tube. Then other 3 mL of chloroform:methanol 2:1 were added to the first tube. The process was repeated twice but agitating by inversion for 3 hours each time, and had its lipid extract volume completed to 10 mL with chloroform:methanol. 2.5 mL of distilled water were added to the tubes and agitated for 15 minutes. Subsequently the tubes were centrifuged for 10 minutes at 2000 rpm at room temperature. The upper phase was discarded, and the volume was completed up to 10 mL with methanol containing BHT (10 mg/L). This process was repeated twice although using 2% NaCl instead of distilled water. The tubes were stored closed with Teflon cap to avoid evaporation.

4.2 Fatty acids profile of diets

The fatty acids were identified and quantified using gas chromatography. In order for this to occur, volatile methyl esters of each fatty acid were prepared by submitting the samples to a trans-esterification reaction with acetyl chloride in methanol (227).

The trans-esterification involves the evaporation of 0.5mL of each lipid extract with a vacuum evaporator *Speed-Vac Plus Savant* (*Thermo Fisher Scientific,* Waltham, MA, USA) followed by resuspension of the residual fat in 300µL of toluene containing BHT (10mg/L). 200µL of each resuspended sample was added to a tube with 2 mL of methanol:toluene (4:1) (v:v) containing BHT (50mg/L) and then 200µL of acetyl chloride was added (under a fume hood during vortex agitation). The trans-esterification reaction was carried out at 80ºC for 2.5 hours in a screw glass tube closed with teflon cap. The reaction products were resuspended in 500µL of toluene containing BHT (10mg/L) and then the reaction was stopped with 5mL of 6% potassium carbonate solution. 10 µL of a methylated fatty acid (methyl 17:0 from *Sigma,* St Louis, MO, USA) was added to confirm the efficiency of the injection in the chromatograph. The tubes were centrifuged at 2000 rpm at room temperature for 10 min and the upper phase was transferred to another tube where it was dried using a vacuum evaporator Speed-Vac Plus Savant. The residue was resuspended in 60µL of toluene containing BHT (50mg/L), and this solution was stored in glass sealed vials at -20ºC.

The samples stored in sealed vials were automatically injected into a capillary column Omegawax (*Supelco*, Bellefonte, PA, USA) with 0.25mm of inner diameter, coating of 0.25µm and 30m in length. The gas chromatograph used was a *Perkin Elmer* (Waltham, MA, USA) with split-splitless injection. The injector was at 260ºC and the flame ionization detector at 280ºC. The oven start temperature was 200ºC, and after 20 minutes the temperature was increased at the speed of four degrees per minute to 240ºC. This temperature was maintained

for the final 15 minutes. Every sample analysis lasted for approximately 45 minutes. Nitrogen circulating through the column at a pressure of 17 psi was used as mobile phase. The methylated fatty acid esters were identified comparing their retention time with the ones from purified patterns (*Sigma*, St Louis, MO, USA). They were quantified using as reference the area of the internal standard (19:1 n-9) and the concentration of it which was added at the beginning of the process.

An analysis of repeatability of the method was carried out and a relative standard deviation lower than 10% was found in all cases in which the fatty acid was found in a proportion higher than 1% in relation to total fatty acids. Additionally, we checked the method linearity and found values higher than 0.999 in the fatty acids which were in a proportion higher than 1%.

4.3 Determination of the amount of fat in the diets

The gravimetric method was used to determine the fat content of the diets. For this, a known volume of each lipid extract, obtained as described in the section 4.1., was pipetted into a weighed vial and dried in an oven at 37ºC. The vials were weighed several times until reaching a constant mass.

5. Plasma and tissues analysis

5.1 Plasmatic parameters

5.1.1 Plasma lipid extraction and fatty acid profile analysis

The plasma samples were subjected to a process of lipid extraction, methylation of fatty acids and analysis as previously described (227).

An aliquot of plasma (75 or 100 µL, depending on the amount of sample available), were placed in tubes containing 2 mL of methanol:toluene (4:1) (v:v)

BHT (50mg/L) and 25 µL of the internal standard 19:1 n-9. 200 µL of acetyl chloride were added to the tubes under fume hood and vortex agitation. The methylation reaction took place in a bath at 100ºC for 1 hour. To stop the reaction, 5mL of 6% potassium carbonate were added followed by 500µL of toluene containing BHT (10mg/L) and 10µL of the methylated fatty acid 17:0. The tubes were centrifuged at 2000rpm for 10 minutes at room temperature. The upper phase was collected and submitted to vacuum evaporation. The residue was resuspended in 60µL of toluene containing BHT (50mg/L). The extract was stored in sealed glass vials with an insert at -20ºC. The fatty acid plasma profile was analysed by gas chromatography as described above in section 4.2.

5.1.2 Determination of glucose in plasma

To quantify plasma glucose a commercial assay kit *Glucose-Trinder GOD-POD* (*Spinreact*, Gerona, Spain) was used. The assay is based on the glucose oxidase enzyme (GOD) which oxidises the glucose from the samples producing gluconic acid and hydrogen peroxide. The hydrogen peroxide is detected when it reacts with phenol-ampyrone by the action of peroxidase enzyme (POD). The intensity of the colour was determined using a plate spectrophotometer (*Bio-tek Intruments,* Winooski, VT, USA) at a wavelength of 500 nm and was proportional to the glucose sample.

$$\beta\text{-}D\text{-}Glucose + O_2 + H_2O \xrightarrow{GOD} Gluconic\ acid + H_2O_2$$

$$H_2O_2 + Phenol + Ampyrone \xrightarrow{POD} Quinone + H_2O$$

A calibration curve was prepared with different glucose concentrations: 0; 12.5; 25; 37.5; 50; 75 and 100 mg/dL. A typical calibration curve is shown in figure 6. Depending on the expected glucose concentration either 5 or 10 µL of each sample were analysed together with the calibration curve and the controls.

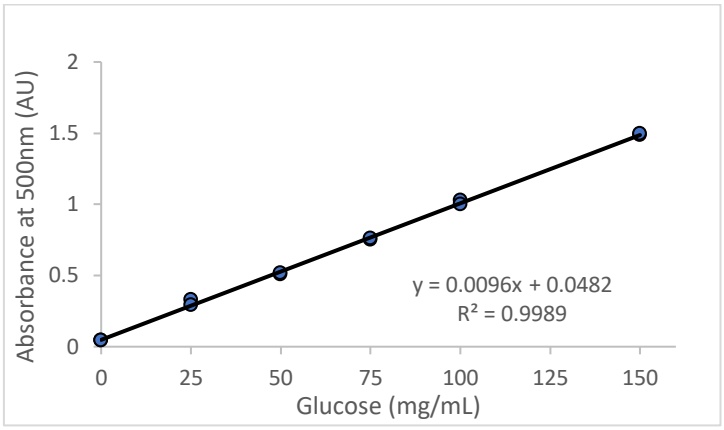

Figure 6: Typical calibration curve for glucose concentration

5.1.3 Determination of triacylgycerol in plasma

To quantify plasma triacylglycerol (TAG) concentrations the commercial kit *Triglycerides-GPO-POD (Spinreact*, Gerona, Spain) was used. The method is based on the activity of the enzyme lipoprotein lipase (LPL) that hydrolyzes triacylglycerols present in the samples producing glycerol and free fatty acids. Glycerol is phosphorylated by glycerol kinase reaction (GK) producing glycerol 3-phosphate in the presence of adenosine 3'-phosphate (ATP). Glycerol 3-phosphate by the action of glycerol phosphate dehydrogenase produces dihydroxyacetone phosphate (DHAP) and hydrogen peroxide. The quantity of hydrogen peroxide is determined through a reaction catalyzed by POD as described in section 5.1.2. The intensity of the colour obtained is proportional to the TAG concentration in the samples.

$$Triacylglycerols + H_2O \xrightarrow{LPL} Glycerol + Free\ Fatty\ Acids$$

$$Glycerol + ATP \xrightarrow{GK} G_3P + ADP$$

$$G_3P + O_2 \xrightarrow{GPO} DHAP + H_2O_2$$

$$H_2O_2 + p\text{-}chlorophenol + 4\text{-}aminophenazone \xrightarrow{POD} Quinone + H_2O$$

A calibration curve was prepared using known concentration dilutions (0; 25; 50; 100 and 200 mg/dL) prepared from a triolein standard solution. A characteristic calibration curve is shown in figure 7.

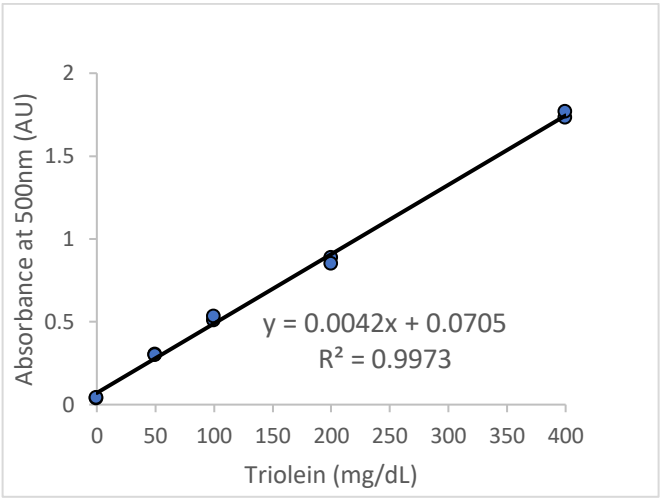

Figure 7: Typical calibration curve for plasma triacylglycerols analysis

5.1.4 Determination of the concentration of glycerol in plasma

To determine plasma free glycerol levels the commercial kit *Free Glycerol Determination Kit* (*Sigma,* St. Louis, MO, USA) was used. Glycerol from the samples is phosphorylated to glycerol-1-phosphate (GP1) by the action of GK in the presence of ATP. GP1 is oxidized in the presence of GPO and DHAP and the hydrogen peroxide produced reacts with POD producing a coloured product that

absorbs light at 550 nm. The intensity of the colour produced is proportional to the glycerol concentration present in the samples.

$$Glycerol + ATP \overset{GK}{\rightarrow} G1P + ADP$$

$$G1P + O_2 \overset{GPO}{\longrightarrow} DAP + H_20_2$$

$$H_2O_2 + N - etyl - N - (3 - sulfopropyl)\, m - anisidina\ sodium + 4 - aminoantypirin \overset{POD}{\longrightarrow} Quinoneimine + H_2O$$

From a glycerol standard solution several solutions were prepared (0; 0.034; 0.068; 0.136; 0.271; 0.543 and 1.086 mmol//L) and used as calibration curve. The absorbance was determined at 550 nm in a plate spectrophotometer (*Bio-tek Instruments,* Winooski, VT, USA). Figure 8 shows a typical calibration curve of this analysis.

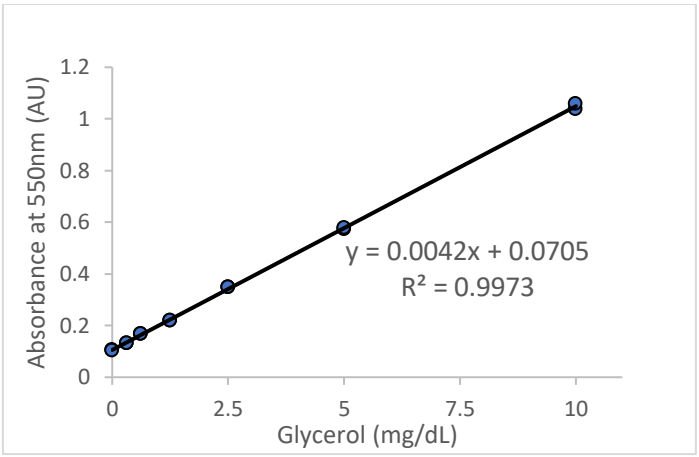

Figure 8: Typical calibration curve for plasma glycerol analysis

5.1.5 Determination of the concentration of non-esterified fatty acids (NEFA) in plasma

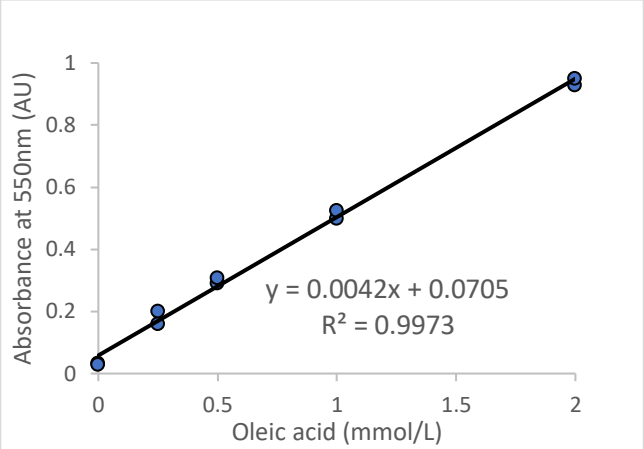

Figure 9: Typical calibration curve for plasma non-esterified fatty acids analysis

The kit *Wako NEFA-HR* (*Wako Chemicals,* Osaka, Japan) was used to determine plasma non-esterified fatty acid concentrations (NEFA). The method is based on the following reaction: NEFA from the samples react with coenzyme A by the action of acyl-CoA-synthetase (ACS) in the presence of ATP which also produces adenosine-5-phosphate (AMP) and pyrophosphoric acid (PPi). Acyl-CoA produced is oxidized by the action of acyl-CoA-oxidase (ACOD) generating hydrogen peroxide that produces a coloured product in the presence of POD. The coloured product absorbs light at 500 nm proportionally to the amount of NEFA in the samples.

$$NEFA + ATP + CoA \xrightarrow{ACS} Acyl - CoA + AMP + PPi$$

$$Acyl - CoA + O_2 \xrightarrow{ACOD} 2{,}3 - trans - enoyl - CoA + H_2O_2$$

$$H_2O_2 + 3 - metyl - N - etyl - N - (\beta - hydroxyetyl) - aniline + 4 - aminoantipiryn \xrightarrow{POD} Quinoneimine + 3H_2O$$

The calibration curve was prepared from an oleic acid standard solution at different concentrations: 0; 0.0625; 0.125; 0.25; 0.5 mmol/L. Depending on the expected NEFA concentrations 5 or 10 µL of each sample was analysed. Plates were incubated at 37ºC for 10 minutes after adding 200 µL of the first reagent in the kit. Afterwards, a first analysis was carried out in a microplate reader (*Bio-tek Instruments*, Winooski, VT, USA) at 550 nm, in which the absorbance obtained was (going to be) subtracted from the second read absorbance values. 100 µL of the second reagent in the kit was added and the plate incubated at 37ºC for 10 min before reading for a second time. Figure 9 shows a typical calibration curve where the absorbance shown in the axis of ordinates correspond to the absorbance difference between the first and second read.

5.1.6 Determination of cholesterol in plasma

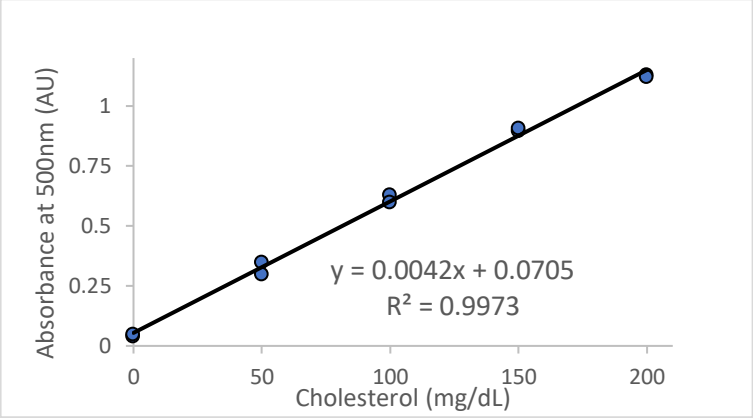

Figure 10: Typical calibration curve for plasma cholesterol analysis

Total cholesterol concentration in plasma samples was determined using the Cholesterol-CHOD-POD kit (*Spinreact,* Gerona, Spain). In this assay, an enzyme known as cholesterol esterase (ChE) hydrolyzes cholesterol esters from the samples to free cholesterol and free fatty acids. Then, the cholesterol oxidase (CHOD) enzyme catalyses the oxidation of these free cholesterol molecules into 4-cholestenone and hydrogen peroxide. The hydrogen peroxide reacts with peroxidase and the reaction product is a coloured compound able to absorb light at a wavelength of 500 nm, and its intensity is directly proportional to sample's cholesterol concentrations.

$$Cholesterol\ esters + H_2O \xrightarrow{ChE} Cholesterol + Fatty\ acids$$

$$Cholesterol + O_2 \xrightarrow{CHOD} 4 - Cholestenone + H_2O$$

$$2H_2O_2 + Phenol + 4 - aminophenazone \xrightarrow{Peroxidase} Quinonimine + 4H_2O$$

To perform this analysis, a calibration curve was prepared (0; 50; 100; 150; 200mg/dL). A volume of 10 µL was collected from both samples and controls and 250 µL of the kit's reagent were added. They were incubated at room's temperature for 10 minutes and their absorbance measured at 500 nm (*Bio-tek Instruments*, Winooski, VT, USA). A typical calibration curve of this analysis can be observed in figure 10.

5.1.7 Determination of insulin in plasma

Insulin concentration was determined by using a rat insulin Enzyme-linked Immunosorbent Assay kit (ELISA) (*Mercodia*, Uppsala, Sweden). This assay presents a detection limit of 0.15 µg/L, and its intra-assay and inter-assay coefficient of variation are 3.1% and 4.4%, respectively.

This assay is based on the sandwich technique, in which two monoclonal antibodies are directed against different epitopes of insulin during a 2 hours incubation. The first antibody is linked to the bottom of the plate wells while the second antibody, peroxidase-conjugated, is added later. After the incubation with both antibodies, several washing steps eliminate the antibodies that remain free in those wells not attached to insulin. Then, 3,3',5,5'-tetramethylbenzidine (TMB) is added and reacts with peroxidase, transforming it into a coloured product. Sulfuric acid (0.5M) is introduced to stop this enzymatic reaction and the absorbance is determined through spectrophotometry using a plate reader (*Bio-tek Instruments*, Winooski, VT, USA) at 450 nm. The colour intensity is directly proportional to the insulin concentration in the samples. A typical calibration curve of this analysis can be observed in figure 11.

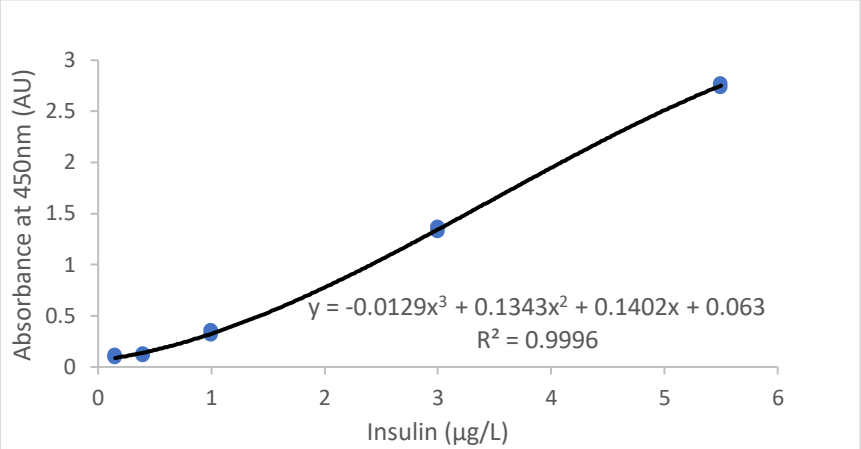

Figure 11: Typical calibration curve for insulin analysis

5.1.8 Determination of leptin in plasma

Plasma leptin concentration was determined by a mouse and rat ELISA kit (*Biovendor*, Czech Republic).

The technique is based on the sandwich technique in which the antigen present in the sample attach an anti-leptin rat antibody. During several incubations and washes, a second polyclonal anti-leptin rat antibody marked with biotin was attached to a streptavidin-horseradish peroxidase conjugate. The attached conjugate antibody was detected by the reaction with TMB substrate that produces colour. The reaction was stopped using an acidic solution and the leptin concentrations were measured spectrophotometrically with a plate reader (*Bio-tek Instruments*, Winooski, VT, USA) at 450nm. The colour intensity is directly proportional to the leptin concentrations in the samples.

A calibration curve (figure 12) was prepared using the calibrators from the commercial kit. To perform the assay 100 µL of the diluted samples (1:20), 100 µL of diluted antibody marked with biotin, 100 µL of the enzymatic conjugate, 100 µL of TMB substrate and 100 µL of the stop solution was added to the plate.

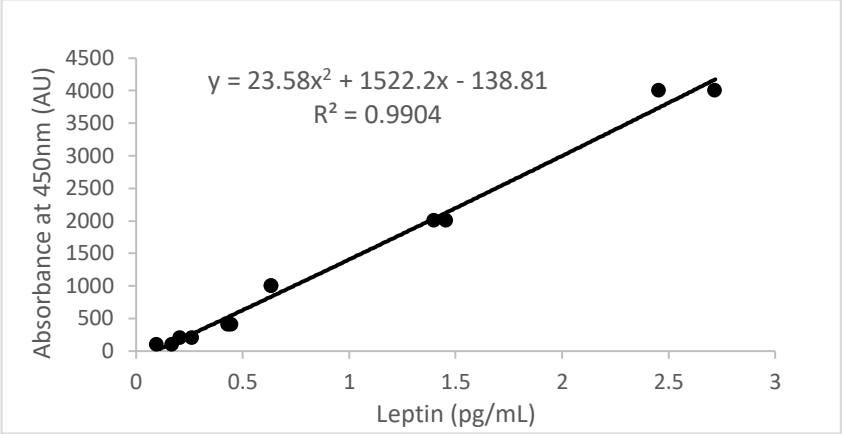

Figure 12: Typical calibration curve for leptin analysis

5.1.9 Determination of adiponectin in plasma

Plasma adiponectin concentration was determined using a rat adiponectin ELISA kit (*Milipore*, USA).

This assay is based on the sandwich technique, in which a monoclonal anti-adiponectin rat antibody present on the reaction plate attaches its antigen in the samples. After several incubations and washes, a second biotinylated monoclonal anti-adiponectin antibody binds the antigen in the plate and later a streptavidin-horseradish peroxidase conjugate is added and binds the immobilized biotinylated antibody. The bounded conjugate is detected due to its reaction with the substrate TMB that produces colour. The reaction is stopped with an acidic solution and the adiponectin concentration is quantified spectrophotometrically using a plate reader (*Bio-tek Instruments*, Winooski, VT, USA) at 450nm. The colour intensity is directly proportional to the adiponectin concentration in the sample.

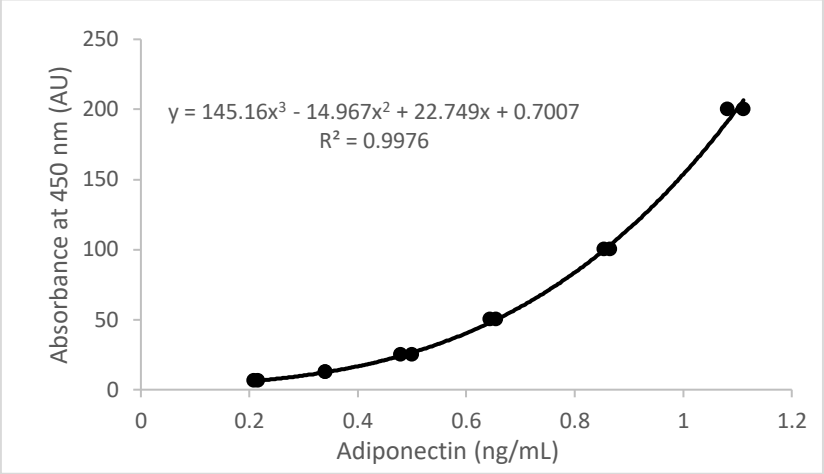

Figure 13: Typical calibration curve for adiponectin analysis

A calibration curve was prepared with the calibrators from the commercial kit. A typical calibration curve is shown in the figure 13. 20 µL of the diluted sample (1:500), 80 µL of assay buffer, 100 µL of the antibody marked with biotin, 100 µL of the enzymatic conjugate, 100 µL of TMB substrate and 100 µL of the stop solution were added to the plate.

5.1.10 Determination of resistin in plasma

Plasma resistin concentration was determined using a Rat Resistin ELISA kit (*Biovendor*, Czech Republic). 100 µL of standards, quality controls and samples (diluted samples 1:20) were incubated in a microplate where the wells were pre-coated with polyclonal anti-rat resistin antibody. Biotin labelled polyclonal anti-rat resistin antibody (100 µL) was added after 60 min of incubation under agitation at 300 rpm and then the plate was washed 3-times. A streptavidin-HPR conjugate (100 µL) was added after washing. The substrate solution (TMB) (100 µL) was added to react with the remaining conjugate from the last washing step. To stop the reaction an acidic solution (100 µL) was added, and the plates were read in a plate reader (*Bio-tek Instruments*, Winooski, VT, USA). First, the

plate was read at 450nm and then at 600nm. The readings at 600nm were subtracted from the readings at 450nm. The absorbance is proportional to resistin levels. A standard curve was prepared from concentrations of calibrators as shown in figure 14.

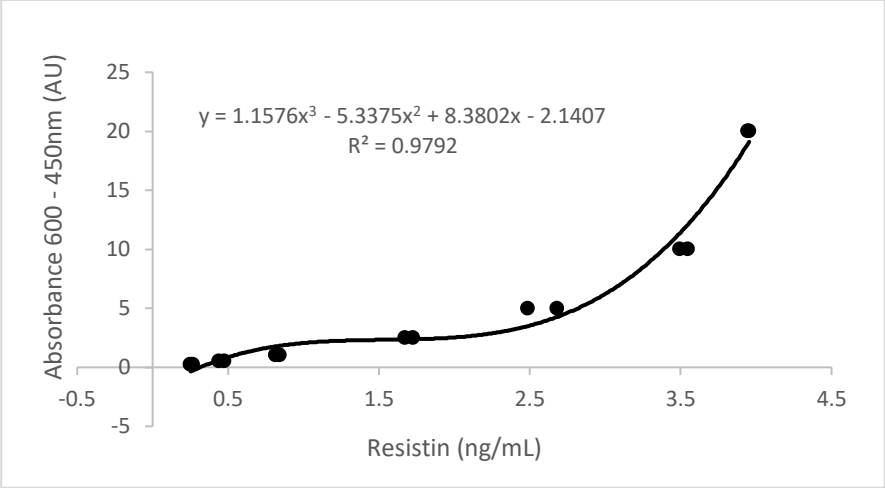

Figure 14: Typical calibration curve for resistin analysis

5.2 Tissue parameters

5.2.1 Tissue lipid extraction

Lipids from liver and adipose tissues were extracted and purified by the Folch method (226) similarly as described for the diets in section 4.1. Depending on the tissue an amount of sample (50 mg for adipose tissue and 200 mg for liver) were added to tubes containing 3 mL of chloroform:methanol 2:1 (v:v) and BHT (50 mg/L). As internal standard either 100 or 200 µL (depending on the tissue) of the fatty acid 19:1 n-9 (*Sigma,* St. Louis, MO, USA) was added. The tubes were agitated by inversion for 4 hours and aspirating through a piece of cotton the liquid was transferred to a new tube. This process was repeated twice and at the end the volume of the lipid extract was completed to 10 mL with methanol containing BHT (50mg/L). The lipid extracts were washed with distilled water and

NaCl at 2% as described in section 4.1. The tubes were stored sealed with a screw and teflon cap.

5.2.2 Fatty acids profile analysis

To identify and quantify the fatty acids in the lipid extract from each tissue through gas chromatography, the samples were submitted to a transesterification reaction as described in section 4.1.

A volume (depending on the tissues) of lipid extract was evaporated and the dry residue was resuspended in toluene with BHT (50mg/L). 200 µL of the resuspended extract was added to 2 mL of methanol:toluene (4:1) (v:v) containing BHT (50mg/L) and 200 µL of acetyl chloride. Esterification reaction with a methyl group was carried out in a bath at 80ºC for 2.5 hours. Afterwards, 500 µL of toluene containing BHT (10mg/L) and 5 mL of 6% potassium carbonate were added. The methylated fatty acid 17:0 (*Sigma*, St. Louis, MO, USA) was added (10 µL) as a reference standard. The tubes were centrifuged for 10 minutes at 2000 rpm at room temperature and the upper phase was collected into another tube. The tubes were submitted to a vacuum evaporation and the residue was resuspended in 60 µL of toluene containing BHT (50mg/mL). The samples were stored in sealed vials at -20ºC to further analysis.

The samples were processed in a gas chromatograph and the fatty acids were identified and quantified using the internal standard 19:1 n-9 (*Sigma*, St. Louis, MO, USA) as reference as described in section 4.1.

5.2.3 Determination of the triacylglycerols in tissues

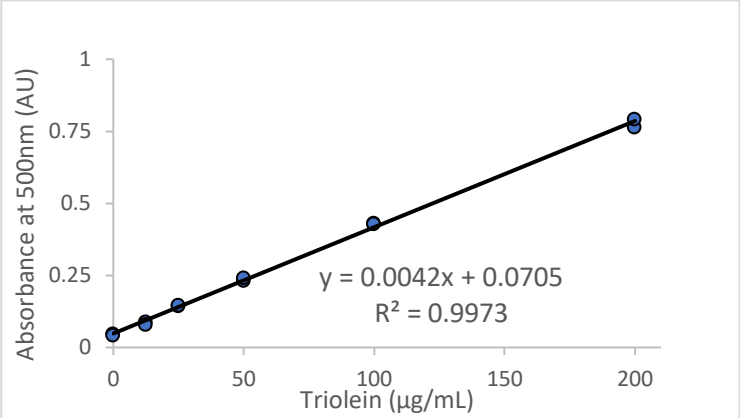

Figure 15: Typical calibration curve for tissue triacylglycerols analysis

To determine TAG in liver we first prepared a lipid extract as described in section 5.2.1 and subsequently the extract was analysed with the kit used to determine plasma TAG (section 5.1.3). However, since the chloroform in the lipid extract could inhibit the enzyme present in the reagent of the kit, it was necessary to eliminate the chloroform from the samples. In order to do so, Triton-X-100 was used as a detergent to solubilize the lipids in deionized water and make the samples soluble in water and compatible with the kit as previously described (228).

For the calibration curve, dilutions of triolein of known concentration were used (0; 12.5; 25; 50; 100; 200 µg/mL) (*Sigma*, St Louis, MO, USA). They were prepared in chloroform and processed in parallel to the samples. 1% Triton-X-100 in chloroform was added to either 0.2 or 0.4 mL of the samples (depending on the expected TAG level) and 1 mL of standards, and all the solutions were evaporated in a vacuum concentrator *Speed-Vac Plus Savant* (*Thermo Fisher Scientific*, Waltham, MA, USA) at medium temperature. The dry residue was resuspended in deionized water and the tubes were agitated in a bath at 37ºC for

15 minutes. The TAG from the samples and standards (50 µL) were quantified in a 96-well plate in duplicate, where 250 µL of the reagent from the commercial kit was added. The plate was incubated for 10 minutes at room temperature and the absorbance was read at 500 nm in a plate reader (*Bio-tek Instruments*, Winooski, VT, USA). A typical calibration curve is shown in figure 15.

5.2.4 Determination of the cholesterol in tissues

Similarly to the method for TAG analysis in tissues described in section 5.2.3, lipids were extracted accordingly to the method described in section 5.2.1. Subsequently, the chloroform was eliminated from the samples in order to solubilize lipids in deionized water using Triton-X-100 (228). Different known concentrations of a cholesterol standard (*Sigma,* St. Louis, MO, USA) were prepared for the calibration curve in chloroform (0; 12.5; 25; 50; 100; 200 µg/mL). Samples and standards were dried after the addition of the detergent and were resuspended in water. 50 µL of the samples and standards were analysed using the commercial kit described in section 5.1.6. A typical calibration curve is shown in figure 16.

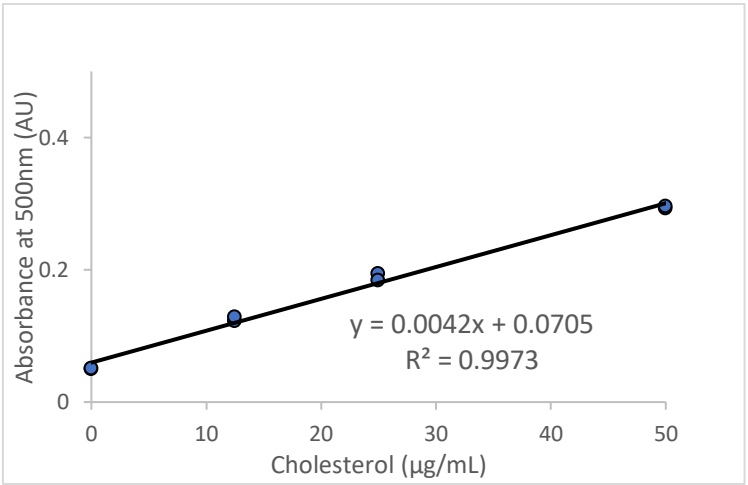

Figure 16: Typical calibration curve for tissue cholesterol analysis

6. Determination of the activity of Glycerol kinase

Glycerol kinase activity was analysed in inguinal, lumbar, peri-renal and mesenteric adipose tissues following the method described by Newsholme et al.(229) For this about 200 mg of each tissue was weighed and homogenized in 0.8 mL of homogenate buffer (1% KCl 1 mM EDTA). The samples were centrifuged at 2000 g for 10 min at 4ºC. After removal of the fat layer the supernatant was placed into another tube.

For the enzymatic assay, 50 μL of these supernatants were added to 50 μL of a reaction mixture prepared with 1 mL of incubation buffer (200mM TRIS, 50mM 2-Mercaptoethanol, 49mM FNa,10mM Cl_2Mg, 2mM EDTA, 1mM Glycerol and 1% Albumin, adjusted to pH 7.5 using HCl), 50 μL of ^{14}C-Glycerol, 0.3 mg of phosphocreatine kinase, 6.6 mg of ATP and 2.5 mg of phosphocreatine. 100 μL of ethanol was used as a blank and kept in cold. All the tubes were agitated with Vortex and incubated at 37ºC for 30 minutes under gently agitation, except the blank tubes. 100 μL of 99% ethanol were added to stop the reaction. Then 75 μL of each tube was filtered in a 96 well plate. The plate was washed 7 times with 150 μL of 1M glycerol and 7 times with distilled water.

The filters where removed from the plates and placed into radioactivity vials and 5 mL of scintillation liquid was added for the radioactive counting in a *Pakard* radioactive counter. A sample of the supernatant was separated for further protein quantification. For this determination, 200 mL of the supernatant were digested with 0.13N NaOH for 1 hour at 60ºC. The protein analysis was performed by following the Lowry method (8) and using a 0.5 mg/mL bovine serum albumin solution as standard. A calibration curve was performed with the following amounts of albumin: 0, 5, 10, 20, 30 and 40 μg/tube; and 10 μL of the digested sample were used.

7. Determination of the activity of Lipoprotein lipase

To analyse the lipoprotein lipase (LPL) activity in adipose tissue the method described by Nilsson-Ehle & Scholz (230) was adapted for its use in tissues by Llobera et al. (231) through previous delipidation of the samples in acetone-ether.

Around 0.2 g of each adipose tissue sample was weighed and homogenized in Tris-HCl buffer. Then the samples were delipidated with cold diethyl ether and acetone to obtain an acetone powder that was redissolved in 0.05M NH_4-$ClNH_4$ buffer. To analyse the enzymatic activity 200 μL of the redissolved solution were used. As substrate an emulsion containing cold triolein (13.8 mg/mL of glycerol), ^{14}C-triolein (2.5 Ci/mL of glycerol) and phosphatidylcholine (0.66 mg/mL of glycerol); stabilized with glycerol and prepared under sonication. This substrate was prepared at least, 24 h before the enzymatic assay was carried out, because it is stable at room temperature protected from the light for 3 months.

The reaction mixture contained one part of buffer made with 0.2M Tris pH 8.2, 6% fatty acid free bovine serum albumin and 0.15M sodium chloride, all adjusted to pH 8.2 with 1N HCl. Additionally, the mixture contained another part of the substrate and half part of pre-heated rat plasma (10 min at 60ºC) in order to add Apoprotein C-II that function as an enzymatic cofactor. Since LPL is inhibited by 1M NaCl and protamine sulphate, the assay was carried out in the presence and absence of 1M NaCl to test its specificity and inhibition.

The enzymatic activity is based on the following reaction:

$$TG(^{14}C - Trioleate\ 1) \rightarrow G\text{lycerol} + FreeFattyAcids(^{14}C - Oleate 1)$$

The free fatty acids released (^{14}C-Oleate 1) were separated by liquid-liquid partition system. To stop the reaction 3.5 mL of a chloroform:heptane:methanol

(1.25:1:1.41) (v:v:v) were added. After mixing, 1 mL of an alkaline buffer was added to facilitate the extraction of marked free fatty acids (borate buffer/0.1M K_2CO_3 pH 10.5). The radioactive counting was performed in 1mL of the supernatant.

To show the enzymatic activity as function of the amount of protein in the tissue (specific activity), we quantified the amount of protein in the adipose tissue homogenates prepared for LPL analysis by the Lowry method (232) as described in section 6.

8. Statistical analysis

In this study, the data are quoted as mean ± standard error of the mean.

The statistical analysis was conducted using the GraphPad Prism software version 6.00 to Windows (GraphPad Software, San Diego, CA, USA) or IBM SPSS Statistics software version 20.0 to Windows (IBM Corporation, Armonk, NY, USA).

Student's t-distribution was applied to compare two different experimental groups of animals. To determine the existence of significant differences between a higher number of groups, a one-way or two-way analysis of variance (ANOVA) was used, followed by a post hoc test (Newman-Keuls or Bonferroni, respectively). Differences were considered as statistically significant if *p*-value was less than 0.05. The measure of the association between two variables was conducted using Pearson correlation coefficient test. Some variables were expressed as their natural logarithms before they were analysed, and these changes are indicated in the figures and tables captions.

RESULTS AND DISCUSSION

1. Experiment I: Gender and long-term effects of cafeteria diet

The experimental design is detailed above in section 1.2.1. Summarizing, female and male Sprague Dawley rats were fed with a cafeteria diet (CD group), rich in fat and simple carbohydrates, for 90 days. Other female and male rats were fed continuously with the standard diet (SD group) and studied in parallel. Half of the animals were sacrificed at day 90 to have their tissues collected and the second half from the CD group started to receive the same diet as the SD group for 110 days more, making a total of 200 days of experiment.

1.1 Lipid composition of the experimental diets.

The figure 17 shows that there was a big difference in the amount of total fat present in the diets, because whereas the CD had 23% of fat the SD contained only 5% of fat. Furthermore, the composition of the diets was very different, the SD contained only standard pellets whereas the CD was made up of different

ingredients (table 1). Therefore, we analysed the fatty acid profile of both diets and they are shown in figure 18. The cafeteria diet showed higher amounts of both saturated and monounsaturated fatty acids, specifically the CD showed a higher concentration of PA (palmitic acid, 16:0), POA (palmitoleic acid, 16:1n-7), SA (stearic acid, 18:0) and OA (oleic acid 18:1n-9) when compared to the standard diet. Nevertheless, there were no differences in the concentration of any of the n-6 and n-3 polyunsaturated fatty acids, whose values were in similar amounts in the standard diet and in the cafeteria diet.

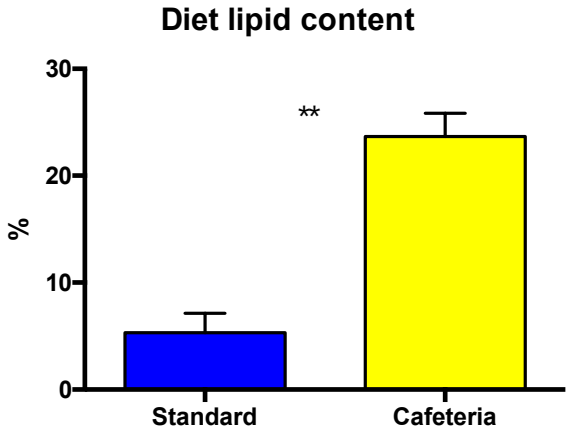

Figure 17: Total lipid content of the diets

Asterisks indicate statistically significant differences between the groups (** = $p < 0.01$).

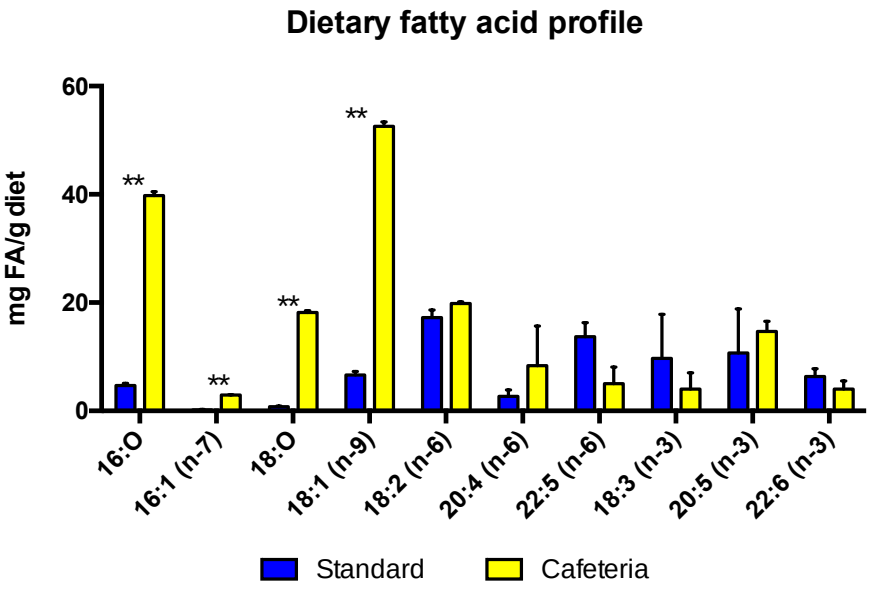

Figure 18: Fatty acid profile of the experimental diets

Asterisks indicate statistically significant differences between the groups (** = $p < 0.01$).

1.2 Food intake and body and tissue weights

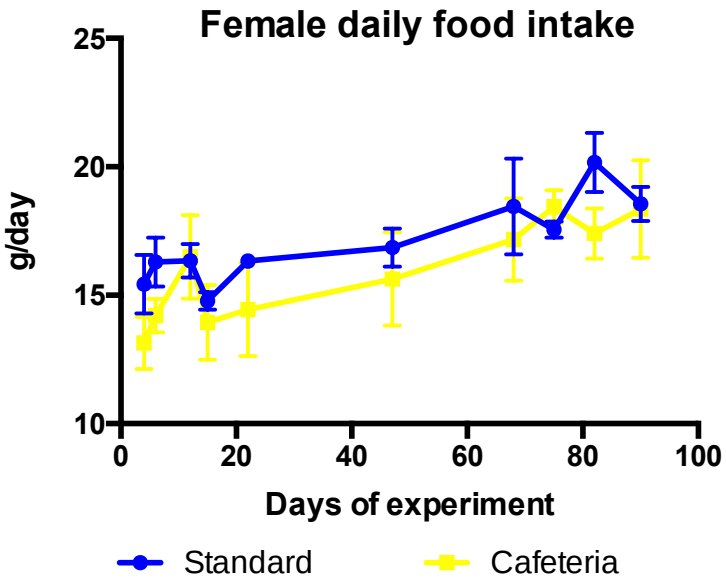

Figure 19: Effects of cafeteria and standard diets in daily food intake of female rats for 90 days.

No statistical difference between the groups at any of the days studied.

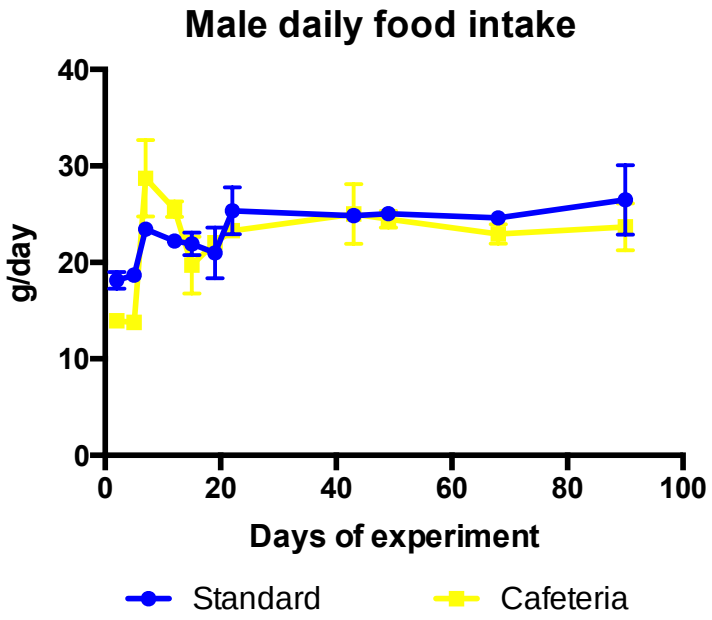

Figure 20: Effects of cafeteria and standard diets in daily food intake of male rats for 90 days.

No statistical difference between the groups at any of the days studied.

Although there was no difference in daily food intake between the SD and CD groups in female and male rats (figures 19 and 20), the energy intake was higher in the CD groups than in the SD groups in both female and male rats as shown in figure 21 and 22. In spite of the difference in the energy intake the body weight (figures 23 and 24) did not show differences due to the dietary treatment in female and male rats. Nevertheless, at 90-day, the weights of the different adipose tissues were higher in the CD than in the SD females as shown in figures 25-29. However, these differences were not observed in males (figures 25-29). Moreover, at 200 days of experiment, with the only exception of inguinal adipose tissue weight in females that remained higher in the CD group than in the SD, all the differences in adipose tissue weights between the two dietary groups in female disappeared. Figure 30 shows liver weight of females and males from SD and CD groups at 90 and 200 days of experiment. Likewise most adipose tissues

weights, CD females liver weight was higher than SD at 90 days but no difference was seen between males from SD and CD. At 200 days those differences disappeared.

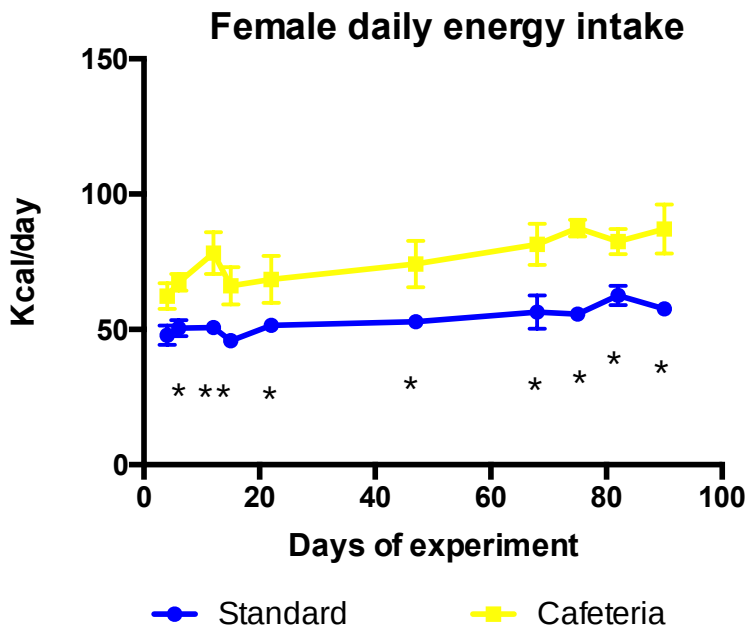

Figure 21: Effects of cafeteria and standard diets on female rats daily energy intake for 90 days

Asterisks indicate statistically significant differences between the groups (* =$p < 0.05$).

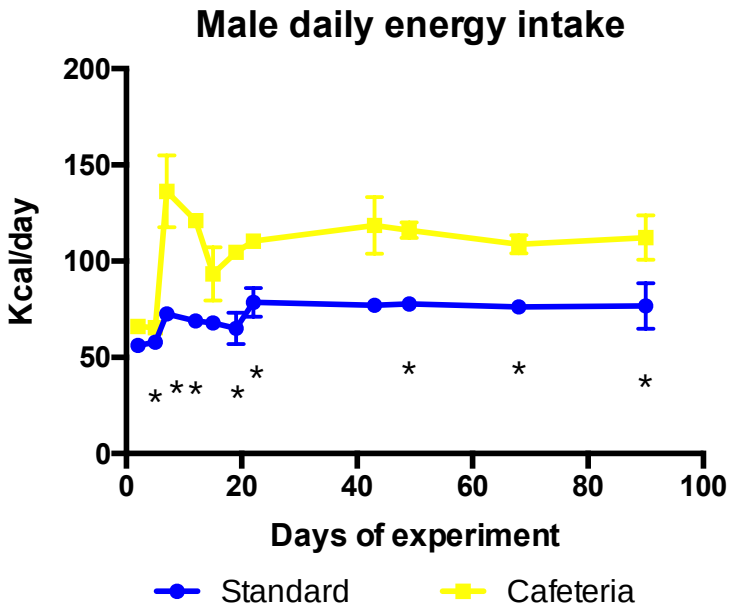

Figure 22: Effects of cafeteria and standard diets on male rats daily energy intake for 90 days

Asterisks indicate statistically significant differences between the groups (* =p < 0.05).

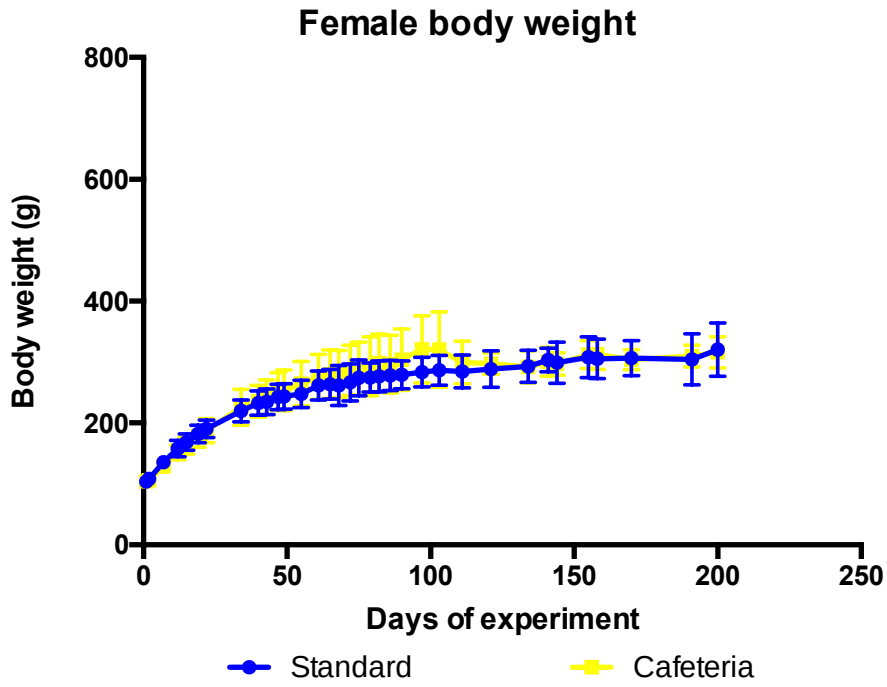

Figure 23: Effects of cafeteria and standard diets on female rats body weight along the 200 days of experiment

No statistical difference between the groups at any of the days studied.

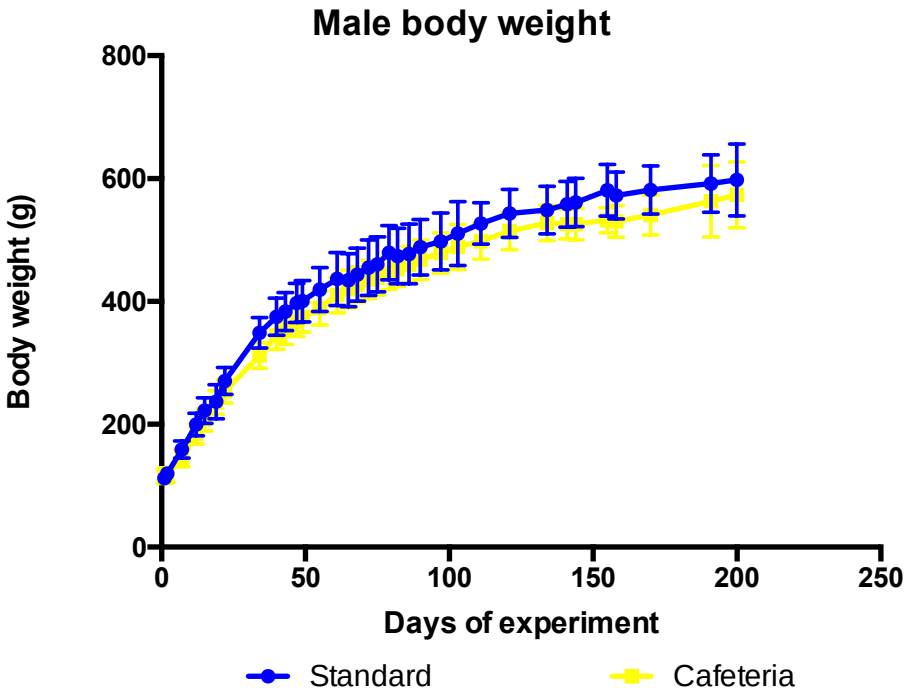

Figure 24: Effects of cafeteria and standard diets on male rats body weight along the 200 days of the experiment

No statistical difference between the groups at any of the days studied.

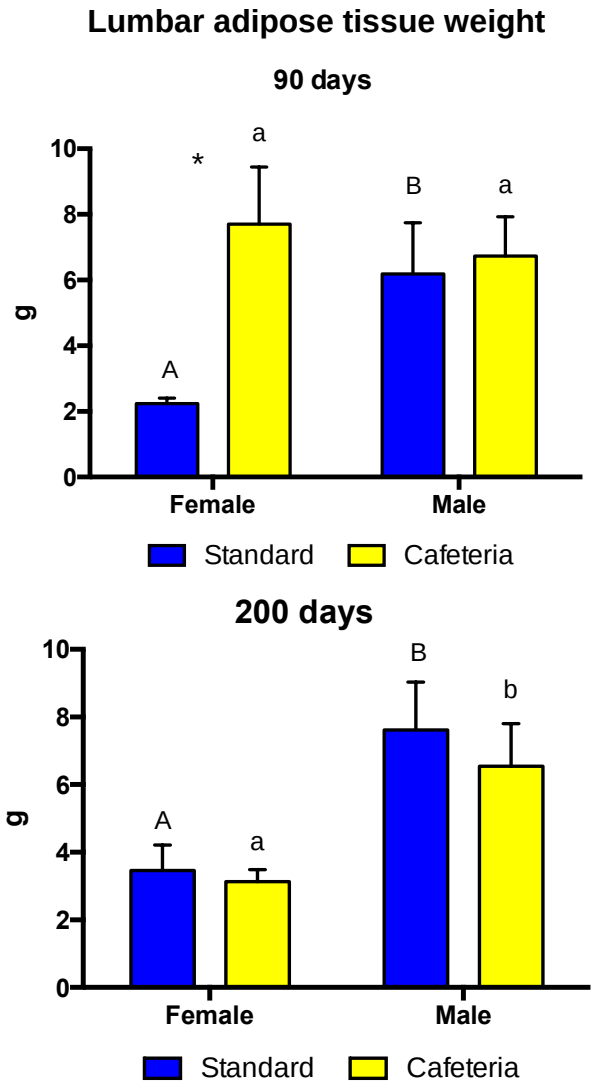

Figure 25: Effects of cafeteria and standard diets on female and male rats' lumbar adipose tissue weight at 90 days (left) and 200 days (right) of experiment

Asterisks indicate statistically significant differences between the cafeteria and standard groups (* =p < 0.05). Different uppercase letters indicate statistical difference between female and male standard groups and different lowercase letters indicate statistical difference between female and male cafeteria groups.

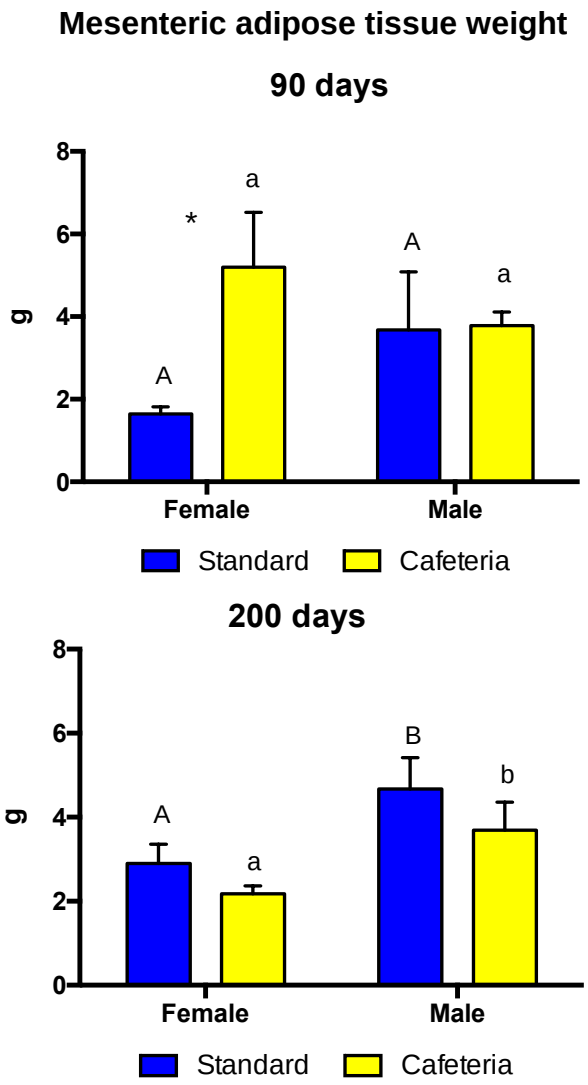

Figure 26: Effects of cafeteria and standard diets on female and male rats' mesenteric adipose tissue weight at 90 days (left) and 200 days (right) of experiment

Asterisks indicate statistically significant differences between the cafeteria and standard groups (* =p < 0.05). Different uppercase letters indicate statistical difference between female and male standard groups and different lowercase letters indicate statistical difference between female and male cafeteria groups.

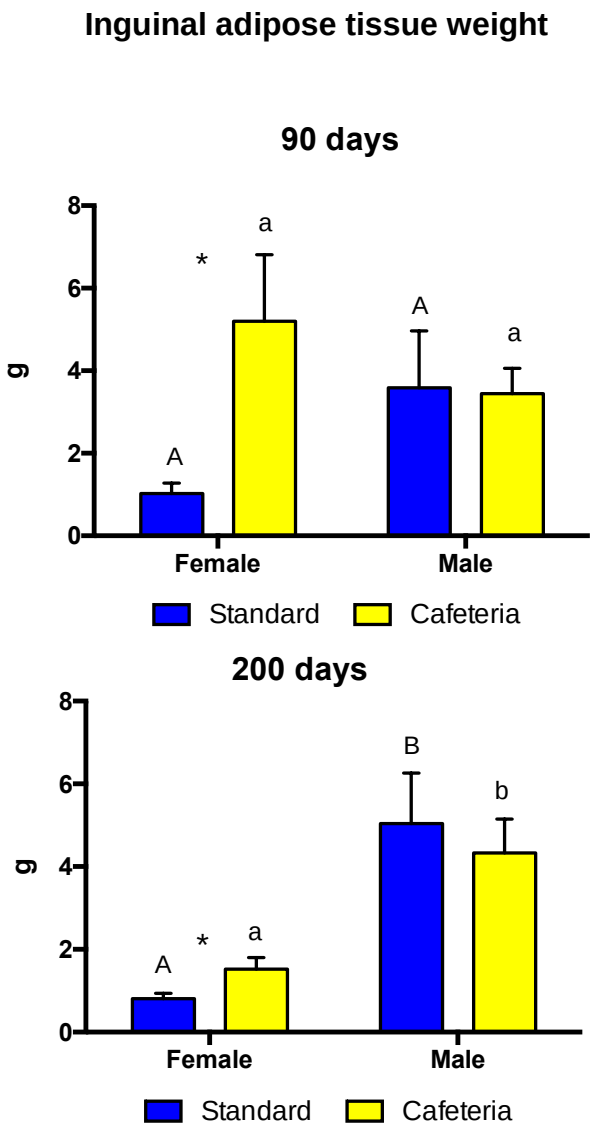

Figure 27: Effects of cafeteria and standard diets on female and male rats' inguinal adipose tissue weight at 90 days (left) and 200 days (right) of experiment

Asterisks indicate statistically significant differences between the cafeteria and standard groups (* =p < 0.05). Different uppercase letters indicate statistical difference between female and male standard groups and different lowercase letters indicate statistical difference between female and male cafeteria groups.

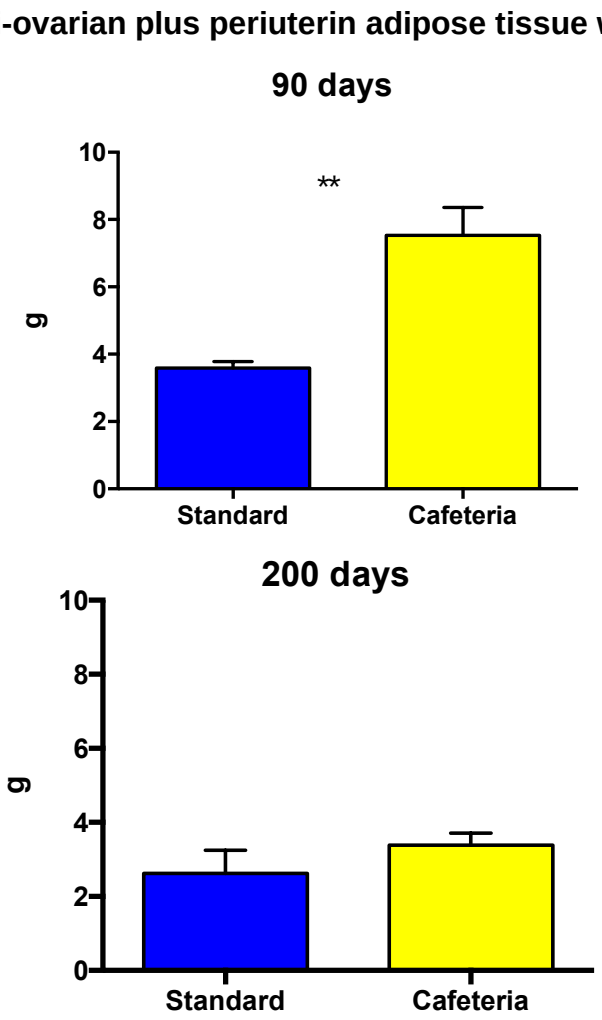

Figure 28: Effects of cafeteria and standard diets on female rats' peri-ovarian plus periuterin adipose tissue weight at 90 days (left) and 200 days (right) of experiment

Asterisks indicate statistically significant differences between the cafeteria and standard groups (** = $p < 0.01$).

Epididymal adipose tissue weight

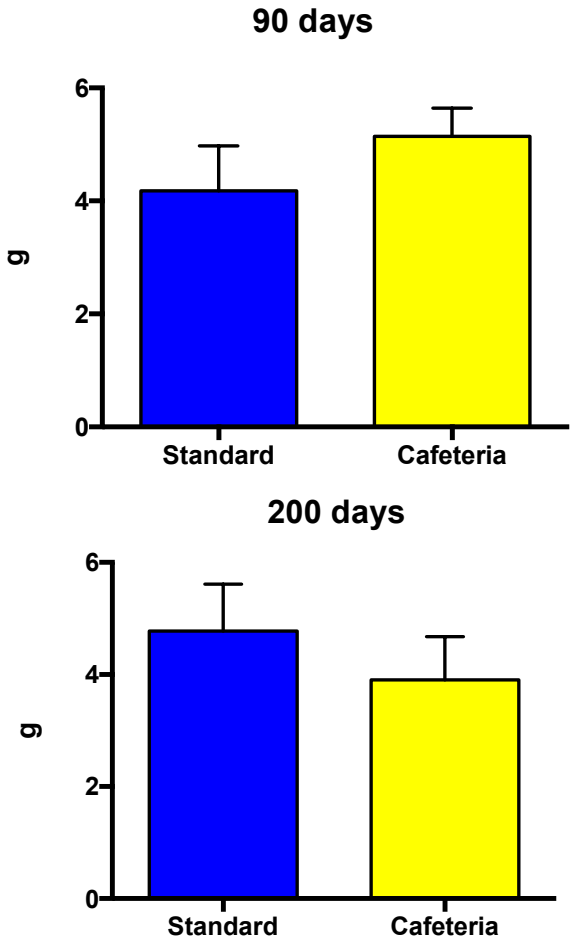

Figure 29: Effects of cafeteria and standard diets on male rats epididymal adipose tissue weight at 90 days (left) and 200 days (right) of experiment

No statistical difference between the groups at any of the days studied.

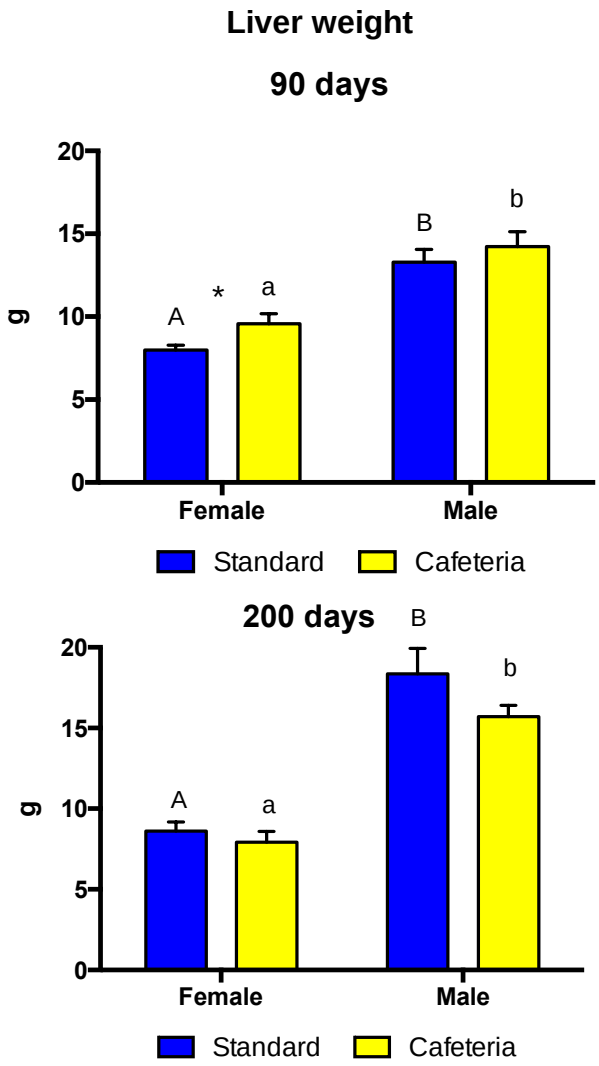

Figure 30: Effects of cafeteria and standard diets on female and male rats' liver weight at 90 days (left) and 200 days (right) of experiment

Asterisks indicate statistically significant differences between the cafeteria and standard groups (* =p < 0.05). Different uppercase letters indicate statistical difference between female and male standard groups and different lowercase letters indicate statistical difference between female and male cafeteria groups.

From these results it clearly seems that the response to the cafeteria diet in white adipose tissue and liver weights is much higher in females than in male rats. The different gender response of adipose tissue weight to the CD may be

due to the known greater reduction in adipose tissue of the stimulatory effect of adrenoceptors on the lipolytic activity in female rats fed a cafeteria diet compared to male rats (66). It is known that the lipolysis regulation in white adipose tissues through adrenergic signal transduction is a target for sex hormones (233). As reviewed by Lafontan and Berlan (234), the control of fat cell function by physiological lipolytic stimulants such as catecholamines involves different adrenergic receptors. There are three β-adrenoceptors (β_1, β_2 and β_3) that activate lipolytic activity that coexist in the same fat cell with one α_2-adrenoceptor that inhibit the lipolytic pathway. The functional importance of these receptors varies according to the species, the sex and the nature of the fat depots, including the response to the cafeteria diet (234). Although gender effects are not limited to the adrenergic controlled lipolytic cascade, sex hormones influence adipose tissue metabolic function through the adrenergic-controlled lipolytic cascade, as found in adult male rats after castration and testosterone treatment (142). Furthermore, it has been shown that the α_2-adrenoceptor is increased in fat-cell hypertrophy and that the α_2/ β_3 adrenoceptor balance is correlated with the obese state (234-236). Moreover, the protein levels of α_{2A}-adrenoceptor have been shown to be up-regulated in rats fed cafeteria diet for just 15 days, the effect being greater in male than in female rats, whereas those of β_3 adrenoceptor were down-regulated by cafeteria diet more in females (237). These findings agree with the lower and the smaller increase in the weight of retroperitoneal adipose tissue found in male than in female rats in response to the cafeteria diet feeding (237). In fact, these results are in accordance with the reported greater body-weight gain in high fat diet-induced obese female α_2-transgenic mice in the absence of β_3 adrenergic than in their male counterparts (238). Therefore, our findings on the changes found in white adipose tissue depot weights in female versus male rats fed the cafeteria diet are in agreement with these different gender adipose tissue responses to the hormonal control of the lipolytic cascade.

In addition, Lladó et al. studied the short-term effects of a cafeteria diet (i.e. 15 days in adult rats) on adrenoceptors expression and lipolytic activity in white adipose tissues in female and male rats and found an overall lower lipolytic activity in female than in male rats which did not show relation to a different energy intake between both sex groups (66). In our study, the cafeteria diet was offered for 90 days to the rats, when adipose tissue samples were collected and weighed. Thus, besides other factors that will be discussed below in this thesis, the augmented white adipose tissue weights only in females but not in males fed with the cafeteria diet compared to animals fed with a control diet could be related to a lower stimulatory adrenoceptors effect on lipolytic activity in the tissue of the formers. However, direct studies are needed to determine the comparative long-term effects of a cafeteria diet in female and male rats in white adipose tissue lipolytic activity.

In our study, from day 90 of being fed either a standard or a cafeteria diet, all the groups were fed with a standard diet until day 200 of experiment. At that moment, the adipose tissues and livers were collected and weighed. Also as shown in the figures 25 – 29, female and male tissue weights did not show differences between cafeteria and standard groups. Therefore, the lower energy content of the standard diet compared to the cafeteria diet (table 1) lead to a reduction of energy intake in animals previously fed cafeteria diet that resulted in lumbar, mesenteric and peri-ovarian plus periuterin fat depots and liver weight similar in CD than in SD female rats.

Even though only studied in male rats, Reynés et al. (239) determined the effects of a cafeteria diet in male rats fed for 60 days followed by subsequent replacement of the cafeteria diet to a control diet. The authors found a rapid increase of body weight caused by the ingestion of a cafeteria diet and after the replacement of it by a control diet they found a reduction in body weight although it did not reach the body weight of the control group. Furthermore, the size of different adipose tissues (excluding retroperitoneal) were completely recovered.

In our study, the higher adipose tissue weights found in female rats after 90 days on cafeteria diet completely disappeared after 110 days of feeding the control diet, which agrees with those reported findings in male rats (239). In conclusion, as it was previously reported in male rats, the increased white adipose tissue weight found in adult female rats on a cafeteria diet can be also reversed by feeding *ad libitum* a balanced control diet.

1.3 Plasma and liver lipid components

As shown in figure 31, at day 90 of experiment, no difference was found in plasma cholesterol concentrations in female rats between the two dietary groups. However, male rats receiving the cafeteria diet showed lower plasma cholesterol levels than those on standard diet (figure 31) whereas the reduction disappeared after 110 days of being fed with a standard diet (i.e. 200 days of experiment). Such reduction of plasma cholesterol in males fed the CD is in contrast to previous reports by other authors where female and male rats fed the cafeteria diet showed an increase in plasma cholesterol (240, 241). Therefore, we thought that our findings in males fed CD could be the result of a retention of cholesterol in the liver. In fact, as shown in figure 36 we found that liver cholesterol concentration was higher in male rats fed the CD for 90 days whereas no difference was seeing in females (figure 36). Since cholesterol is carried in plasma associated to lipoproteins but plasma very low density lipoproteins (VLDL), which are secreted by the liver, are specifically rich in triacylglycerols (242) we also measured plasma triacylglycerol values.

As shown in figure 32 at day 90 of treatment, plasma triacylglycerol levels did not differ between the two dietary groups in females, but they were higher in males fed the CD than in their controls whereas this difference disappeared at 200 days of experiment. In order of knowing if these changes in plasma values could be also related to liver triacylglycerol concentrations, they were also

measured in liver. As shown in figure 35 there were no differences between the two dietary groups in females, but in males at day 90 liver triacylglycerols were higher in those on CD than in their controls, the difference disappearing at 200 days.

These findings could be related to those of de Melo et al. (243) that studied male adult rats fed a cafeteria diet for 21 days and found a higher concentration of liver triacylglycerols that was associated to an increased liver VLDL secretion. On the other hand, the accumulation of lipids in liver in spite of an enhanced VLDL secretion could be the result of a reported increase in liver lipogenesis (243) in the presence of an enhanced arrival to the liver of plasma non-esterified fatty acids (NEFA). This latter possibility is supported by our results showing an increased plasma level of NEFA in males fed with the cafeteria diet compared to their controls without differences in females and this effect was maintained even 110 days after shifting the animals to a standard diet (figure 33).

This finding in males could be the result of an enhanced adipose tissue lipolytic activity in these animals which would also fit with the lack of change found in adipose tissue weight in male rats fed the CD in contrast to the increase found in female rats (figures 25-29). An enhanced lipolytic activity in adipose tissue of male rats is compatible with unchanged plasma levels of glycerol (figure 34), since besides its efficient utilization as gluconeogenic substrate in the liver (244), its plasma levels can be also influenced by its intracellular reutilization in adipose tissue, as will be discussed below.

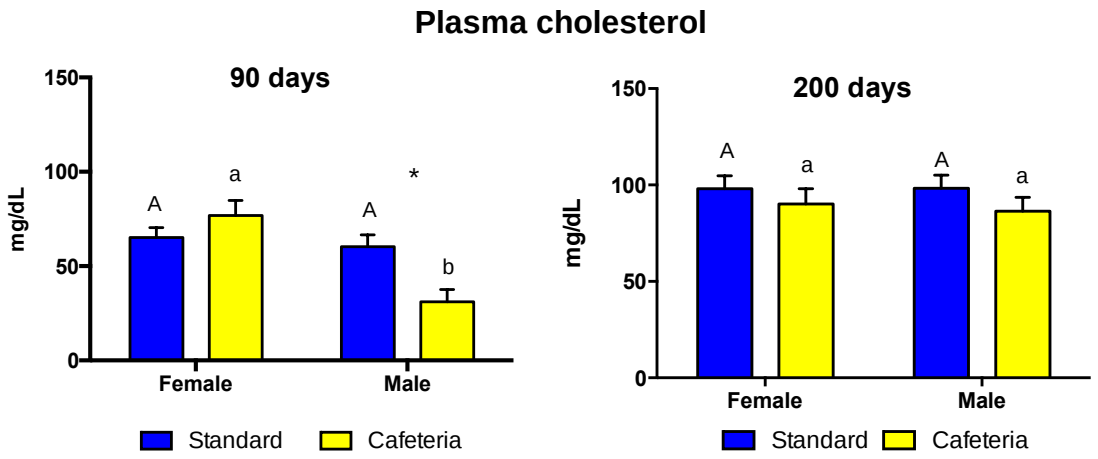

Figure 31: Effects of cafeteria and standard diets on plasma cholesterol of female and male rats at 90 days (left) and 200 days (right) of experiment

Asterisks indicate statistically significant differences between the groups (* =p < 0.05, ** = p < 0.01). Different uppercase letters indicate statistical difference between female and male standard groups and different lowercase letters indicate statistical difference between female and male cafeteria groups.

Furthermore, the liver accumulation of both cholesterol and triacylglycerols in male rats fed the CD could be related to the development of the non-alcoholic fatty liver disease (NAFLD) (245). The prevalence of this disease in humans has been shown to increase among overweight and non-diabetic obese individuals (246, 247) and is the leading cause of chronic liver disease (248). In fact, the prevalence of NAFLD has been described to be more frequently detected in men than in women (249, 250), although the opposite has been shown in post-menopausal women (251).

Moreover, at 200 days of experiment, no differences were found in liver triacylglycerols and cholesterol concentrations (figures 35 and 36). Therefore, a shift from cafeteria diet to standard diet was able to restore the effects of the cafeteria diet in liver triacylglycerols and cholesterol accumulation (figures 35 and 36).

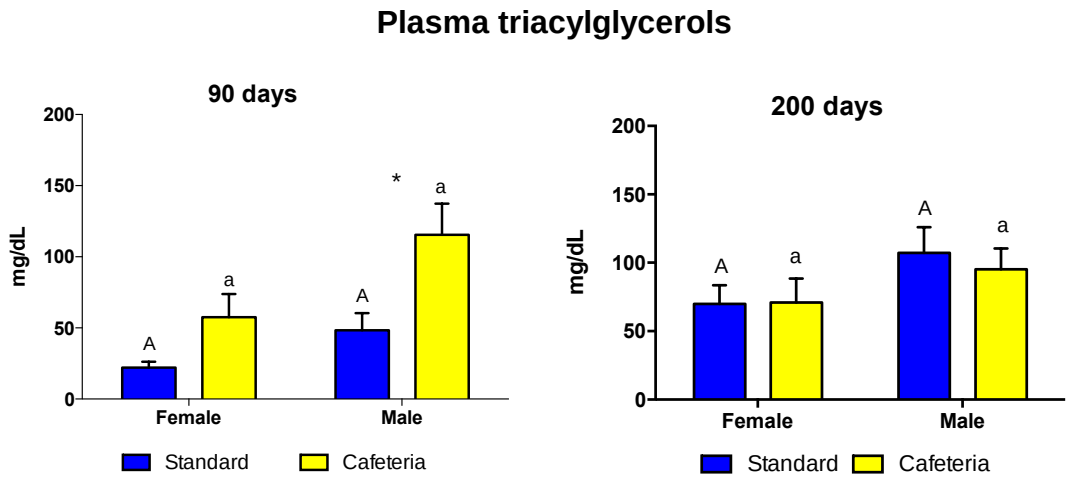

Figure 32: Effects of cafeteria and standard diets on female and male rats on plasma triacylglycerols at 90 days (left) and 200 days (right) of experiment

Asterisks indicate statistically significant differences between the cafeteria and standard groups (* =p < 0.05). Different uppercase letters indicate statistical difference between female and male standard groups and different lowercase letters indicate statistical difference between female and male cafeteria groups.

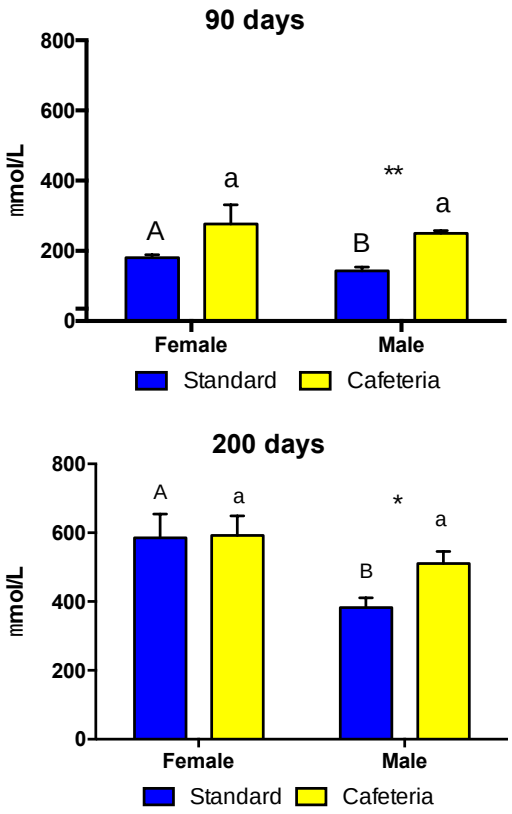

Figure 33: Effects of cafeteria and standard diets on female and male plasma non-esterified fatty acids at 90 days (left) and 200 days (right) of experiment.

Asterisks indicate statistically significant differences between the cafeteria and standard groups (* =p < 0.05, **=p < 0.01)). Different uppercase letters indicate statistical difference between female and male standard groups and different lowercase letters indicate statistical difference between female and male cafeteria groups.

Plasma glycerol

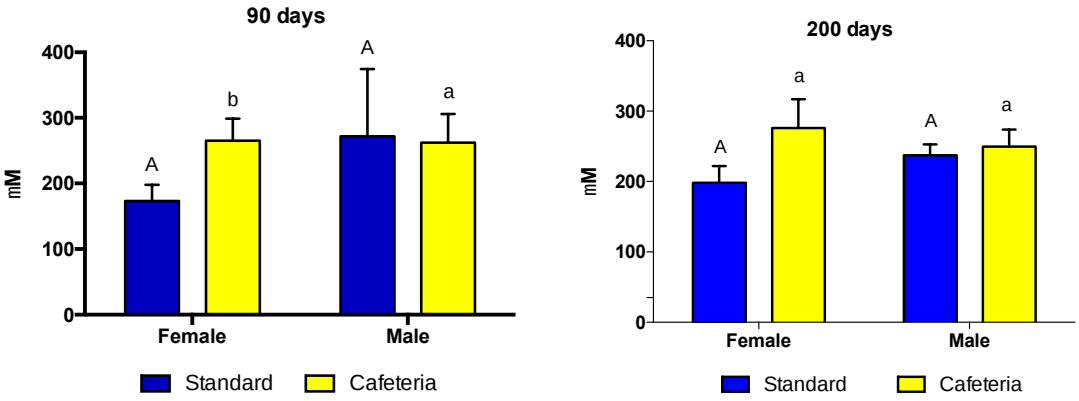

Figure 34: Effects of cafeteria and standard diets on female and male rats on plasma glycerol at 90 days (left) and 200 days (right) of experiment.

No statistical differences were found in rats on cafeteria diet versus standard diet. Different uppercase letters indicate statistical difference between female and male standard groups and different lowercase letters indicate statistical difference between female and male cafeteria groups.

Liver triacylglycerols

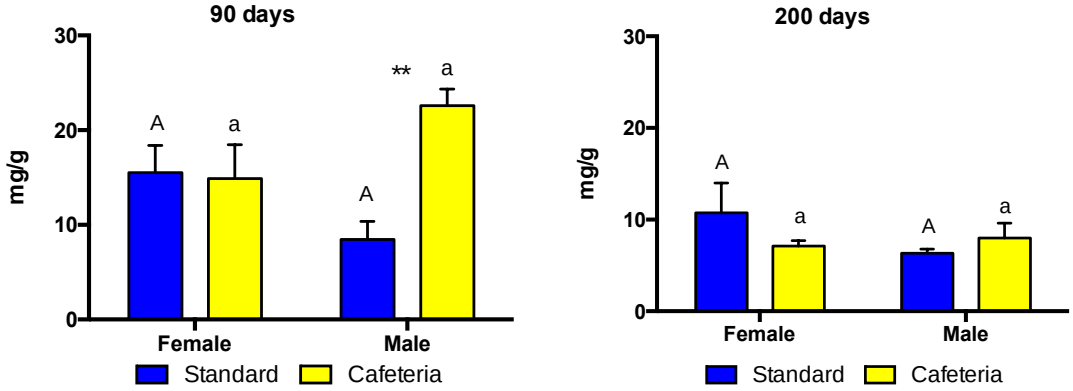

Figure 35: Effects of cafeteria and standard diets on female and male rats on liver triacylglycerols at 90 days (left) and 200 days (right) of experiment

Asterisks indicate statistically significant differences between the cafeteria and standard groups (** = $p < 0.01$). Different uppercase letters indicate statistical difference between female and male standard groups and different lowercase letters indicate statistical difference between female and male cafeteria groups.

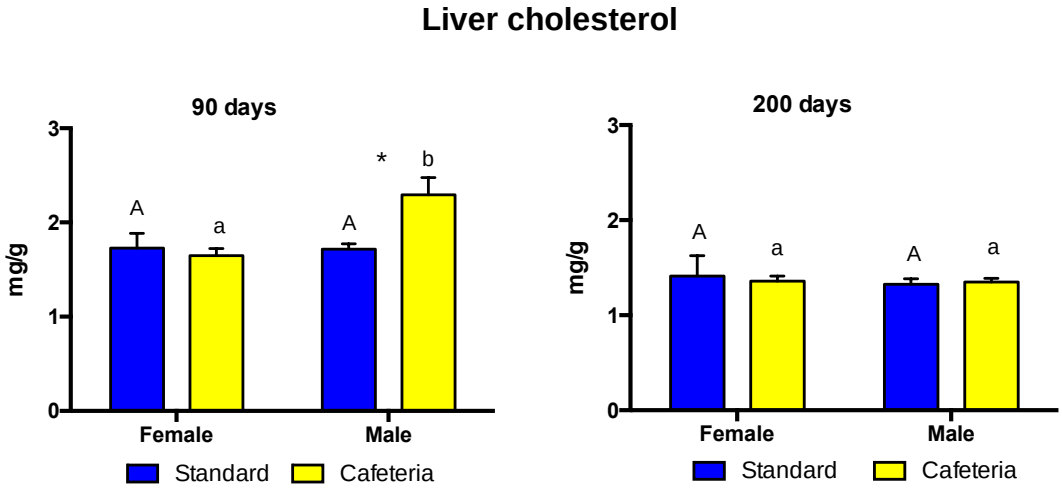

Figure 1: Effects of cafeteria and standard diets on female and male rats on liver cholesterol at 90 days (left) and 200 days (right) of experiment.

Asterisks indicate statistically significant differences between the cafeteria and standard groups (* =p < 0.05). Different uppercase letters indicate statistical difference between female and male standard groups and different lowercase letters indicate statistical difference between female and male cafeteria groups.

1.4 Determination of the activity of adipose tissue glycerol kinase

For decades the glycerol kinase activity in adipose tissue was not considered relevant due to its low activity (124, 125, 252). However previous studies in our laboratory have demonstrated that the utilization of glycerol by rat adipose tissue is higher than thought (126-128, 253, 254). Furthermore, Stern et al. (255) found in genetically obese rats higher glycerol kinase activity in adipose tissue when compared to lean rats. In this study, the effects of high carbohydrate and high fat on adipose tissue cell size and glycerol kinase activity were analysed. The authors found an elevated glycerol kinase activity in animals fed a high fat diet than in rats fed a high carbohydrate diet in females and males. Moreover, an overall significant correlation was found between glycerol kinase activity and adipose tissue cell size which was independent of body weight (255).

Therefore, in the present study, the glycerol kinase activity was measured in order to understand the mechanisms by which the cafeteria diet increases adipose tissue weight in rats in a gender dependent manner. Thus, the glycerol kinase activity of inguinal, lumbar, mesenteric and peri-renal tissues collected at 90 days were analysed (figures 37 – 40). Figures 37, 39 and 40 show that glycerol kinase activity was statistically higher in inguinal, mesenteric and peri-renal adipose tissues of CD female rats when compared with SD females. In males, although in inguinal and lumbar fat pads glycerol kinase activity tended to be higher in CD than in SD, the difference did not reach statistical difference (figures 37 – 40).

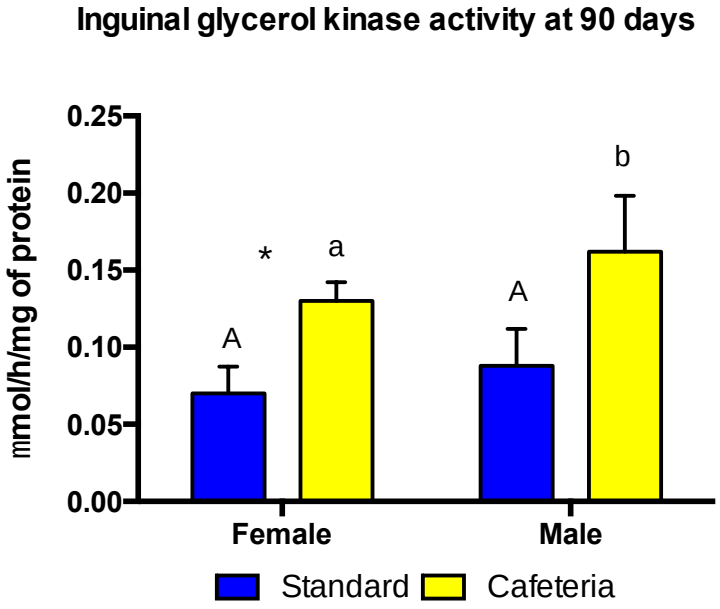

Figure 37: Effects of cafeteria and standard diets on inguinal adipose tissue glycerol kinase activity (GK) in female and male rats at 90 days of experiment.

Asterisks indicate statistically significant differences between the cafeteria and standard groups (* =p < 0.05). Different uppercase letters indicate statistical difference between female and male standard groups and different lowercase letters indicate statistical difference between female and male cafeteria groups.

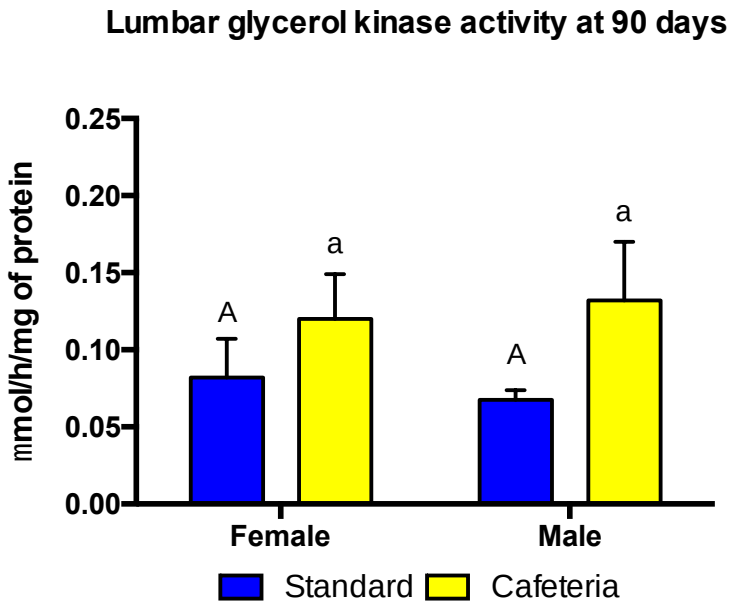

Figure 38: Effects of cafeteria and standard diets on lumbar adipose tissue glycerol kinase activity (GK) in female and male rats at 90 days of experiment.

No statistically differences were found between values of Standard vs. Cafeteria diet in either female or male groups No statistical differences were found between Male vs. Female rats on any of the dietary treatment.

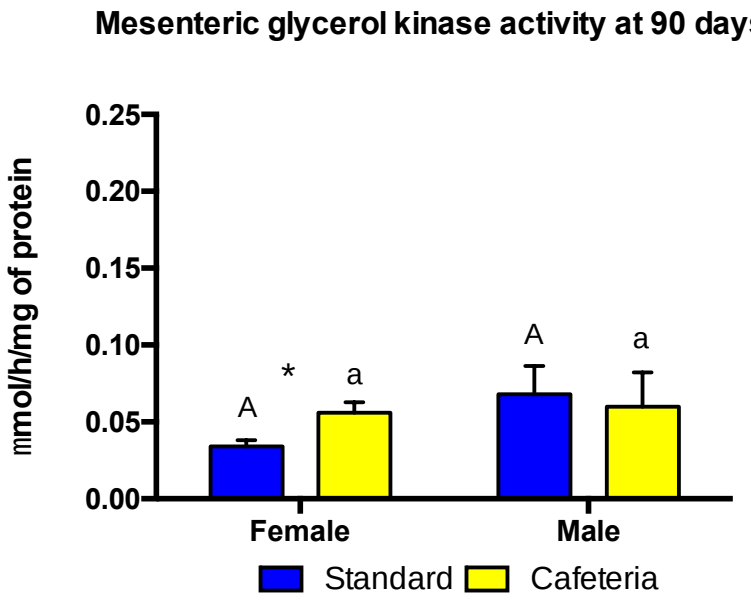

Figure 39: Effects of cafeteria and standard diets on mesenteric adipose tissue glycerol kinase activity (GK) in female and male rats at 90 days of experiment.

Asterisks indicate statistically significant differences between the cafeteria and standard groups (* =p < 0.05). No statistical differences were found between Male vs. Female rats on any of the dietary treatment.

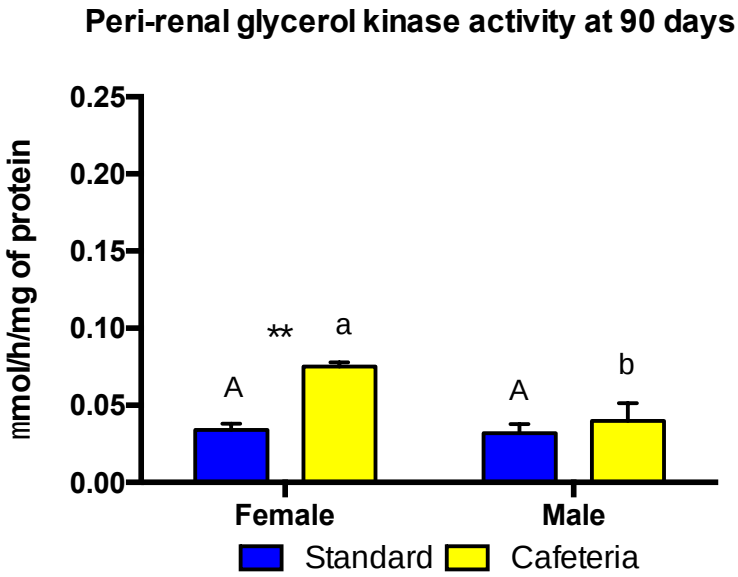

Figure 40: Effects of cafeteria and standard diets on peri-renal adipose tissue glycerol kinase activity (GK) in female and male rats at 90 days of experiment.

Asterisks indicate statistically significant differences between the cafeteria and standard groups (** = $p < 0.01$). Different uppercase letters indicate statistical difference between female and male standard groups and different lowercase letters indicate statistical difference between female and male cafeteria groups.

An elevated glycerol kinase activity was found in the CD female rats in which most of the adipose tissue weights were also increased as shown above in figures 25 – 29. Therefore, we may suggest that in female rats fed with cafeteria diet, the activity of glycerol kinase in adipose tissues produced an increase in the intracellular glycerol utilization for TAG synthesis that would facilitate the increased adipose tissue weight. Moreover, no significant differences were found in plasma non-esterified fatty acids and glycerol between SD and CD female rats at 90 days (figures 33 and 34). This lack of change in plasma glycerol found in these animals could be explained by the increase in the glycerol utilization by the glycerol kinase in the adipose tissue as it is explained above. Similarly, plasma non-esterified fatty acids did not show statistical differences between CD and SD female rats (figure 33) which could be due to their re-esterification in the adipose

tissues together with that of glycerol to form triacylglycerols in adipose tissue of the rats fed CD for 90 days.

In males fed with a cafeteria diet, neither the adipose tissue weights nor glycerol kinase activity showed statistical differences when compared to animals fed with the standard diet at 90 days as commented above (figures 25 – 29 and 37 – 40). As it has been already commented, these findings fit with a lower intracellular re-esterification activity of NEFA in CD males versus females probably as a consequence of this unchanged glycerol kinase activity.

1.5 Determination of the activity of adipose tissue lipoprotein lipase (LPL)

Lipoprotein lipase (LPL) is present in the luminal surface of the capillary endothelium of extrahepatic tissues and catalyses the hydrolysis of plasma circulating TAG associated to chylomicrons and VLDL to release fatty acids and glycerol that are taken up by the subjacent tissue (for reviews see (256, 257)). This enzyme is especially active in adipose tissue. It is considered that LPL activity plays a crucial role in the storage of plasma TAG in the adipose tissue since the products of its action, non-esterified fatty acids, can be re-esterified within the tissue as TAG for energy storage (258). In fact although glycerol-3-phosphate for the esterification of fatty acids within adipose tissue is normally synthesized from glucose as a deviation of glycolysis, we know that there are circumstances where phosphorylated glycerol may be provided from the hydrolysis of the glyceride glycerol from TAG-rich lipoproteins and taken up by rat adipose tissue (259). Therefore, for a better understanding of the effects of the cafeteria diets on adipose tissue metabolism we evaluated the LPL activity in inguinal and lumbar adipose tissues at 90 and 200 days and in mesenteric adipose tissue at 90 days of experiment. As shown in figure 41, at day 90 of treatment, a higher LPL activity was found in inguinal adipose tissue in CD female rats when compared with SD, the effect disappearing at 200 days. No difference

between the two groups was seen in males in this fat depot at 90 and 200 days of treatment nor in any of the other groups or fat depots studied in male or female rats (figures 41 – 43). It is therefore proposed that the high LPL activity found in inguinal adipose tissue of CD female rats at day 90 of treatment together with the above reported high glycerol kinase activity may contribute to the increased adipose tissue mass found in these rats.

In this regard, a high adipose tissue LPL activity has been also reported in both human and rat obese subjects (260, 261). In our study, the increased adipose tissue LPL activity in the presence of increased glycerol kinase activity in female rats fed the CD may justify not only the intracellular reutilization of glycerol producing their augmented adipose tissue weight when compared with rats fed the SD but also the lack of differences in plasma glycerol levels that were found (figure 34). This effect stimulating the tissue utilization of circulating TAG in females fed the CD may also contribute to the lack of increased plasma TAG level in these animals at day 90 of treatment whereas values in CD males were significantly augmented (see figure 32). However, more research is needed to understand the mechanism involved in these changes.

Inguinal adipose tissue LPL activity

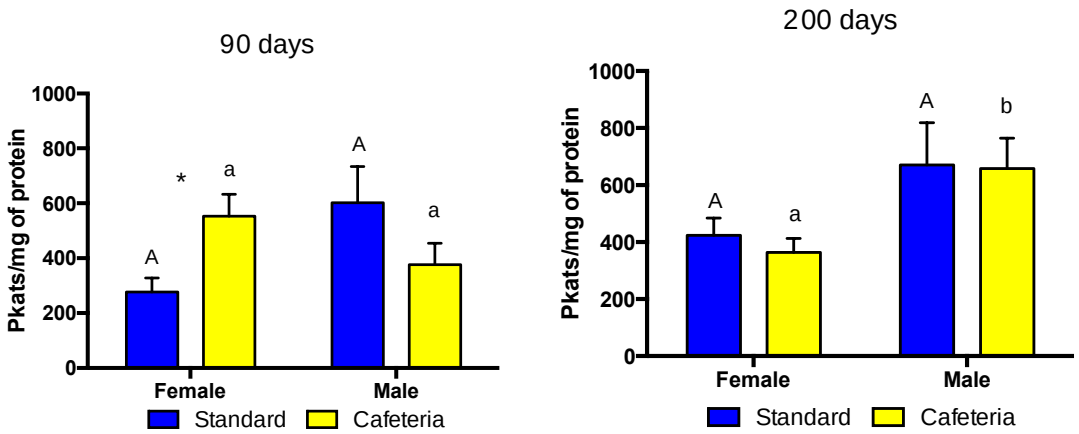

Figure 41: Effects of cafeteria and standard diets on inguinal adipose tissue lipoprotein lipase (LPL) in female and male rats at 90 days (left) and 200 days (right) of experiment.

Asterisks indicate statistically significant differences between the cafeteria and standard groups (* =p < 0.05). Different uppercase letters indicate statistical difference between female and male standard groups and different lowercase letters indicate statistical difference between female and male cafeteria groups.

Lumbar adipose tissue LPL activity

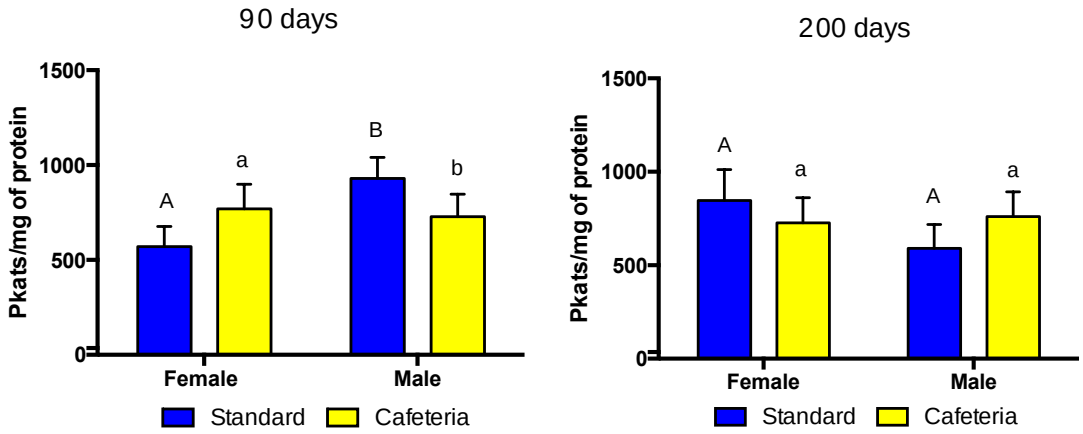

Figure 42: Effects of cafeteria and standard diets on lumbar adipose tissue lipoprotein lipase (LPL) in female and male rats at 90 days (left) and 200 days (right) of experiment.

No statistical differences were found between Cafeteria vs. Standard dietary groups. Different uppercase letters indicate statistical difference between female and male standard groups and different lowercase letters indicate statistical difference between female and male cafeteria groups.

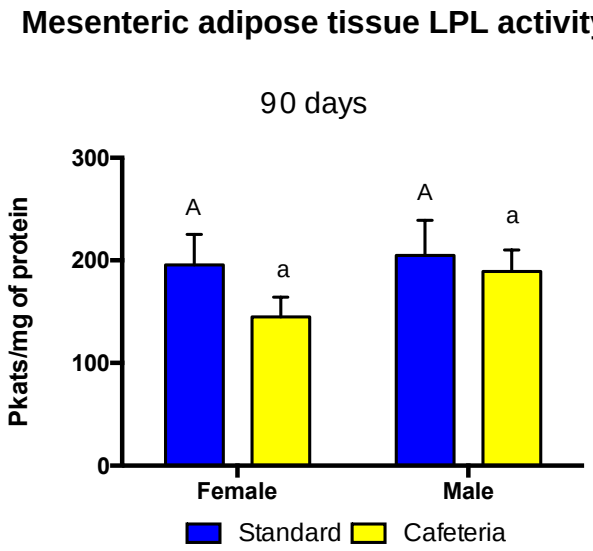

Figure 43: Effects of cafeteria and standard diets on mesenteric adipose tissue lipoprotein lipase (LPL) in female and male rats at 90 days of experiment.

No statistical differences were found between Cafeteria vs. Standard dietary groups. Different uppercase letters indicate statistical difference between female and male standard groups and different lowercase letters indicate statistical difference between female and male cafeteria groups.

1.6 Fatty acids profiles in plasma and in different tissues

In order to have a complete understanding of the effect of the cafeteria diet in the lipid metabolism of the rats we also analysed the fatty acid composition of different tissues. Regarding the composition of fatty acids in adipose tissue, it is known that most of the fatty acids stored in adipose tissue depots result from lipogenic processes that occur predominantly in liver and adipose tissue but also from fatty acids derived from the diet (262, 263). As described in section 1.2, not only the amount of fat of the cafeteria diet offered in the present study was different from the standard diet, but also the fatty acid profile was modified. As shown in figure 18 in the cafeteria diet there was a much higher amount of saturated and monounsaturated fatty acids than in the standard diet, whereas no differences were found in polyunsaturated fatty acids. Thus, we analysed the

effects of both diets in plasma, liver and lumbar adipose tissue regarding the fatty acid profiles in rats at day 90 of being fed with their corresponding diet and also in plasma and liver at day 200 of experiment, when both groups had been receiving SD for 110 days.

Plasma fatty acids profile at 90 days

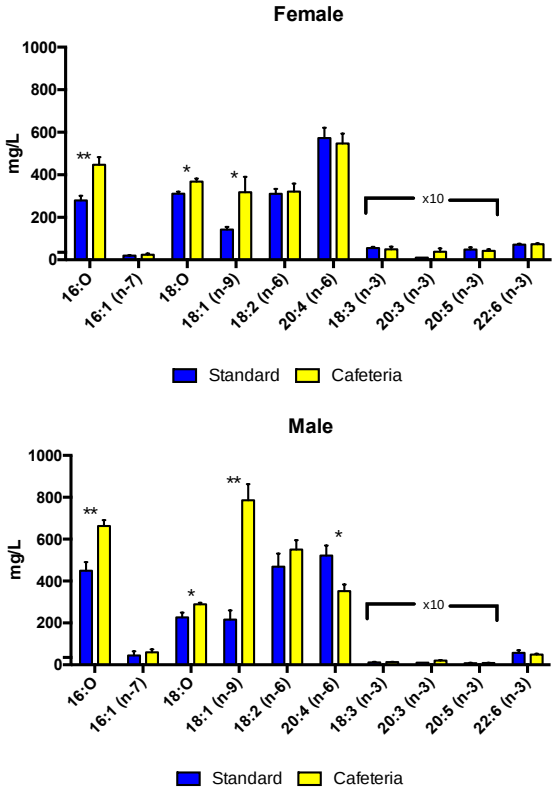

Figure 44: Effects of cafeteria and standard diets on plasma fatty acid profile in female and male rats at 90 days of experiment.

Asterisks indicate statistically significant differences between the dietary groups (* =p < 0.05, ** = p < 0.01).

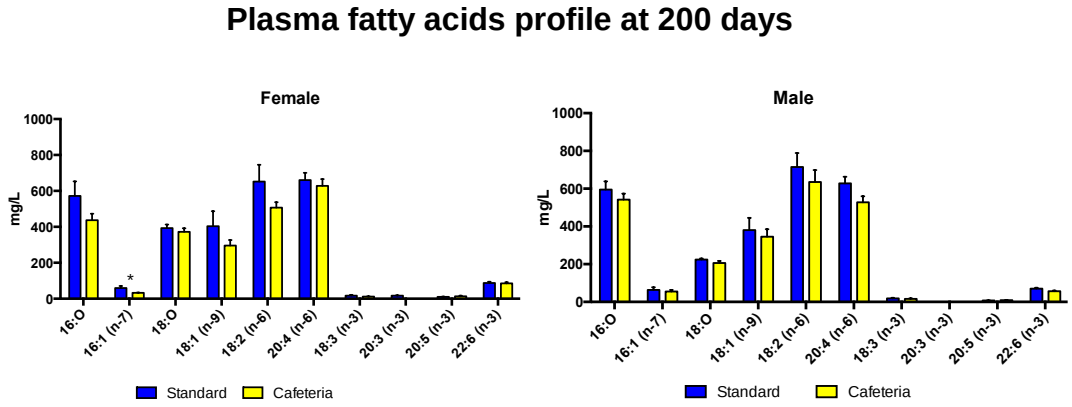

Figure 45: Effects of cafeteria and standard diets on plasma fatty acid profile in female and male rats after 110 days on standard diet (total 200 days of experiment).

Asterisks indicate statistically significant differences between the dietary groups (* =p < 0.05).

As shown in figure 44, in plasma, female and male rats fed for 90 days with CD showed a higher concentration of saturated and monounsaturated fatty acids as these were the fatty acids that were in a higher amount in the CD diet (figure 18). Specifically, the concentrations of PA (palmitic acid 16:0), SA (stearic acid 18:0) and OA (oleic acid 18:1 n-9) were higher in the group fed with CD than those fed with SD. However, male rats fed CD showed lower levels of AA (arachidonic acid 20:4 n-6) than those fed the SD, and this is a surprising result since the concentration of this fatty acid is similar and very low in both diets. Moreover, AA can be endogenously synthesized from its precursor LA (linoleic acid 18:2 n-6) and also this precursor was in a similar concentration in both diets. In relation with n-3 polyunsaturated fatty acids there were no differences in the amount of any of these fatty acids in both males and females as a consequence of the different diets.

The differences already commented above about the plasma fatty acid profile between the cafeteria and standard groups completely disappeared at day 200 of experiment, when all the rats were fed with the SD for 110 days (figure

45). In liver at day 90 of experiment (figure 46) there were less differences in the fatty acid profiles between the two dietary groups than in plasma. Only OA (oleic acid 18:1 n-9) was higher in liver of females and males from the CD group when compared to those fed with SD at 90 days. However, as shown in figure 47, in females at 200 days, lower levels of PA, POA (palmitoleic acid 16:1 n-7), OA and LA (linoleic acid 18:2 n-6) were found in CD than in SD group. In males, higher levels of PA, SA and LA were found in the CD than in SD group and no differences were found in the other fatty acids. In lumbar adipose tissue at 90 days on their respective diet (figure 48) females and males showed lower concentrations of POA, LA, AA and ALA (alpha-linolenic acid 18:3 n-3) in CD than in SD group and higher concentrations of SA and OA in CD than in SD group. No differences were found in other fatty acids.

Liver fatty acids profile at 90 days

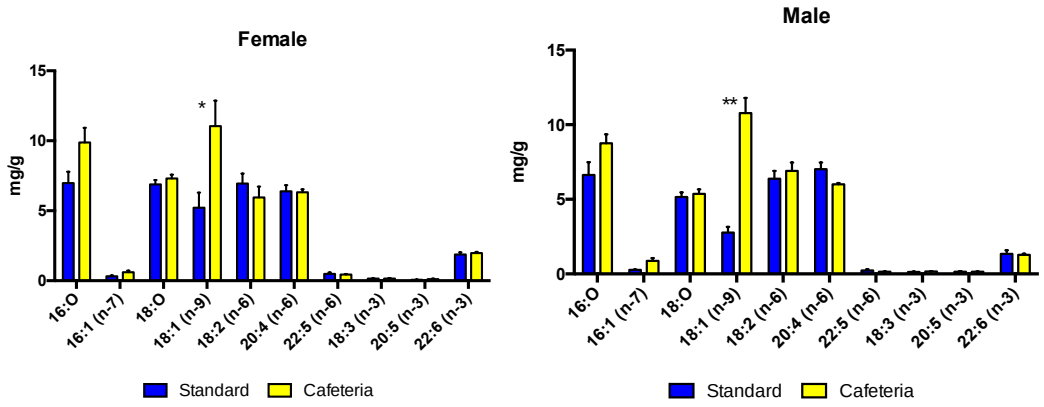

Figure 46: Effects of cafeteria and standard diets on liver fatty acids profile in female and male rats at 90 days of experiment.

Asterisks indicate statistically significant differences between the dietary groups (* =p < 0.05, ** = p < 0.01).

Liver fatty acids profile at 200 days

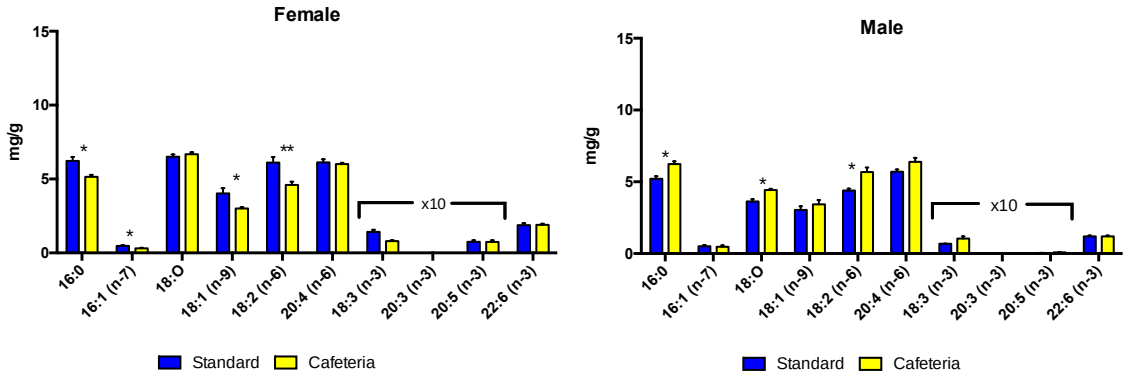

Figure 47: Effects of cafeteria and standard diets on liver fatty acids profile in female and male rats at after 110 days on standard diet (total 200 days of experiment).

Asterisks indicate statistically significant differences between the dietary groups (* =p < 0.05, ** = p < 0.01).

Lumbar adipose tissue fatty acids profile at 90 days

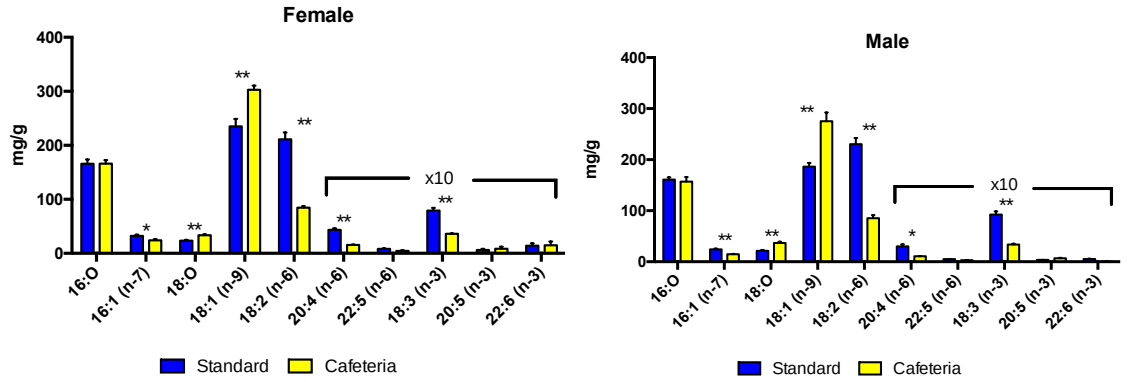

Figure 48: Effects of cafeteria and standard diets on lumbar adipose tissue fatty acids profile in female and male rats at 90 days of experiment.

Asterisks indicate statistically significant differences between the dietary groups (* =p < 0.05, ** = p < 0.01).

In order to understand better the fatty acid metabolism in the studied samples, since long chain polyunsaturated fatty acids are synthesized from their precursors we calculated the ratio of different fatty acids to have an index of the different desaturases and elongases that have a role in their conversion: 20:4 n-6/20:3 n-6 (as an index of Δ^5 desaturase), 18:3 n-6/18:2 n-6 (as an index of Δ^6 desaturase), and 20:3 n-6/18:3 n-6 (as an index of elongase 5). On the other hand, due to the fact that the Δ^9 desaturase index has been used as an indicator of lipogenesis and is the desaturase involved in the endogenous synthesis of oleic acid (18:1 n-9), we also calculated the 18:1 n-9/18:0 ratio in plasma, liver and adipose tissue of all the animals studied. Also, in relation with the synthesis of OA we calculated the 18:0/16:0 ratio as an index for elongase 6 activity in plasma, liver and adipose tissue.

In plasma, a reduced Δ^5 desaturase activity index was found in females and males fed with CD at 90 days (figure 49) with females showing higher values than males in both dietary treatments. Those differences disappeared after 110 days of receiving both groups the standard diet (figure 49). No differences were found between SD and CD in female and male rats on Δ^6 desaturase activity index at 90 days and after 110 days on SD, although reduced values were found in males compared to females fed with CD after 110 days on SD diet (figure 50). On the other hand, Δ^9 desaturase activity index was significantly higher in males from the CD group than those from the SD group, and in a similar way, this index tended to increase in females from CD in comparison to the ones in the SD group after 90 days on dietary treatment (figure 51). Besides, it showed higher values in males than in females of both dietary groups (figure 51). After 110 days more, when all the groups received the SD, both dietary groups did not differ in Δ^9 desaturase activity index within the same gender, although males showed higher values than females (figure 51).

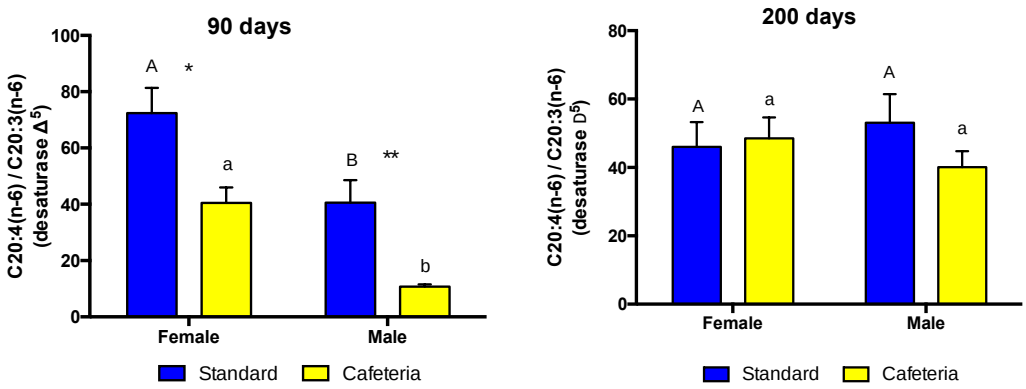

Figure 49: Effects of cafeteria and standard diets on Δ5 desaturase activity index in plasma of female and male rats at 90 days (left) and those rats followed by 110 days on just standard diet (right) (total 200 days of experiment).

Asterisks indicate statistically significant differences between the dietary groups (* =p < 0.05, ** = p < 0.01). Different uppercase letters indicate statistical difference between female and male standard groups and different lowercase letters indicate statistical difference between female and male cafeteria groups.

Plasma Δ^6 desaturase activity index

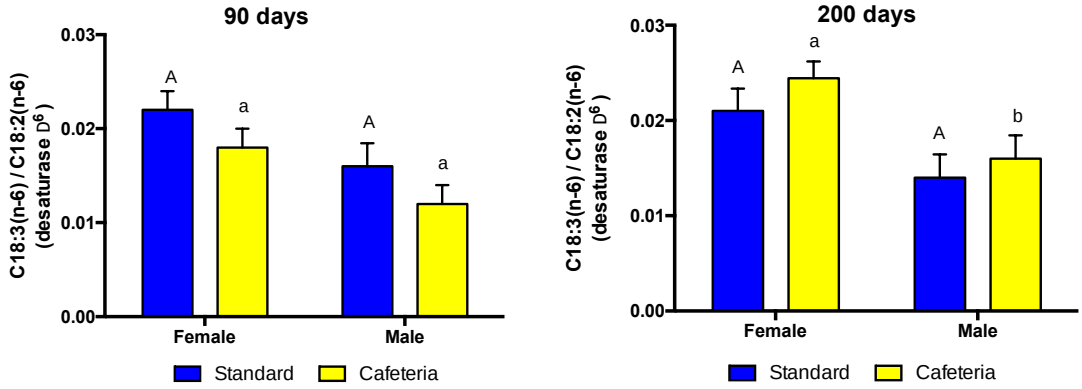

Figure 50: Effects of cafeteria and standard diets on Δ^6 desaturase activity index in plasma of female and male rats at 90 days (left) and those rats followed by 110 days on just standard diet (right) (total 200 days of experiment).

No statistical differences were found between Cafeteria vs. Standard diet groups. Different uppercase letters indicate statistical difference between female and male standard groups and different lowercase letters indicate statistical difference between female and male cafeteria groups.

Plasma Δ^9 desaturase activity index

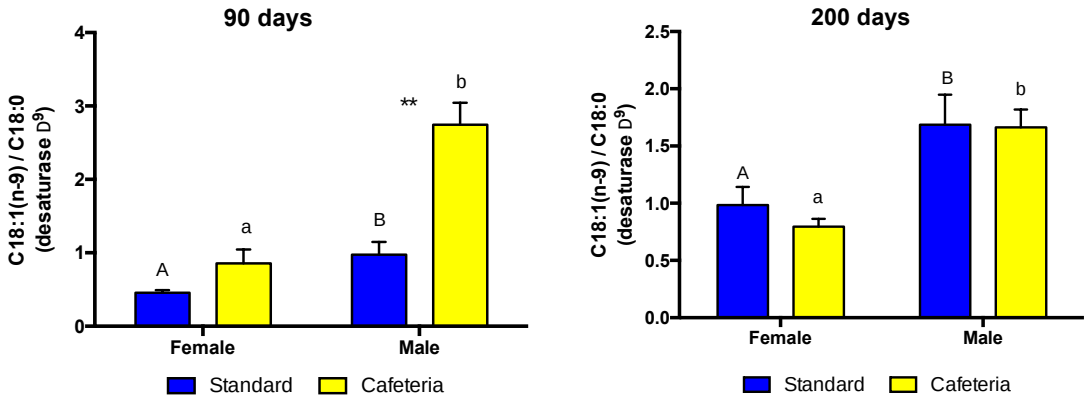

Figure 51: Effects of cafeteria and standard diets on Δ^9 desaturase activity index in plasma of female and male rats at 90 days (left) and those rats followed by 110 days on just standard diet (right) (total 200 days of experiment).

Asterisks indicate statistically significant differences between the dietary groups (** = $p < 0.01$). Different uppercase letters indicate statistical difference between female and male standard groups and different lowercase letters indicate statistical difference between female and male cafeteria groups.

Similarly to what we had found in plasma, liver Δ^5 desaturase activity index decreased in males fed CD compared to those fed SD for 90 days, while in female there was just a tendency to be reduced in the CD group (figure 52). When we compared the values between males and females we found that females showed higher values than males only in the CD groups. After the treatment of both groups with SD for 110 days, no differences were found in liver Δ^5 desaturase activity index between the groups (figure 52). Again similar to what occurred in plasma, liver Δ^6 desaturase activity index did not show differences between SD and CD in females nor in males at 90 days, but in this case Δ^6 desaturase activity index was augmented in females vs males on CD (figure 53) as it occurred and it has been already commented in the case of the Δ^5 desaturase activity index in liver (figure 52). At 200 days of experiment, no differences were found neither between dietary treatments nor gender comparison in Δ^6 desaturase activity index in liver (figure 53). In relation with Δ^9 desaturase index, an increased Δ^9 desaturase activity index was found in liver of males from CD group compared to SD and no gender differences was found at 90 days of experiment (figure 54). However, after receiving SD for 110 days females previously fed with CD showed decreased Δ^9 desaturase activity index while no difference was found in male rats (figure 54). Also, values were higher in males than in females from the same dietary experimental group (figure 54).

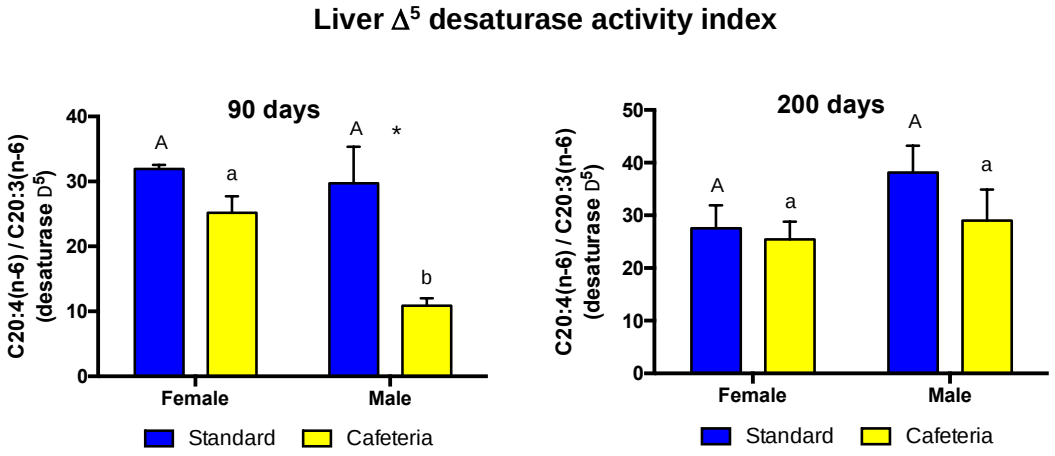

Figure 52: Effects of cafeteria and standard diets on Δ^5 desaturase activity index in liver of female and male rats at 90 days (left) and those rats followed by 110 days on just standard diet (right) (total 200 days of experiment).

Asterisk indicates statistically significant differences between the dietary groups (* =$p < 0.05$). Different uppercase letters indicate statistical difference between female and male standard groups and different lowercase letters indicate statistical difference between female and male cafeteria groups.

Liver Δ^6 desaturase activity index

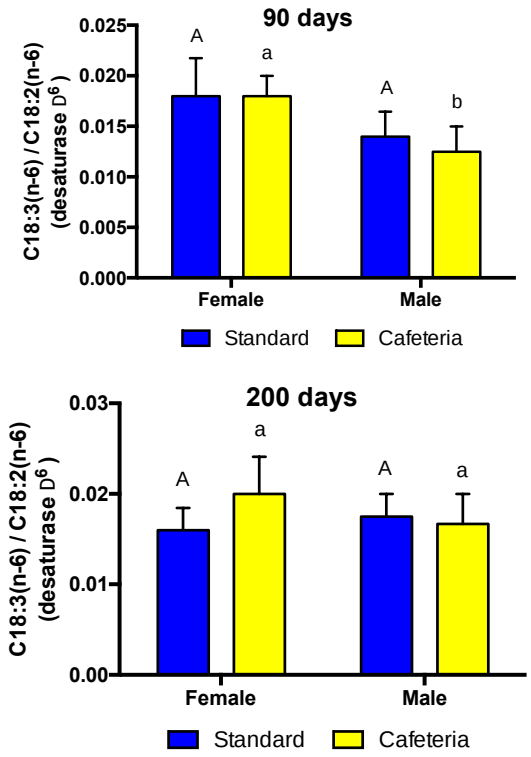

Figure 53: Effects of cafeteria and standard diets on Δ^6 desaturase activity index in liver of female and male rats at 90 days (left) and those rats followed by 110 days on just standard diet (right) (total 200 days of experiment).

No statistical differences were found between Cafeteria vs. Standard diet groups. Different uppercase letters indicate statistical difference between female and male standard groups and different lowercase letters indicate statistical difference between female and male cafeteria groups.

Liver Δ^9 desaturase activity index

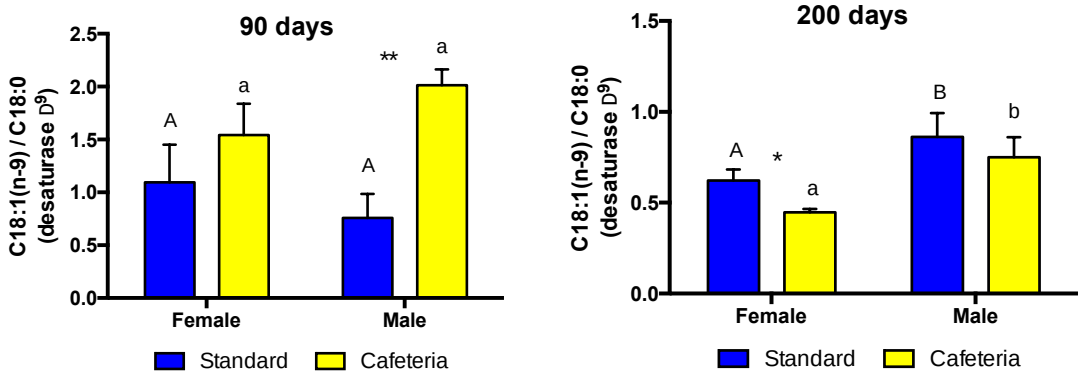

Figure 54: Effects of cafeteria and standard diets on Δ^9 desaturase activity index in liver of female and male rats at 90 days (left) and those rats followed by 110 days on just standard diet (right) (total 200 days of experiment).

Asterisks indicate statistically significant differences between the dietary groups (* = $p < 0.05$, ** = $p < 0.01$). Different uppercase letters indicate statistical difference between female and male standard groups and different lowercase letters indicate statistical difference between female and male cafeteria groups.

In lumbar adipose tissue at 90 days on either SD or CD diet, female and male rats fed with CD showed lower Δ^5 desaturase activity index than those on SD, the same to what occurred with this desaturase in plasma. When we compared the activity of Δ^5 desaturase between females and males we found again higher values in females than males from both dietary groups (figure 55). Nevertheless, Δ^6 desaturase activity index was not affected by the dietary treatments in females and males (figure 56). Males from SD group showed lower values for Δ^6 desaturase activity than females while animals fed CD did not show gender differences (figure 56). When we analysed the Δ^9 desaturase activity index (figure 57) we found a lower desaturase activity in males fed CD for 90 days than those on SD but we did not find such change in females. Additionally, in CD groups male animals had lower values compared to females, although no differences were observed in SD groups between females and males (figure 57).

Lumbar adipose tissue Δ^5 desaturase activity index

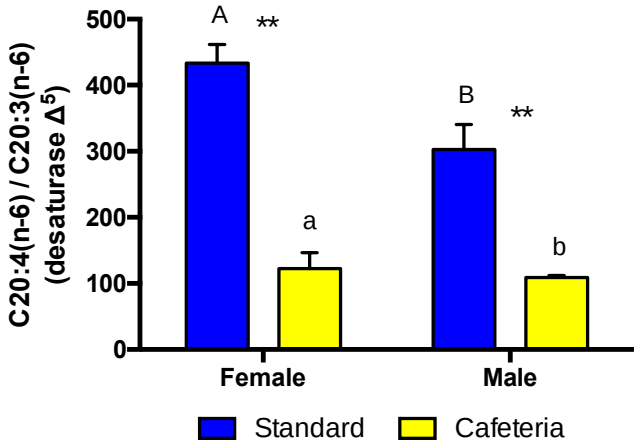

Figure 55: Effects of cafeteria and standard diets on Δ^5 desaturase activity index in lumbar adipose tissue of female and male rats at 90 days of experiment.

Asterisks indicate statistically significant differences between the dietary groups (** = $p < 0.01$). Different uppercase letters indicate statistical difference between female and male standard groups and different lowercase letters indicate statistical difference between female and male cafeteria groups.

Lumbar adipose tissue Δ^6 desaturase activity index

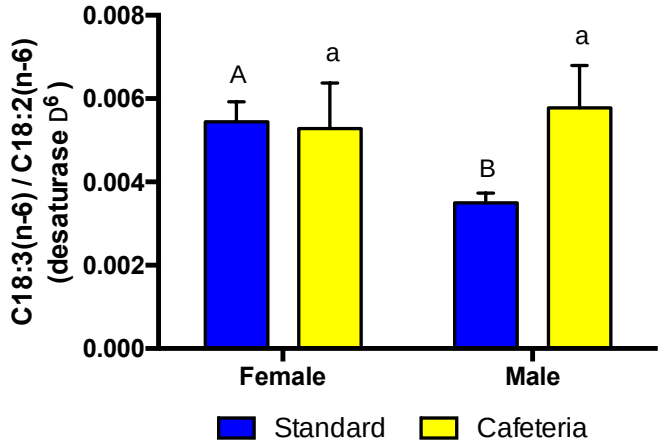

Figure 56: Effects of cafeteria and standard diets on Δ^6 desaturase activity index in lumbar adipose tissue of female and male rats at 90 days of experiment.

No statistical differences were found between Cafeteria vs. Standard diet groups. Different uppercase letters indicate statistical difference between female and male standard groups and different lowercase letters indicate statistical difference between female and male cafeteria groups.

Lumbar adipose tissue Δ^9 desaturase activity index

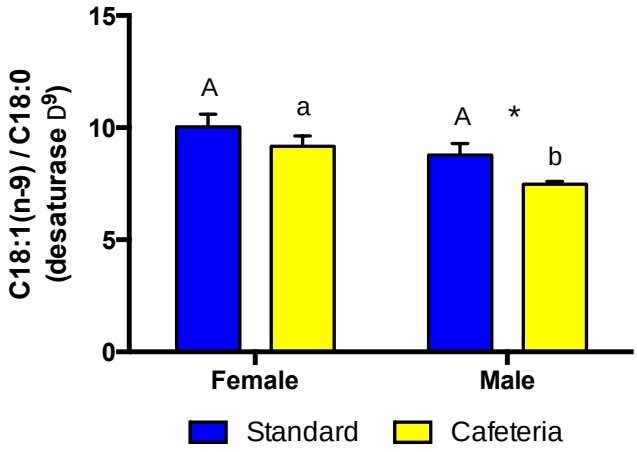

Figure 57: Effects of cafeteria and standard diets on Δ^9 desaturase activity index in lumbar adipose tissue of female and male rats at 90 days of experiment.

Asterisk indicates statistically significant differences between the dietary groups (* =p < 0.05). Different uppercase letters indicate statistical difference between female and male standard groups and different lowercase letters indicate statistical difference between female and male cafeteria groups.

To summarize all the results surrounding the fatty acid composition of the tissues and the desaturase indexes we can point out that the cafeteria diet offered in the present study modified the fatty acid profile of plasma, liver and lumbar adipose tissue. The lower concentration of AA found in plasma and adipose tissue of the CD group was surprising since there were no differences in the AA concentrations in the diets and the amount of it in both diets was very low. A possible explanation for this low concentration of AA in the CD groups could be that a reduction on the AA (arachidonic acid, 20:4 n-6) precursor, LA (linoleic acid 18:2 n-6), occurred and impaired the AA endogenous biosynthesis. Nevertheless, a similar amount of LA was offered through the diets and no differences were found in LA concentrations in plasma, liver and adipose tissue between cafeteria and standard groups. Furthermore, there is a possibility that AA production was

reduced as a consequence of a decreased Δ^5 desaturase activity (enzyme involved in the endogenous biosynthesis of AA from LA) as suggested by the low Δ^5 activity index found by us in plasma, liver and lumbar adipose tissue of animals fed with cafeteria diet. This result is in accordance with a previous study carried out in our laboratory that also found lower AA levels in tissues from rats fed with cafeteria without changes in LA levels when compared to animals fed with standard diet (264). The reason for a reduced Δ^5 desaturase activity could be related with the higher amount of OA found in plasma and tissues of these animals (figures 44 and 45). In fact, an inversely proportional association between the concentration of OA and AA and the activity of the Δ^5 and Δ^6 desaturases has been described in humans (265) which could have occurred to our animals from the CD groups since a high amount of OA was offered in the cafeteria diet (figure 18). Even though the mechanisms by which this association occurs remain unknown.

Regarding the activity of the Δ^9 desaturase, it is known that the Δ^9 desaturase activity index can be positively correlated with lipogenesis and adipogenesis (266-269). Additionally, in our study in males fed with the CD the Δ^9 desaturase activity index was higher in plasma and liver vs those on SD (figures 51 and 54, respectively). Thus, it is possible that our CD caused an increased lipogenesis in males fed with this diet. One of the reasons for the increased Δ^9 activity index could be the higher amounts of OA in the CD used here compared to SD (figure 18). This relationship agrees with the previously described by Field *et al.* that found a higher Δ^9 activity index as consequence of a diet rich in OA (270). However, more direct studied are necessary to confirm this relation.

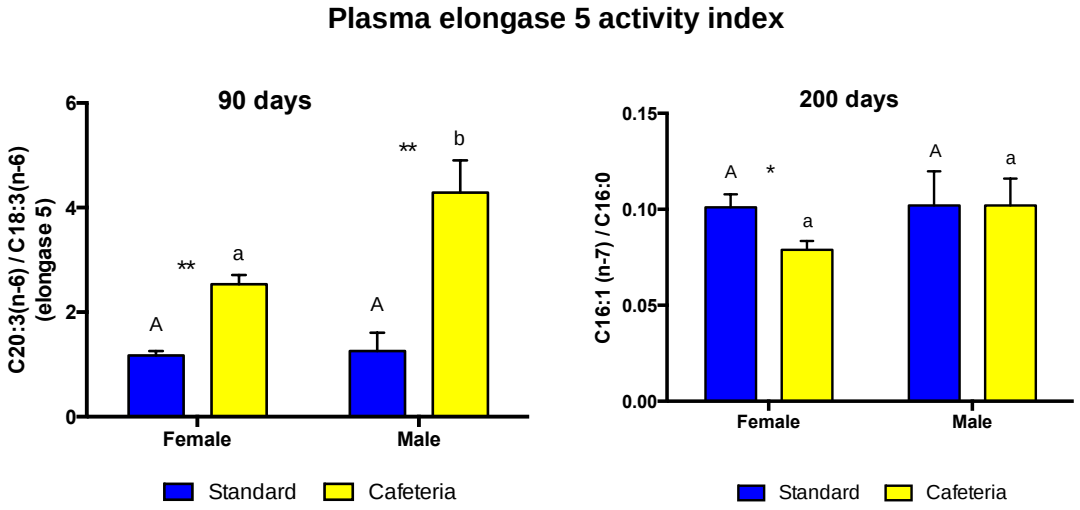

Figure 58: Effects of cafeteria and standard diets on elongase 5 activity index in plasma of female and male rats at 90 days (left) and those rats followed by 110 days on just standard diet (right) (total 200 days of experiment).

Asterisks indicate statistically significant differences between the dietary groups (** = $p < 0.01$). Different uppercase letters indicate statistical difference between female and male standard groups and different lowercase letters indicate statistical difference between female and male cafeteria groups.

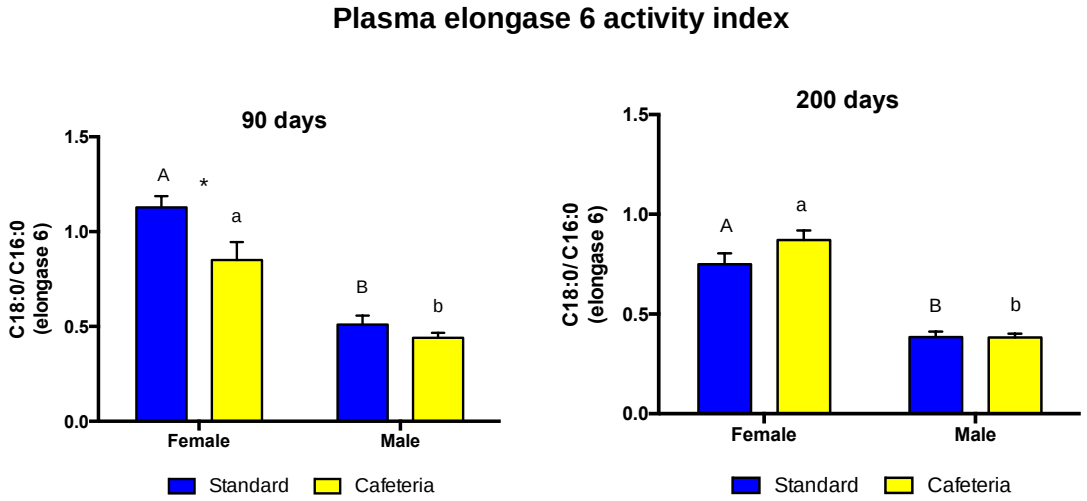

Figure 59: Effects of cafeteria and standard diets on elongase 6 activity index in plasma of female and male rats at 90 days (left) and those rats followed by 110 days on just standard diet (right) (total 200 days of experiment).

Asterisks indicate statistically significant differences between the dietary groups (* = $p < 0.05$). Different uppercase letters indicate statistical difference between female and male standard groups and different lowercase letters indicate statistical difference between female and male cafeteria groups.

Figure 58 shows increased elongase 5 activity index in plasma of CD female and male rats at 90 days of experiment in comparison to those fed SD. Additionally, whereas in animals fed with SD no differences were found between males and females, male rats fed with the CD had higher elongase 5 activity index than CD females (figure 58). At 200 days of experiment no differences were found in dietary treatments nor between gender. Different to elongase 5, plasma elongase 6 index was decreased in females from CD group but no change in males was found at 90 days of experiment (figure 59). Moreover, females from SD and CD showed higher values of elongase 6 index in plasma than males at 90 and 200 days of experiment, when no differences were seen in dietary treatment (figure 59).

Similar to plasma, in liver, elongase 5 activity index is increased in females and males fed with CD vs those on SD and males showed higher values than

females (figure 60) at 90 days. After 110 days on SD, no differences were observed in liver of female and male animals (figure 60). However, the liver elongase 6 activity index in liver at 90 days did not show differences neither in dietary treatment nor in gender comparisons (figure 61). However, after 110 days on SD, females from the CD group showed higher elongase 6 activity index in liver than females in SD group, and comparing genders we found higher values in the elongase activity in females than in males on both dietary groups (figure 61).

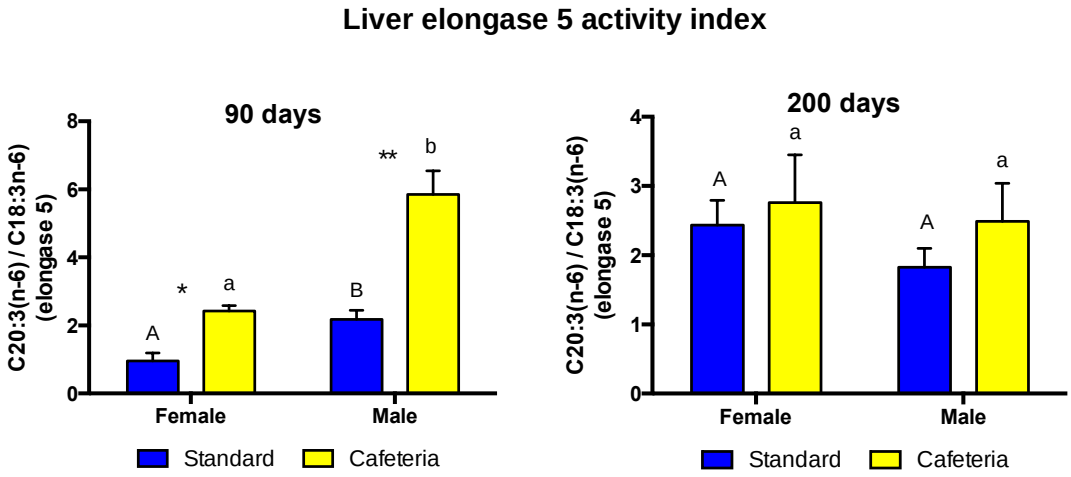

Figure 60: Effects of cafeteria and standard diets on elongase 5 activity index in liver of female and male rats at 90 days (left) and those rats followed by 110 days on just standard diet (right) (total 200 days of experiment).

Asterisks indicate statistically significant differences between the dietary groups (* = $p < 0.05$, ** = $p < 0.01$). Different uppercase letters indicate statistical difference between female and male standard groups and different lowercase letters indicate statistical difference between female and male cafeteria groups.

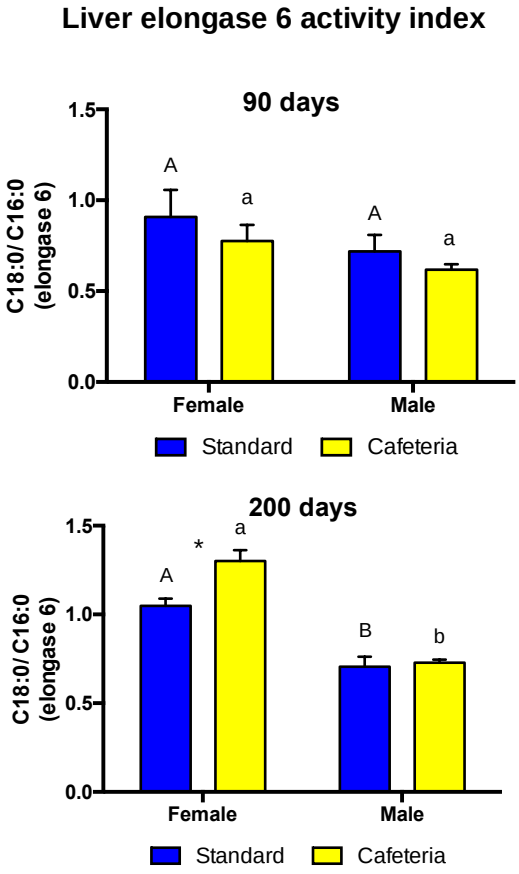

Figure 61: Effects of cafeteria and standard diets on elongase 6 activity index in liver of female and male rats at 90 days (left) and those rats followed by 110 days on just standard diet (right) (total 200 days of experiment).

Asterisks indicate statistically significant differences between the dietary groups (* = $p < 0.05$). Different uppercase letters indicate statistical difference between female and male standard groups and different lowercase letters indicate statistical difference between female and male cafeteria groups.

Also as plasma and liver, lumbar adipose tissue elongase 5 activity index was increased in males fed CD but not significantly in females at 90 days of experiment and no differences were found between females and males (figure 62). However, different to plasma and liver, in the case of elongase 6 activity index in lumbar and adipose tissue, CD groups showed significantly higher values in female and male animals at 90 days (figure 63). No differences were observed in lumbar adipose tissue elongase 6 activity index in the SD groups between

female and male, whereas in the CD groups, males showed higher values than females at day 90 of experiment (figure 63).

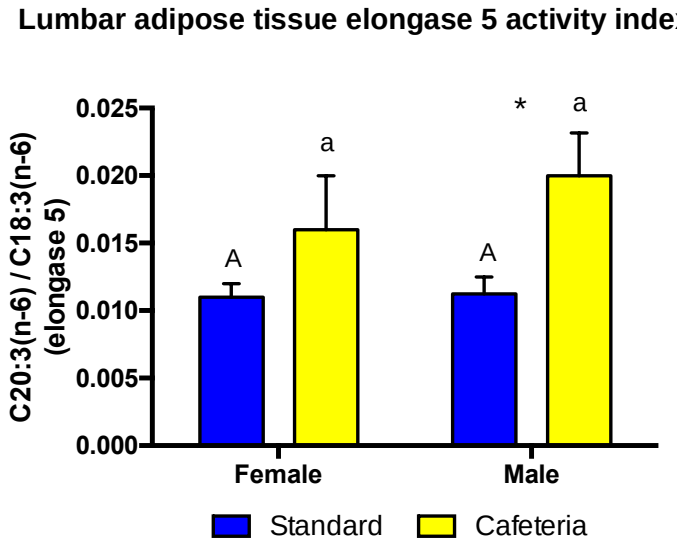

Figure 62: Effects of cafeteria and standard diets on elongase 5 activity index in lumbar adipose tissue of female and male rats at 90 days of experiment.

Asterisk indicates statistically significant differences between the dietary groups (* =p < 0.05). Different uppercase letters indicate statistical difference between female and male standard groups and different lowercase letters indicate statistical difference between female and male cafeteria groups.

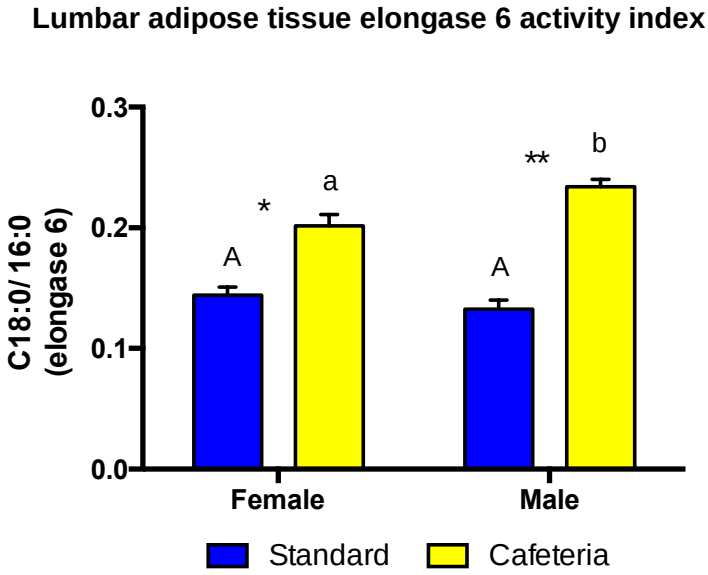

Figure 63: Effects of cafeteria and standard diets on elongase 6 activity index in lumbar adipose tissue of female and male rats at 90 days of experiment.

Asterisks indicate statistically significant differences between the dietary groups (* =p < 0.05, ** p < 0.01). Different uppercase letters indicate statistical difference between female and male standard groups and different lowercase letters indicate statistical difference between female and male cafeteria groups.

To summarize the results about elongases and as commented above, the elongase 5 activity index in animals fed with CD was increased in plasma, liver and lumbar adipose tissue at 90 days compared to those fed the SD. A previous study also found in male rats that feeding a diet rich in carbohydrates caused an increase of elongase 5 mRNA expression in liver compared to the standard diet group (271). Also, it is described that LXRα-SREBP-1c in liver regulates elongase 5 expression (272) and that glucose is able to stimulate the transcriptional activity of LXR (273). These findings are in agreement to the increased elongase 5 activity index found in the present study in rats fed the CD since this diet is rich in both carbohydrate and fat.

However, we have to recognize that our results concerning the changes found in the rats fed the CD in the calculated desaturases and elongases activity indexes are not completely consistent as also has been described by other

authors (271, 274, 275). In fact, these enzymatic activities seem to vary accordingly to gender, tissue studied, the amount and type of fatty acids offered by the experimental diets during the studied period. Additionally, in the present study, at 200 days a rebound effect seems to occur in some of the calculated fatty acid ratios when the CD was shift to SD.

1.7 Oral Glucose Tolerance Test (OGTT)

Type 2 diabetes is characterized by a failure on insulin secretion and action that results in hyperglycaemia. Several authors have described a strong relationship among diet, obesity and type 2 diabetes development (276). It is known that diets rich in fat are a cause of obesity and also of insulin resistance. This phenomenon is related to the fact that increased fat size in obesity, especially visceral fat, plays an important role in the impaired insulin action on reducing plasma glucose levels (277).

For a better understanding of the potential effects of CD on glucose metabolism, some indexes related with insulin sensitivity (HOMA and QUICKI) were calculated, and also an Oral Glucose Tolerance Test was performed in the rats at 90 and 200 days of experiment. First of all, we measured the concentrations of basal plasma glucose and insulin in the rats and found no differences after both dietary treatments at 90 and 200 days of experiment in basal glucose (figure 64) whereas basal insulin increased in CD vs SD females at 200 days of experiment (figure 65). When compared by gender, males fed CD showed higher concentrations of glucose than females at 90 and 200 days (figure 64). In relation with insulin, although no differences due to gender were found at 90 days of treatment at 200 days of experiment males from SD showed higher insulin concentration than females (figure 65).

Taking HOMA value as an indicator of insulin resistance (figure 66) and QUICKI as index of insulin sensitivity (figure 67), no difference was found in both HOMA and QUICKI values between CD and SD in female and male rats at 90 and 200 days of experiment. However, in relation with the gender effect in HOMA, male rats fed CD showed higher HOMA values than females at 90 day, indicating an increased insulin resistance of CD male rats when compared to female, but no differences were found at day 200 when all groups received standard diet (figure 66). In relation with the gender effect in QUICKI, SD males showed a lower QUICKI value when compared to females at 90 and 200 days (figure 67) but this effect disappeared in males vs females in the CD groups at 200 days of experiment, indicating that when the cafeteria fed rats changed to a standard diet for 110 days the differences in insulin sensitivity due to the gender disappeared.

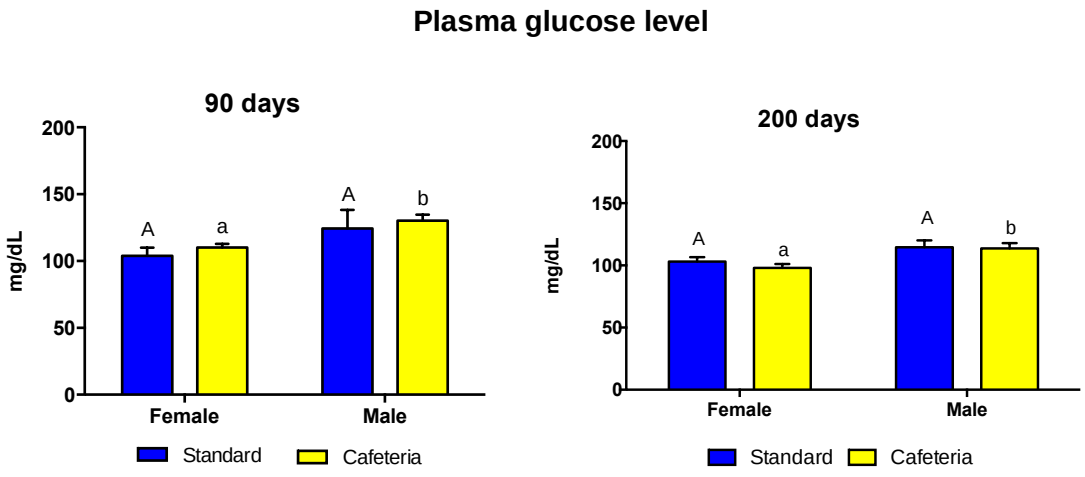

Figure 64: Effects of cafeteria and standard diets on female and male rats on plasma glucose level at 90 days (left) and 200 days (right) of experiment

Different uppercase letters indicate statistical difference between female and male standard groups and different lowercase letters indicate statistical difference between female and male cafeteria groups.

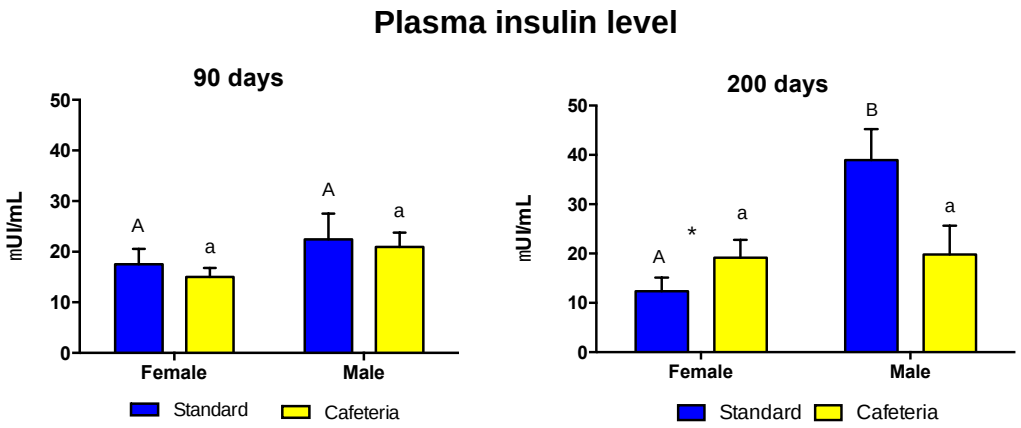

Figure 65: Effects of cafeteria and standard diets on female and male rats on plasma insulin level at 90 days (left) and 200 days (right) of experiment glucose

Asterisk indicate statistically significant differences between the groups (* =p < 0.05). Different uppercase letters indicate statistical difference between female and male standard groups and different lowercase letters indicate statistical difference between female and male cafeteria groups.

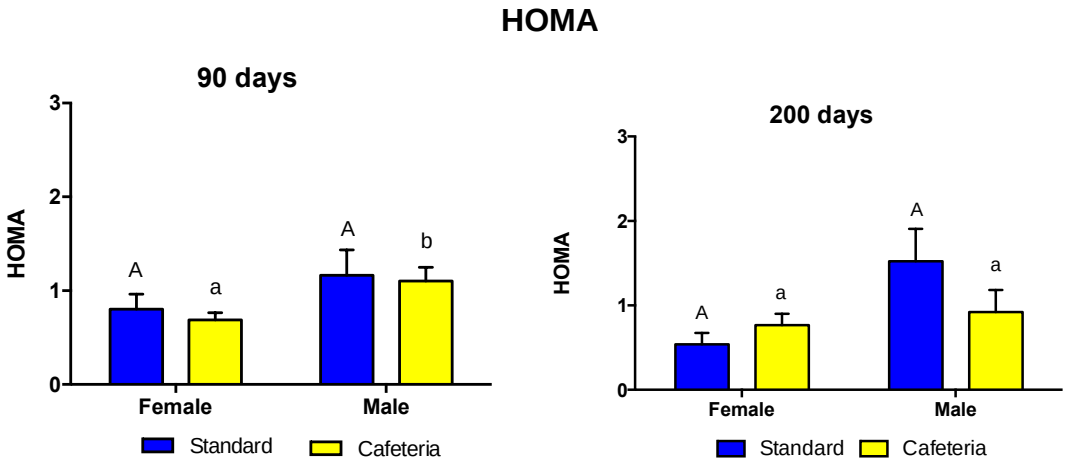

Figure 66: Effects of cafeteria and standard diets on female and male rats on HOMA at 90 days (left) and 200 days (right) of experiment glucose

Different uppercase letters indicate statistical difference between female and male standard groups and different lowercase letters indicate statistical difference between female and male cafeteria groups.

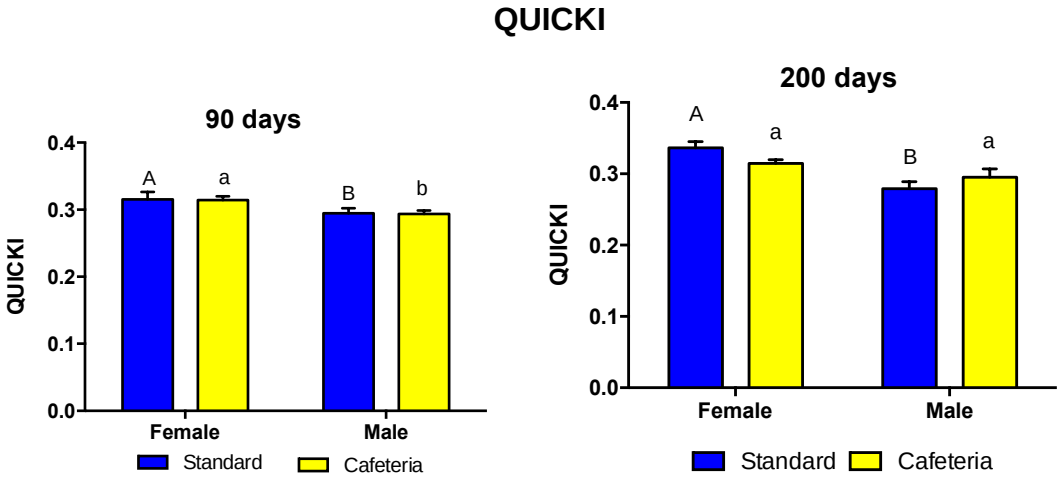

Figure 67: Effects of cafeteria and standard diets on female and male rats on QUICKI at 90 days (left) and 200 days (right) of experiment glucose

Different uppercase letters indicate statistical difference between female and male standard groups and different lowercase letters indicate statistical difference between female and male cafeteria groups.

In relation to the Oral Glucose Tolerance Test (OGTT), this is based on the fact that plasma glucose level is regulated by both the amount of insulin released by the pancreas and the insulin sensitivity of different tissues. Thus, through this OGTT it is possible to identify an altered post-prandial glucose metabolism and insulin resistance not revealed by the glucose and insulin basal fasting conditions (278).

In our study, after the oral glucose load we calculated the concentrations of glucose and insulin in the animals at different time points, expressed as the area under the curve (AUC) and related this with the insulin resistance in the rats. We found that the AUC for insulin of CD females at 90 days was higher than in SD females but no differences were found between males (figure 68). When females and males from the same dietary group were compared, SD males showed higher insulin AUC than SD females whereas no differences were found between females and males from CD (figure 68). For glucose, CD females showed higher AUC than SD group at day 90 but males did not show differences between dietary treatments (figure 69). When females and males receiving the

same diet were compared, females showed lower glucose AUC than males in both SD and CD groups (figure 69). These values allowed us to determine the insulin sensitivity index (ISI) (225). When the ISI was calculated at day 90, CD females showed lower values than SD but no differences were found between males (figure 70). Comparing females and males in the same dietary group, females from SD showed higher ISI than males but CD groups showed no differences between females and males at 90 days.

Insulin resistance is often described as an inability of insulin to produce its effects promoting glucose uptake and utilization by muscle and other tissues, including an inhibition in triglyceride synthesis from glucose in adipose tissue and other effects (279). There are several mechanisms described as possibly responsible for insulin resistance development including dietary habits and adiposity (280). An increased amount of body fat is normally associated to larger adipocytes that are themselves resistant to insulin action. In the adipose tissue, insulin acts inhibiting lipolysis and therefore a decrease in insulin signalling lead to an increased fatty acids and glycerol release (281). In our study, at day 90 of treatment, CD females showed higher adipose tissue weight than SD (figures 25 – 28). Additionally, the glycerol kinase activity in most adipose tissues was higher in females fed the CD than in those fed a SD for 90 days (figures 37 – 40). Therefore, it is possible that the insulin action inhibiting lipolysis in the presence of increased glycerol kinase activity have caused an increased phosphorylation of released glycerol and subsequently re-esterified within the tissue, contributing to the increased adipose tissue size, as described above in section 1.4. More direct studies are necessary to prove this hypothesis.

The reduced insulin sensitivity in females fed CD could be partially caused by the reduced amount of polyunsaturated fatty acids present in the CD diet when compared to SD diet (figure 18). It is described that polyunsaturated fatty acids could act as ligands of PPARγ (282) and also modulate its expression and consequently increase insulin dependent glucose transporter GLUT4

transcription and synthesis (283). A higher number of GLUT4 glucose transporter is associated with an improvement of insulin resistance. In our study, a lower polyunsaturated fatty acid content in the CD diet in comparison with the standard diet could lead to a reduction on the GLUT4 transcription and synthesis stimulus contributing to the insulin resistance shown in CD females at day 90 of treatment (figure 18). However, the action of specific fatty acids on PPARγ is not completely understood and it is currently the aim of numerous investigations (284).

However, in the case of male rats fed CD for 90 days, no differences were found concerning the fasting plasma glucose nor the response to the Oral Glucose Tolerance Test. This could be due to the fact that male rats fed CD did not increase their adipose tissue weight as shown in figures 25 – 27 and 29. Additionally, male animals fed the SD showed lower insulin sensitivity than females (figure 70). Since fat depots are regulated by sex hormones (285) female rats are more sensitive to insulin than males of the same age even though females showed a higher fat amount than males in both humans and rats. This observation is in accordance with our results. Nevertheless, females fed a cafeteria diet for 90 days showed insulin sensitivity similar to males (figure 70). It is therefore possible that, in our study, the higher adipose tissue amount (figures 25 – 28) shown by CD females reduced their insulin sensitivity to the male´s level.

From 90th day of experiment the cafeteria diet was replaced with a standard diet up to 200 days. At 200 days, no differences were found in glucose and insulin AUC of females and males between cafeteria and standard groups (figure 69). Thus, ISI values did not change between dietary groups of females and males as shown in figure 70. On the other hand, when compared by gender, both glucose and insulin AUC in the OGTT were higher in males than in females fed with SD and also higher in males than in females fed with CD. However, when ISI values were compared by gender, females fed SD showed higher insulin sensitivity than males fed the equal diet. Nevertheless, no difference was found in ISI values between females and males fed with CD, not even after 110 days of

shifting to the CD, this could indicate that the impairment in the insulin sensitivity produced by the CD was not recovered at 200 days of experiment.

It is possible that our female animals did not recover from the effects of the cafeteria diet on glucose metabolism due to the fact that the diet was given to them too early in life. The metabolic programming is described as an adaptation to a nutritional insult environment during a critical period of development that last later in life even in the absence of the insult (286). Thus, it is possible that a degree of metabolic programming occurred to our female rats which were fed a CD since they were 35 days old, and such early age impaired the recovery of certain metabolic parameters when the CD rats were fed with a SD for the additional 110 days. A study that evaluated the effects of a post-cafeteria diet using older animals (2 and 4 months old) found an improvement on insulin resistance after 60 days on a control diet (239). However, the effects of metabolic programming vary accordingly to the duration of the insult exposure and life stage, i.e., too early or late in life. Boullu-Ciocca et al (287) programmed male rats during the lactation period reducing the number of pups per mother. From weaning, the pups received control diet to 5 months old. The authors found metabolic disturbances in programmed animals that were fed control diet later in life. Therefore, in our study, it is possible that using recent weaned animals (35 days old) a metabolic programming occurred.

Oral Glucose Tolerance Test

Insulin Area Under the Curve (AUC)

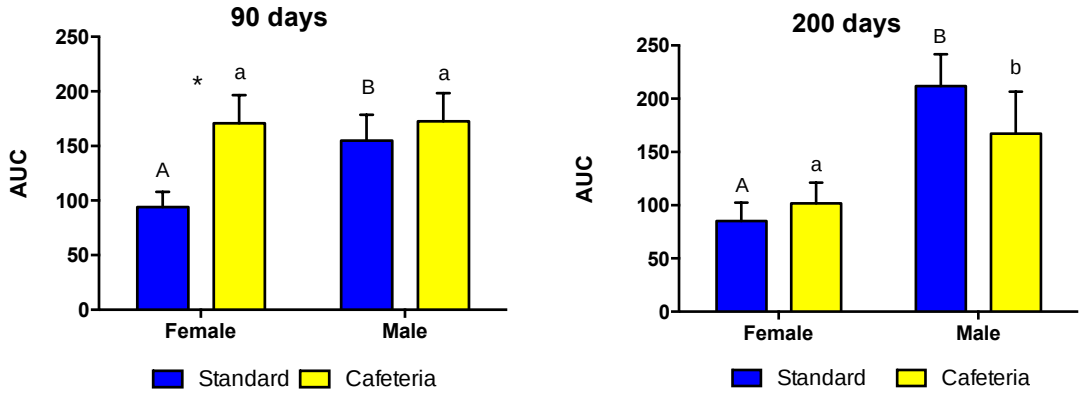

Figure 68: Effects of cafeteria and standard diets on female and male rats on the Area Under the Curve for insulin throughout the Oral Glucose Tolerance Test at 90 days (left) and 200 days (right) of experiment

Asterisk indicate statistically significant differences between the groups (* =$p < 0.05$). Different uppercase letters indicate statistical difference between female and male standard groups and different lowercase letters indicate statistical difference between female and male cafeteria groups.

Oral Glucose Tolerance Test

Glucose Area Under the Curve (AUC)

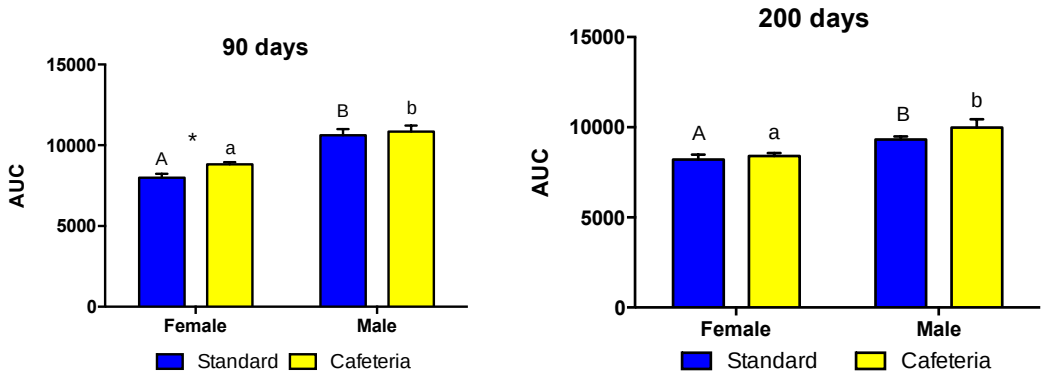

Figure 69: Effects of cafeteria and standard diets on female and male rats on the Area Under the Curve for glucose throughout the Oral Glucose Tolerance Test at 90 days (left) and 200 days (right) of experiment

Asterisk indicate statistically significant differences between the groups (* =p < 0.05). Different uppercase letters indicate statistical difference between female and male standard groups and different lowercase letters indicate statistical difference between female and male cafeteria groups.

Insulin Sensitivity Index (ISI)

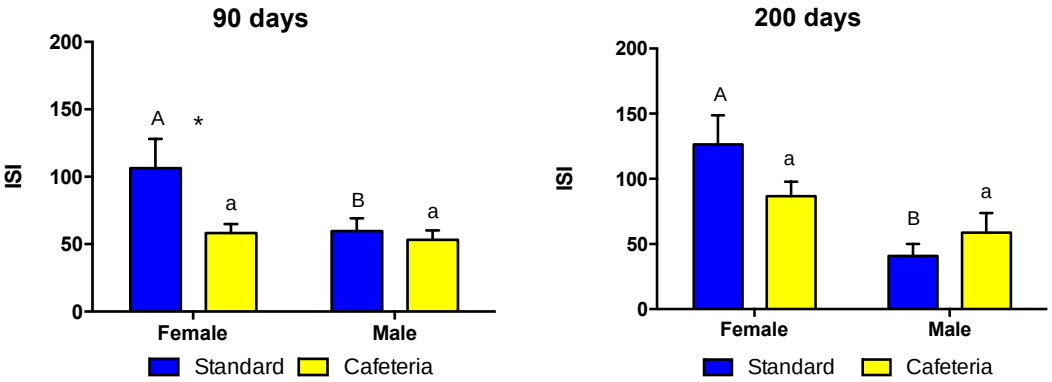

Figure 70: Effects of cafeteria and standard diets on female and male rats in Insulin Sensitivity Index at 90 days (left) and 200 days (right) of experiment

Asterisks indicate statistically significant differences between the groups (* =p < 0.05). Different uppercase letters indicate statistical difference between female and male standard groups and different lowercase letters indicate statistical difference between female and male cafeteria groups.

1.8 Plasma leptin

In 1994, the first adipokine was discovered indicating that the adipose tissue produces several molecules responsible for the regulation of numerous biological process (288). This first adipokine was named leptin by Halaas et al (289). Leptin is a 16kD protein mainly produced and released by white adipose tissue to plasma, but the lymphoid tissue, skeletal muscle, ovary, placenta and bone marrow could also express leptin. Its blood concentrations are positively correlated to body fat (131). The leptin secretion is influenced by estrogen, insulin, glucocorticoids, glucose, fatty acids and other factors. Since leptin was discovered, the scientific community had high expectations on curing obesity throughout the understanding of leptin metabolism (290).

To exert its effects leptin binds a receptor which belongs to a long-chain cytokines superfamily. Leptin receptor is widely distributed through the body and intermediates leptin actions on body weight and energy homeostasis effects which occurs in central nervous system (131). In the brain, leptin receptor is highly expressed in the arcuate, dorsomedial, ventromedial and ventral premamillary nuclei of hypothalamus. Therefore, leptin signalling acts directly in those neurons reducing energy intake and stimulating energy expenditure through neuroendocrine system and cognition (291).

Additionally, it is also known that leptin acts in peripheral tissues promoting energy expenditure. Leptin inhibits glucagon and insulin secretion by the pancreas (292, 293) and in the skeletal muscle it increases glucose uptake and fatty acid oxidation (291). Also, leptin is able to improve insulin sensitivity in the liver and protects mice fed a diet that could lead to glucose intolerance to develop insulin resistance (294).

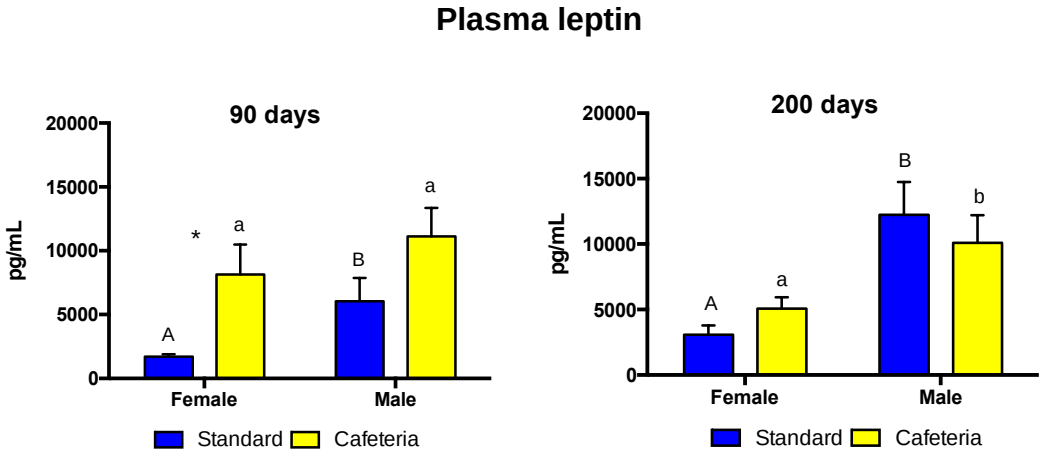

Figure 71: Effects of cafeteria and standard diets on female and male rats in plasma leptin at 90 days (left) and 200 days (right) of experiment

Asterisks indicate statistically significant differences between the groups (* =$p < 0.05$). Different uppercase letters indicate statistical difference between female and male standard groups and different lowercase letters indicate statistical difference between female and male cafeteria groups.

In our study, female rats fed the CD for 90 days showed both a higher adipose tissue weights (figures 25 – 28) and higher plasma leptin (figure 71) levels when compared to female rats fed the SD. In male rats fed the CD no differences were found neither in adipose tissue weight nor in plasma leptin concentration when compared to male rats fed the SD for 90 days. These results are in accordance with the literature since it is described that leptin levels are positively correlated to adipose tissue weight (131). When comparing female and male rats fed with the same diet, males fed the SD showed higher leptin levels compared to females (figure 71). However, no differences were found in leptin concentrations between female and male fed the CD for 90 days because of the higher increase caused by CD in females. It is interesting to point out that the higher effect of CD on plasma leptin levels that we found in females as compared to males agrees with the gender differences in serum leptin levels reported in humans, where it is consistently found higher leptin levels in women than in men per unit of mass of adipose tissue (295, 296).

Although female rats fed the CD showed higher leptin levels compared with SD, as commented above CD females showed reduced insulin sensitivity during the Oral Glucose Tolerance Test (OGTT) as shown in figures 68 – 70. As described above leptin effects in neuroendocrine system could ameliorate fat accumulation, insulin sensitivity and control food intake (290). In fact, in humans an analog of leptin was developed and was used as a treatment for obesity (297). Nevertheless, leptin administration did not have a successful effect since most of the obese individuals have increased leptin concentrations (298). This phenomenon is known as leptin resistance.

Leptin resistance is described as a multifactorial disorder. Several mechanisms have been described to elucidate how this phenomenon occurs which includes impairment in leptin signalling in the cell, deficiency on leptin transportation throughout the blood-brain-barrier and low-grade of chronic inflammation that often occurs in obese individuals (299). Wilsey & Scarpace studied obese rats induced by a high fat/high sucrose diet and found a reduction in the levels of mRNA leptin receptor when compared with animals fed a standard diet (300). However, further studies are necessary to access the possibility of leptin resistance in rats fed a cafeteria diet.

In this study, we evaluate also the effects of shifting from feeding rats a CD for 90 days to feeding a SD for 110 days more (i.e. 200 days of experiment). At this time, no differences in plasma leptin levels were found in female or male rats of the CD group versus the SD group (figure 71). Also, figures 25 – 29 show no difference in adipose tissue weight of CD and SD female and male rats at 200 days of experiment, with values consistently higher in males than in females, as it was so also for plasma leptin levels (figure 71). These findings are in accordance with previous studies that found a positive correlation between the amount of body fat and plasma leptin levels (131). Therefore, we may conclude that the intake of the SD for 110 days was able to recover both adipose tissue

weight and plasma leptin levels in female animals previously fed the CD for 90 days.

1.9 Plasma adiponectin and resistin

Adiponectin is an adipocytokine released exclusively by the adipose tissue that was described for the first time by four different research groups in mouse and human tissues (144). This adipokine is similar to complement factor C1q (or complement component 1q) in terms of structure and it can exist as full-length or globular fragment, though a smaller amount of globular adiponectin as found in human plasma (139).

Concentration of adiponectin in plasma is reported to be associated with obesity and diabetes in both mice and humans (301, 302). However, while increased adipokines such as resistin and tumor-necrosis fator-α (TNF-α) are related to insulin resistance, adiponectin levels in plasma is reduced in obese and insulin resistant rodent models and humans (302). Therefore, the main described effect of adiponectin is the improvement of insulin sensitivity enhancing both glucose uptake and fatty acid oxidation. (303)

Furthermore, it is known that a reduction in adiponectin plasma levels can be caused by a high-fat diet or genetic factors (144). For this reason, we decided to measure plasma levels of adiponectin in female and male rats fed with a CD for 90 days and also after shifting to a SD for 110 days. At day 90, no significant differences were found in adiponectin plasma levels between CD and SD in both female and male rats and within the same dietary groups between female and male rats (figure 72). Similarly, at 200 day of experiment when all the groups were receiving standard diet no differences were found when comparing diets or gender (figure 72).

Although CD female groups showed a higher adipose tissue weight, body weight of female and male CD groups were not affected by feeding a cafeteria

diet for 90 days. It is described that adiponectin plasma concentration is reduced in individuals with increased body weight (303). In our study no differences in adiponectin plasma concentrations were found between animals fed SD and CD from female and male groups. The lack of differences in adiponectin plasma concentrations between SD and CD groups could be due to the fact that the animals fed with CD did not increased their body weight as shown in figures 23 and 24. Therefore, since CD animals were not obese, this could be the reason for similar adiponectin concentration between CD and SD groups.

On the other hand, it is possible to suggest that a resistance to adiponectin action occurred. Even though adiponectin levels are similar in rats fed the CD or the SD, CD female rats showed decreased Insulin Sensitivity Index (ISI) when compared to SD female rats at day 90 as shown in figure 70. It is known that adiponectin acts regulating insulin sensitivity by several mechanisms. One of them was reported by Yamauchi et al.(301) that studied mice treated with adiponectin and found a higher tyrosine phosphorylation of insulin receptor, insulin receptor substrate-1 and Akt induced by insulin when compared to non-treated mice. In our female CD rats an impaired adiponectin signalling could reduce insulin sensitivity without changes in adiponectin plasma levels when compared to SD female rats at day 90. However, more direct studies are necessary to confirm this possibility.

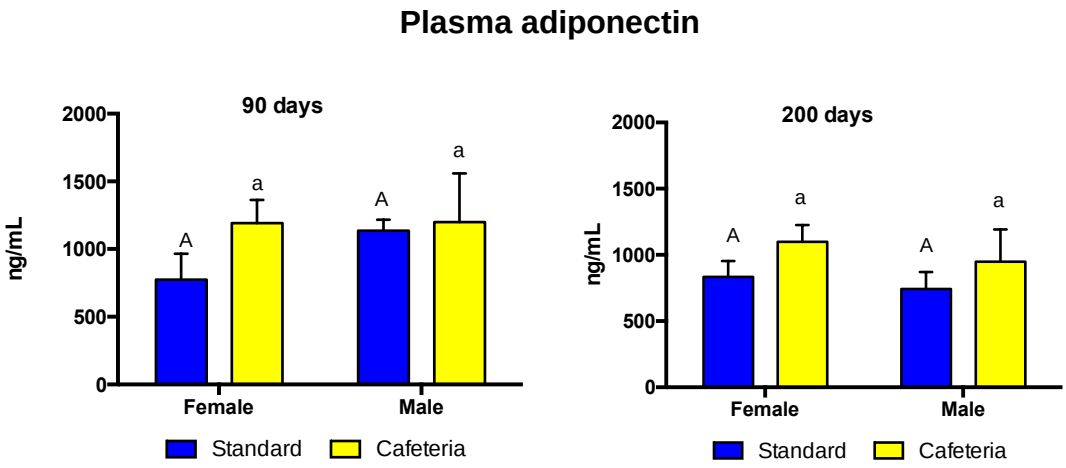

Figure 72: Effects of cafeteria and standard diets on female and male rats on plasma adiponectin at 90 days (left) and 200 days (right) of experiment

Different uppercase letters indicate statistical difference between female and male standard groups and different lowercase letters indicate statistical difference between female and male cafeteria groups.

Resistin is another adipocytokine derived from adipose tissue. It was first identified in 2001 (149, 304) and was initially shown to be up-regulated in rodent models of obesity and insulin resistance and down regulated by an insulin-sensitizer factor (149). These findings brought much scientific attention on resistin that was initially characterized as a potential link between obesity and diabetes, with a functional role contributing to the induction of insulin resistance, being implicated in the pathogenesis of obesity-mediated insulin resistance and type II diabetes mellitus, as reviewed by Kusminski et al (305). However, despite of those antecedents there was a considerable controversy surrounding this adipocytokine in understanding its physiological relevance.

These considerations brought us to analyze plasma resistin levels in our female and male rats fed for 90 days the CD or the SD as well as both groups after 200 days of being fed the SD. The results are shown in figure 73. After 90 days on CD in female rats resistin levels did not differ to those found in rats fed the SD, and as it would be expected, similar plasma resistin levels were found between both groups in female rats fed the SD for 200 days. These findings

contrast with the higher weight of most adipose tissues found in these female rats 90 days after the CD (see figures 25 – 28). However, this finding agrees with the fact that although some studies have reported increased plasma resistin levels in rodent models of obesity (149, 306, 307), other studies failed to demonstrate such correlations between obesity and plasma resistin levels, reporting either reduced (308-311) or no alteration (312, 313).

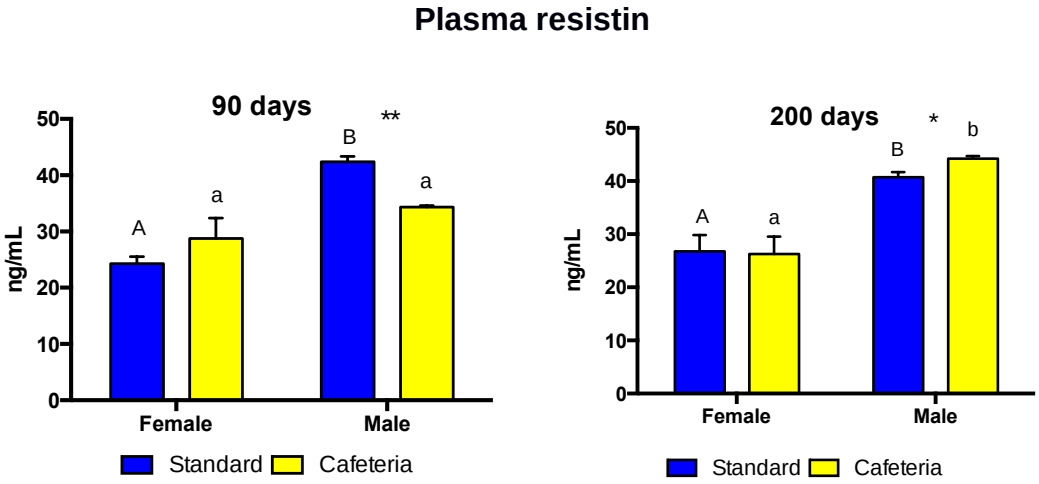

Figure 73: Effects of cafeteria and standard diets on female and male rats on plasma resistin at 90 days (left) and 200 days (right) of experiment

Asterisks indicate statistically significant differences between the groups (* =p < 0.05, ** =p < 0.01). Different uppercase letters indicate statistical difference between female and male standard groups and different lowercase letters indicate statistical difference between female and male cafeteria groups.

As also shown in figure 73, in male rats fed either the CD or the SD the plasma levels of resistin were higher than in females, and this finding agrees with the reported higher mRNA expression of resistin in male than in female rats (314, 315). However, as shown in figure 73 the effect of feeding CD for 90 days in males was to decrease the plasma resistin levels, whereas 200 days of feeding the SD caused a greater increase in those male rats that were under the CD during the earlier stage. We don't have an explanation for this rebound effect of

SD after the CD treatment. This variety in the response, however, agrees with the recognized conclusion recently attained that the significance of gender on the degree of resistin expression in rodents and humans remains unclear (157, 305), and we hope that current results would help to attain a better understanding of the subject.

2. Experiment II: Effects of cafeteria diet supplemented with Blackberry extract in male and female rats

As discussed in section 1, the effects of the cafeteria diet on experiment I concerning insulin resistance (HOMA) and insulin sensitivity (ISI) were not particularly different from animals fed standard diet for 90 days especially for males. We think that the reason for these results is that the animals started to receive the cafeteria diet when weaned, being 35 days old. It is known that when rats receive an unbalanced diet from early life to adult phase a metabolic adaptation occurs that could lead to normal metabolism even though the animals were fed with an over/undernourished diet (316). Thus, in the experiment II we decided to offer cafeteria diet to 70 days old female and male rats. Additionally, since anti-obesity and anti-diabetic effects of berries have been reported (317, 318), besides of offering a cafeteria diet to older rats we decided to evaluate the effects of supplementing standard and cafeteria diets with blackberry extract (*Rubus*) on lipid and glucose metabolism in female and male rats. For this, during 80 days, female and male Sprague Dawley rats were fed one of the following diets: standard diet (SD), standard plus *Rubus* diet (SD+R), cafeteria diet (CD) or cafeteria plus *Rubus* diet (CD+R) as detailed above in section 1.2.2 of Material and Methods. The animals were then sacrificed to have their tissues and plasma collected for further analysis (section 5 of Material and Methods).

2.1 Experimental diet lipid composition and body and fat pad weights

Figure 74 shows the lipid content of the experimental diets. A higher lipid content was found in both CD supplemented or not with *Rubus* extract when compared to both SD with and without *Rubus* extract. No differences were seen in lipid content when *Rubus* extract was added. As shown in table 2, not only the amount of fat of the cafeteria diets were different from the standard diets but also

the source of fat used in each diet. Thus, we quantified the fatty acid profile of the diets. As seen in figure 75, the addition of a *Rubus* extract to the experimental diets did not modify the fatty acid profile of the diets since CD and CD+R diets show a similar fatty acid profile as well as SD and SD+R diets. When comparing the standards diets (SD and SD+R) versus the cafeteria diets (CD and CD+R), the last ones showed higher levels of the saturated and monounsaturated fatty acids PA, POA, SA and OA. And this is similar to what happened with the fatty acid profile of the experimental diets used in experiment I (figure 18 and 75).

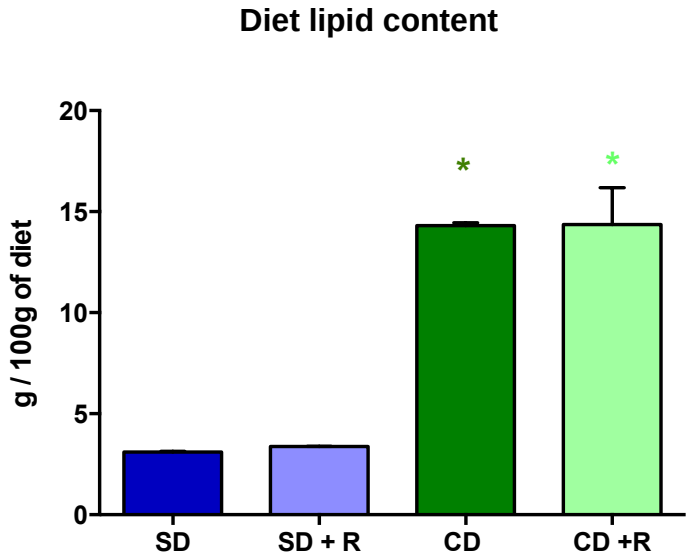

Figure 74: Total lipid content of the diets

Significant differences by two-way ANOVA *, SD *vs* CD; * SD +R *vs* CD +R ($p < 0.05$). SD: standard diet, SD+R: standard diet plus *Rubus*, CD: cafeteria diet, CD+R: cafeteria diet + *Rubus*.

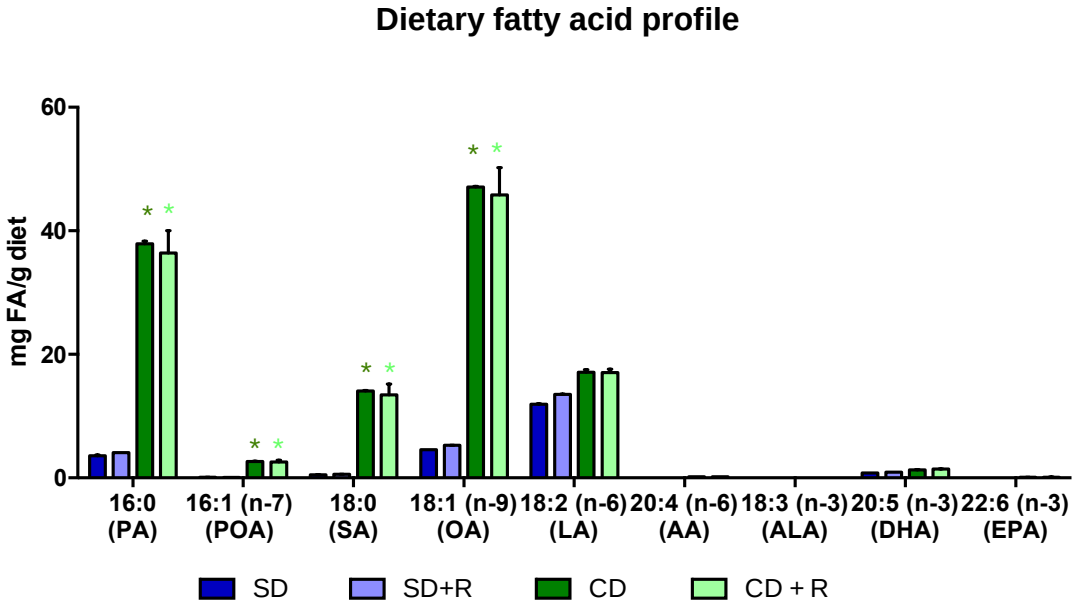

Figure 75: Fatty acid profile of experimental diets

Significant differences by two-way ANOVA *****, SD *vs* CD; *, SD+R *vs* CD+R ($p < 0.05$). SD: standard diet, SD+R: standard diet plus *Rubus*, CD: cafeteria diet, CD+R: cafeteria diet + *Rubus*.

Daily food intake is shown in figure 76. Animals fed with standard diets show higher amount of daily food intake than those fed with the cafeteria diets in female and male groups, but no differences in the amount of daily food intake between rats fed with *Rubus* supplemented diets and not supplemented in female and male groups. However, since CD has a higher caloric content than SD (table 2), daily energy intake of animals fed with cafeteria diet supplemented or not with *Rubus* extract showed higher caloric intake than those fed with the standard diets (figure 77).

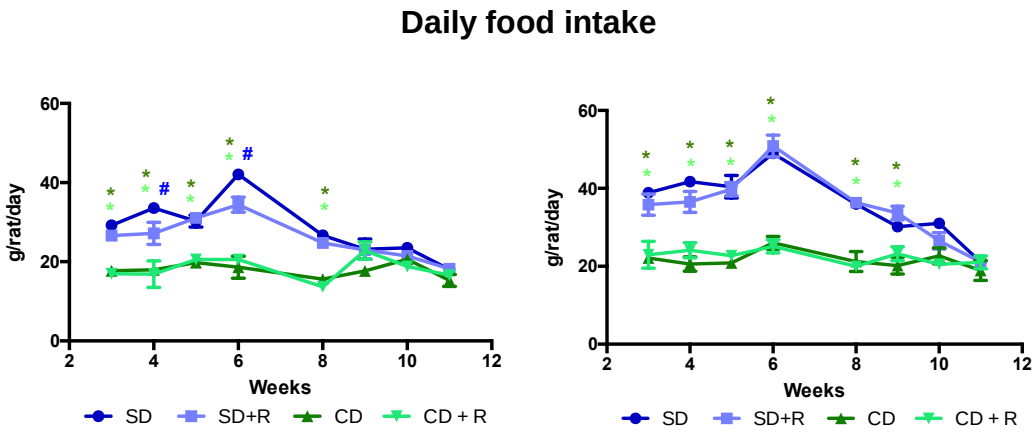

Figure 76: Effects of standard and cafeteria diets supplemented or not with *Rubus* extract in the food intake of females (left) and male (right) rats at day 80 of experiment

Significant differences by two-way ANOVA *, SD *vs* CD; *, SD+R *vs* CD+R, #, SD *vs* SD+R ($p < 0.05$). SD: standard diet, SD+R: standard diet plus *Rubus*, CD: cafeteria diet, CD+R: cafeteria diet + *Rubus*.

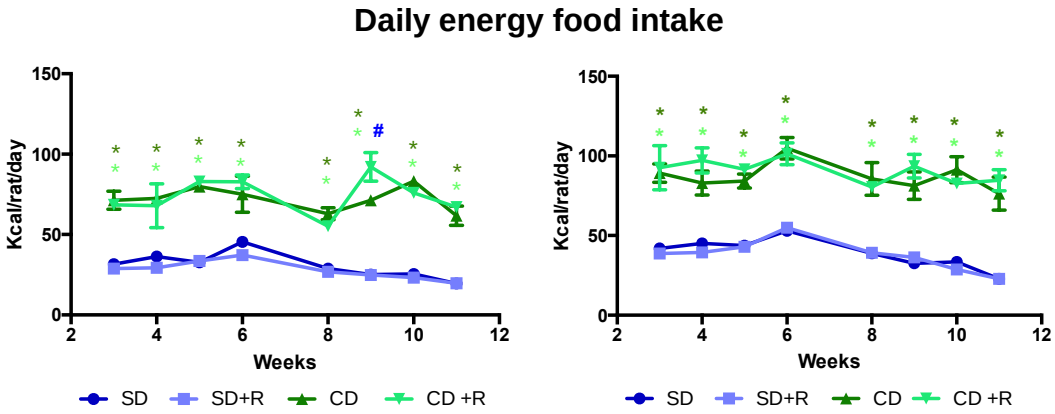

Figure 77: Effects of standard diet, standard diet supplemented with *Rubus* extract, cafeteria diet and cafeteria diet supplemented with *Rubus* extract in energy daily food intake of female (left) and male (right) rats for 80 days.

Significant differences by two-way ANOVA *, SD *vs* CD; *, SD+R *vs* CD+R, #, SD *vs* SD+R ($p < 0.05$). SD: standard diet, SD+R: standard diet plus *Rubus*, CD: cafeteria diet, CD+R: cafeteria diet + *Rubus*.

Figures 78 and 79 shows daily fatty acid intake at day 42. Female and male showed similar results concerning the fatty acid daily intake. Animals fed with CD and CD + R showed higher intake of PA, SA and OA than SD and SD + R. Inversely, CD and CD+R groups of females and males showed lower LA intake than SD and SD+R. Although no difference was seen in LA concentrations among the diets (figure 75), animals from CD and CD+R groups showed lower LA intake than SD and SD+R probably due to the lower food intake of CD and CD+R compared to SD and SD+R as shown in figure 76.

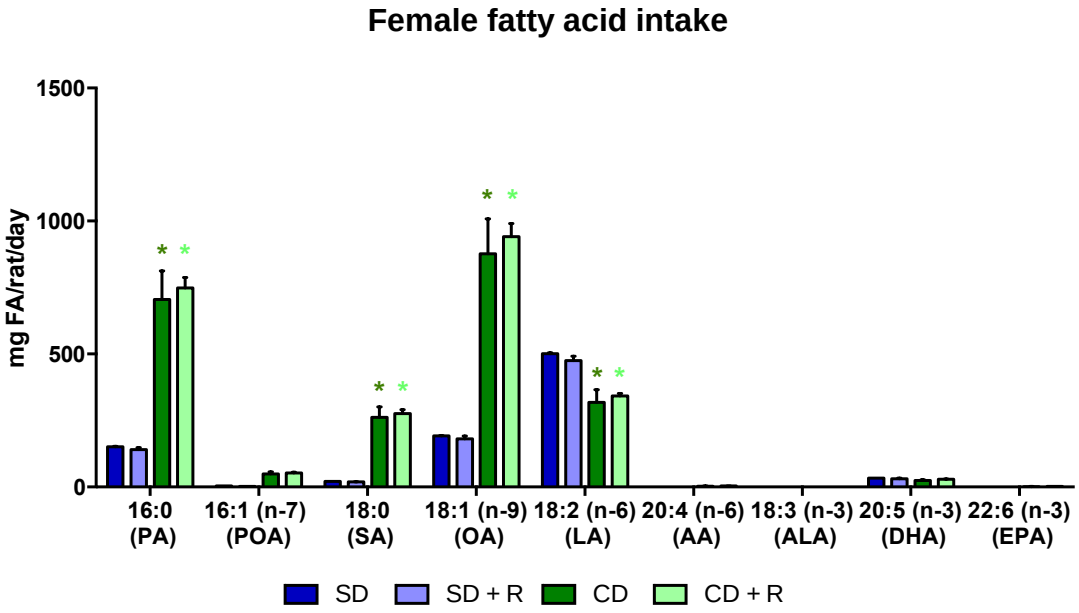

Figure 78: Effects of standard diet, standard diet supplemented with *Rubus* extract, cafeteria diet and cafeteria diet supplemented with *Rubus* extract in fatty acid daily intake of females at day 42 of experiment.

Significant differences by two-way ANOVA *, SD *vs* CD; *, SD+R *vs* CD+R ($p < 0.05$). SD: standard diet, SD+R: standard diet plus *Rubus*, CD: cafeteria diet, CD+R: cafeteria diet + *Rubus*.

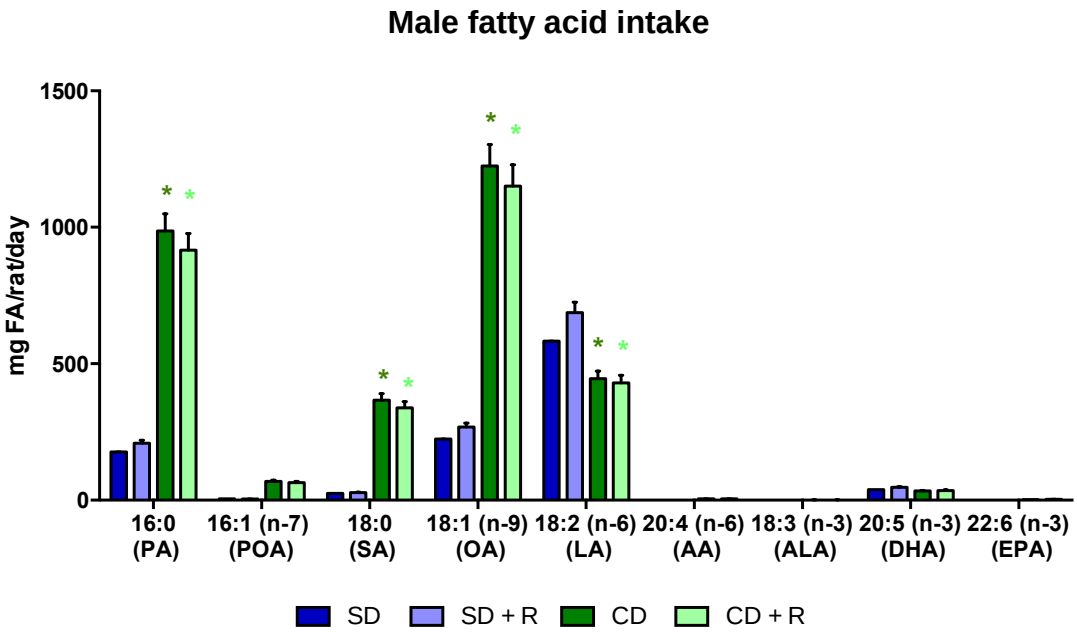

Figure 79: Effects of standard diet, standard diet supplemented with *Rubus* extract, cafeteria diet and cafeteria diet supplemented with *Rubus* extract in fatty acid daily intake of males at day 42 of experiment.

Significant differences by two-way ANOVA *, SD *vs* CD; *, SD+R *vs* CD+R ($p < 0.05$). SD: standard diet, SD+R: standard diet plus *Rubus*, CD: cafeteria diet, CD+R: cafeteria diet + *Rubus*.

Body weights throughout the experiment were measured and are shown in figure 80. No differences were found in body weight between supplemented and non-supplemented groups. However, animals fed cafeteria diets (CD and CD + R) showed higher body weight than those fed with standard diets (SD and SD+R). In fact, females fed with CD and CD + R showed higher body weight than those fed with SD and SD + R from week 5 to the end of experiment while males fed with CD and CD+R showed higher body weight than rats fed with SD and SD + R only from week 9 to week 11 (day 80). Also, male body weight was higher than female as shown in table 3 at day 80 of experiment.

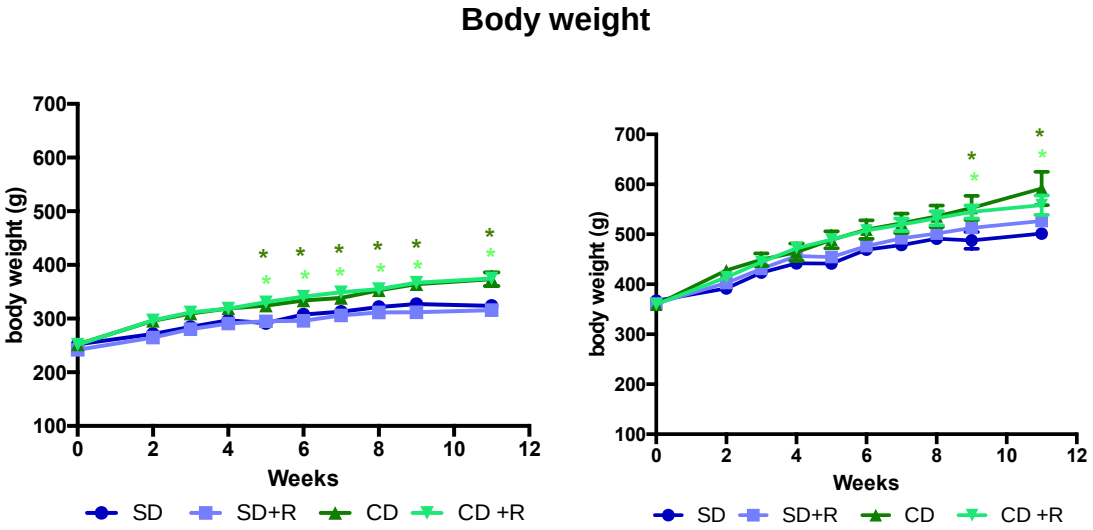

Figure 80: Effects of standard diet, standard diet supplemented with *Rubus* extract, cafeteria diet and cafeteria diet supplemented with *Rubus* extract in body weight of female (left) and male (right) rats for 80 days.

Significant differences by two-way ANOVA *, SD *vs* CD; *, SD+R *vs* CD+R ($p < 0.05$). SD: standard diet, SD+R: standard diet plus *Rubus*, CD: cafeteria diet, CD+R: cafeteria diet + *Rubus*.

Table 3: Body, liver and adipose tissue of rats that were fed standard diet, standard diet supplemented with *Rubus* extract, cafeteria diet or cafeteria diet supplemented with *Rubus* extract for 80 days

	Standard diet		Cafeteria diet		ANOVA (*p*-values)		
	No-*Rubus* (SD)	*Rubus* (SD+R)	No-*Rubus* (CD)	*Rubus* (CD+R)	D	R	D x R
Females							
body weight (g)	338±8	294±7$^{\#}$	379±10*	381±17 *	0.000	0.062	0.043
liver (g)	8.8±0.3	6.1±0.1$^{\#}$	10.0±0.3*	9.7±0.5*	0.000	0.000	0.001
Adipose tissue							
lumbar (g)	3.0±0.3	2.2±0.3	6.1±0.6*	6.2±0.6*	0.000	0.451	0.297
inguinal (g)	2.6±0.4	1.5±0.2	5.9±0.9*	3.9±0.8$^{\#}$*	0.000	0.018	0.454
mesenteric (g)	1.9±0.2	1.3±0.2	3.3±0.4*	2.8±0.4*	0.000	0.088	0.796
adiposity index (%)	3.2±0.3	2.9±0.4	5.9±0.5*	5.4±0.4*	0.000	0.360	0.861
Males							
body weight (g)	483±17	489±7	580±29*	562±17*	0.000	0.749	0.532
liver (g)	14.3±0.4	11.2±0.3$^{\#}$	17.5±1.6*	18.2±0.9*	0.000	0.211	0.063
Adipose tissue							
lumbar (g)	2.9±0.4	4.2±0.5	11.8±2.2*	8.9±1.1*	0.000	0.562	0.116
inguinal (g)	2.8±0.3	2.9±0.3	8.8±1.6*	8.1±1.1*	0.000	0.752	0.703
mesenteric (g)	1.7±0.3	3.4±0.5	6.9±1.4*	5.5±0.5	0.000	0.846	0.053
epididymal (g)	2.1±0.1	3.4±0.3	5.2±0.8*	4.3±0.3	0.000	0.675	0.022
adiposity index (%)	2.1±0.2	3.1±0.2	5.8±0.7*	5.1±0.4*	0.000	0.737	0.064

Significant differences by two-way ANOVA *****, SD *vs* CD, #, No-*Rubus* vs *Rubus* ($p < 0.05$). SD: standard diet, SD+R: standard diet plus *Rubus*, CD: cafeteria diet, CD+R: cafeteria diet + *Rubus*.

To have a better perspective of the body composition of these animals, at day 80 of treatment, when they were sacrificed, we collected and weighed their liver and fat depots and calculated the adiposity index as shown in table 3. Females fed with CD and CD + R when compared to SD and SD + R showed higher body, liver, and lumbar, inguinal and mesenteric adipose tissue weights and also higher adiposity index (table 3). Moreover, supplemented standard diet with *Rubus* (SD+R) offered to females lead to a lower body and liver weights in females when compared to the non-supplemented standard diet group. Between CD and CD + R groups only inguinal adipose tissue weight was significantly different being lower in CD + R than in CD. Therefore, in females the *Rubus* extract supplemented to the standard diet reduced body and liver weights and when offered with cafeteria diet, it reduced only inguinal weight after 80 days of treatment.

Male rats fed with cafeteria diets supplemented or not with *Rubus* extract showed higher body, liver and lumbar, inguinal, mesenteric and epididymal adipose tissue weights and higher adiposity index than rats fed with standard diets supplemented or not with *Rubus* extract (table 3), similarly to the results found in females. Related to the supplementation of Rubus extract in the diets, there was only an effect on liver weight that was lower in SD+R when compared to SD male rats at 80 days of experiment as it was also the case in females.

In relation to the high weight of both adipose tissues and adiposity index in female and male animals fed cafeteria compared to those fed the standard diet it is important to point out that it is well know that the white adipose tissue increases its size when a positive energy balance occurs due to either an excess of food intake or to a reduced physical activity occurs. Fundamentally, one of the typical functions of the white adipose tissue is to work as an energy store that can release fatty acids for oxidation by other tissues when energy deprivation occurs. The release of fatty acids leads to a decrease of the lipid droplet in the adipocyte which could reduce the amount of adipose tissue. Moreover, adipose

tissue is also considered an important endocrine organ that regulates different metabolic pathways as well as inflammatory processes, food intake, insulin sensitivity and others (319). It is also known that other factors rather than energy deprivation/excess such as specific nutrients present in the diet could modify the adipose tissue size and its endocrine function. There are key transcription factors that act as nutrient sensors such as the peroxisome-proliferator-activated receptors ($PPAR_{\alpha/\delta/\lambda}$), sterol regulatory element binding proteins (SREBP-1/2) and liver X receptors ($LXR_{\alpha/\beta}$). These transcription factors regulate genes involved in glucose and lipid metabolism including lipolysis and lipogenesis pathways (320).

Increased adiposity such as the one found in our cafeteria fed animals, has been associated for decades with type 2 diabetes development mainly due to the ability of obesity to lead to insulin resistance (321). Furthermore, insulin resistance is related to several other metabolic conditions, i. e., hyperlipidemia, atherosclerosis, hypertension and others (322). On the other hand, it is important to consider that the liver plays an important role on energy metabolism together with adipose tissue. Thus, in order to understand better the effects on lipid and glucose variables of standard and cafeteria diets supplemented with *Rubus* extract given to female and male rats for 80 days, we analyzed plasma and liver parameters.

2.2 Plasma components

Adipose tissue lipolysis is a catabolic process that produces non-esterified fatty acids (NEFA) and glycerol from triacylglycerols (323). It is strictly regulated by humoral factors, such as catecholamines and glucocorticoids, natriuretic peptides and insulin. Catecholamines regulate lipolysis in an unusual manner since these molecules can both activate and inhibit lipolysis. The catecholamines

adrenaline and noradrenaline are the main stress hormones that mediate the adrenergic signalling in the adipocyte surface. When β-adrenergic receptors are activated, lipolysis is stimulated whereas the activation of α_2-adrenergic receptors in adipose tissue leads to lipolysis inhibition. The natriuretic peptides (such as cardiac hormones atrial and B-type natriuretic peptide) regulate positively lipolysis in the adipose tissue while insulin is a strong inhibitor of the lipolysis pathway in the adipocyte (324). In the present study we measured circulating lipolytic products, NEFA and glycerol, in order to evaluate the effects of *Rubus* extract given with SD and CD to female and male rats on *in vivo* lipolytic activity.

Plasma NEFA concentration is shown in figure 81. In females, there were no differences between animals fed with SD and CD, but comparing SD and SD +R diets, SD+R showed higher NEFA concentrations than animals fed with SD. However, in the cafeteria diet groups, CD + R female animals showed lower NEFA concentration than CD. Additionally, figure 82 shows plasma glycerol concentration. In females, CD rats showed higher plasma glycerol concentration than SD animals. In animals supplemented with *Rubus* extract, SD + R females showed higher plasma glycerol concentration than in SD (figure 82). These findings of plasma glycerol levels follow the same trend as NEFA concentrations in females, all of it indicating a significant effect of *Rubus* increasing the lipolytic activity in rats fed the standard diet. This effect completely disappears when rats were fed the cafeteria diet. It is well known that the main fate of circulating NEFA is the liver, where they are partially oxidized and used for ketone body synthesis. Liver synthesized ketone bodies are secreted into the circulation, and therefore we also quantified plasma 3-hydroxybutyrate. As shown in figure 83 the changes in plasma 3-hydroxybutyrate levels parallel those commented for NEFA. No differences were seen between SD and CD in plasma 3-hydroxybutyrate of females but SD + R group showed higher plasma 3-hydroxybutyrate concentration when compared to SD group. Comparing SD + R and CD + R females rats we found lower plasma 3-hydroxybutyrate concentration in CD+R

females at 80 days of experiment (figure 83). Other metabolites were measured in those female animals, like plasma cholesterol concentration that is shown in figure 84 and no differences were seen in female experimental groups. Likewise, plasma triacylglycerol (TG) concentrations (figure 85) also did not show differences among the female groups studied.

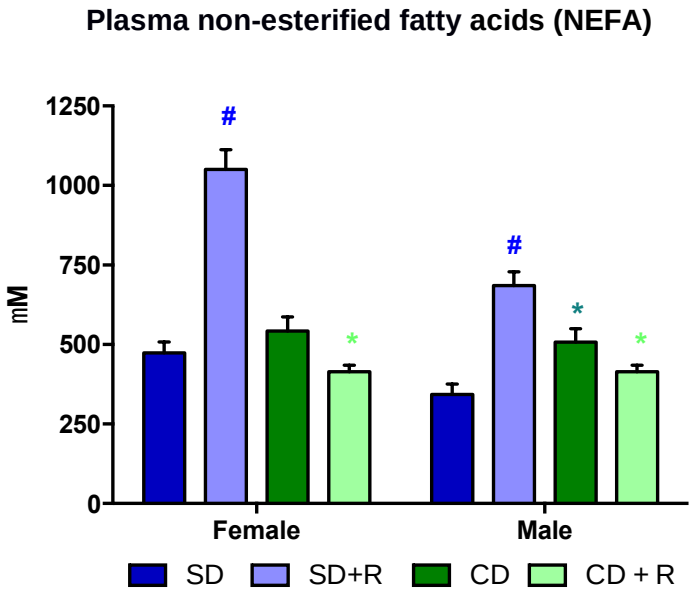

Figure 81: Effects of standard diet, standard diet supplemented with *Rubus* extract, cafeteria diet and cafeteria diet supplemented with *Rubus* extract in plasma non-esterified fatty acids of female (left) and male (right) rats for 80 days.

Significant differences by two-way ANOVA *****, SD *vs* CD; *, SD+R *vs* CD+R, #, SD *vs* SD+R, #, CD *vs* CD+R ($p < 0.05$). SD: standard diet, SD+R: standard diet plus *Rubus*, CD: cafeteria diet, CD+R: cafeteria diet + *Rubus*.

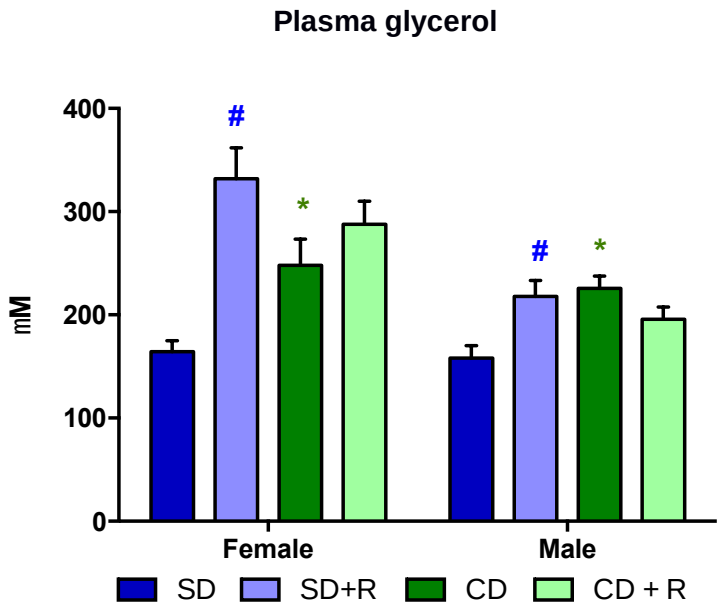

Figure 82: Effects of standard diet, standard diet supplemented with *Rubus* extract, cafeteria diet and cafeteria diet supplemented with *Rubus* extract in plasma glycerol of female (left) and male (right) rats for 80 days.

Significant differences by two-way ANOVA *****, SD *vs* CD; *, SD+R *vs* CD+R, #, SD *vs* SD+R, #, CD *vs* CD+R ($p < 0.05$). SD: standard diet, SD+R: standard diet plus *Rubus*, CD: cafeteria diet, CD+R: cafeteria diet + *Rubus*.

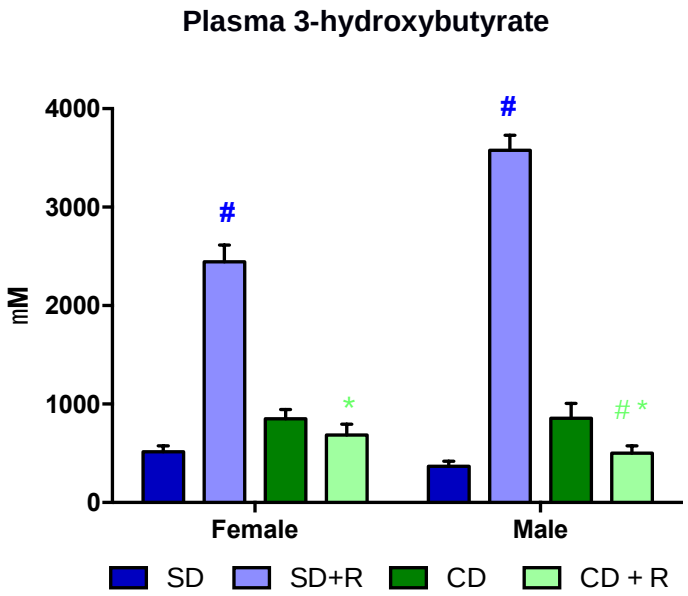

Figure 83: Effects of standard diet, standard diet supplemented with *Rubus* extract, cafeteria diet and cafeteria diet supplemented with *Rubus* extract in plasma 3-hydroxybutyrate of female (left) and male (right) rats for 80 days.

Significant differences by two-way ANOVA *, SD *vs* CD; *, SD+R *vs* CD+R, #, SD *vs* SD+R, #, CD *vs* CD+R ($p < 0.05$). SD: standard diet, SD+R: standard diet plus *Rubus*, CD: cafeteria diet, CD+R: cafeteria diet + *Rubus*.

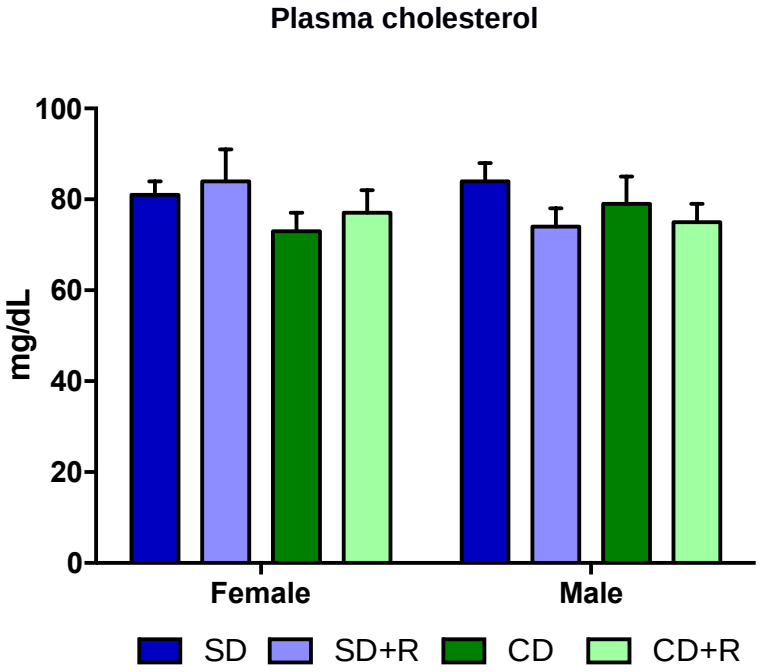

Figure 84: Effects of standard diet, standard diet supplemented with *Rubus* extract, cafeteria diet and cafeteria diet supplemented with *Rubus* extract in plasma cholesterol of female (left) and male (right) rats for 80 days.

No statistically differences were found among SD vs. CD, SD+R *vs* CD+R, SD *vs* SD+R, CD *vs* CD+R ($p < 0.05$) by two-way ANOVA in either females and males groups. SD: standard diet, SD+R: standard diet plus *Rubus*, CD: cafeteria diet, CD+R: cafeteria diet + *Rubus*.

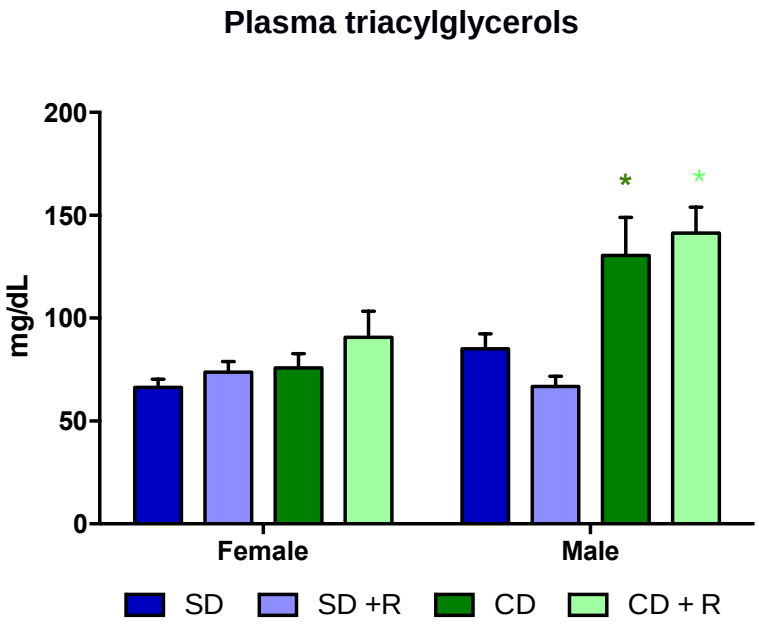

Figure 85: Effects of standard diet, standard diet supplemented with *Rubus* extract, cafeteria diet and cafeteria diet supplemented with *Rubus* extract in plasma triacylglycerols of female (left) and male (right) rats for 80 days.

Significant differences by two-way ANOVA *, SD *vs* CD; *, SD+R *vs* CD+R ($p < 0.05$). SD: standard diet, SD+R: standard diet plus *Rubus*, CD: cafeteria diet, CD+R: cafeteria diet + *Rubus*.

In males the pattern is similar to the one commented in females. To summarize, in male rats fed with CD, NEFA concentration was statistically higher than in those fed with SD (figure 81). When male animals SD were compared to SD + R, the latest showed higher NEFA concentrations than SD male rats as they did in females. Also, males fed CD + R showed lower NEFA concentrations than SD + R. In relation to plasma glycerol concentration males showed equal results as females: higher plasma glycerol in CD when compared to SD and higher plasma glycerol concentrations in SD + R when compared to SD. Moreover, plasma 3-hydroxybutyrate concentrations in males showed similar results to those found in females in the comparison between SD and SD + R, being higher in SD + R than in SD male animals. But differently from females, males fed CD + R showed lower levels of plasma 3-hydroxybutyrate concentrations than CD male rats. Comparing the supplemented groups, CD + R showed lower plasma 3-

hydroxybutyrate concentrations than SD + R. As found in females, no differences were seen in plasma cholesterol concentrations among the studied groups. However, in the case of plasma triacylglycerol concentrations males fed CD showed higher plasma TG concentrations than SD (figure 85). When males SD + R were compared to CD + R, the latest group showed higher TG concentrations differently from the results found in females where no statistical differences were found (figure 85).

To explain all these results, it is important to mention that the adipose tissue is considered the most important source of plasma NEFA throughout lipolysis that is mainly mediated by the enzyme hormone sensitive lipase (HSL) action. It is described that NEFAs circulating in plasma bounded to albumin are transported from adipose tissue to other tissues, mainly liver, to be used there as a source of energy. Thus, in a fasted state, other tissues are able to use these fatty acids as a source of energy (325). On the other hand, a previous study suggests that in obesity, since circulating NEFA are derived mainly from adipose tissue, an important increase in plasma NEFA occurs in fasted obese individuals due to a high amount of adipose tissue shown by these obese individuals and such change itself could lead to insulin resistance (326). However, Karpe et al. (327) analysing data from two cohorts, Paris Prospective Study (5,790 individuals) and Oxford Biobank (1,591 individuals), described that plasma NEFA concentrations did not increase in the same proportion as the adipose tissue content. Therefore, the fasting plasma NEFA concentrations seem not to be proportionally increased as a result of a high amount of adipose tissue in the body, as discussed by Karpe et al. (327). Indeed, in the present study, no differences were seen in NEFA levels between females from SD and CD groups (figure 81), but males fed with CD showed NEFA concentrations slightly augmented when compared to males fed with SD (figure 81). However, in the case of the effects of the *Rubus* extract supplement we found that the *Rubus* extract doubled NEFA plasma concentrations in SD + R compared to SD at day

80 of experiment in both female and male rats but not in animals fed CD + R compared to CD animals. Therefore, we may conclude that our *Rubus* extract when offered with a balanced diet (SD) leads to an important increase in one of the lipolytic products when compared to animals who received not supplemented diet (SD *vs* SD + R).

Together with NEFA released from adipose tissue, plasma glycerol, which forms part of the triacylglycerols, is also released to plasma by adipose tissue lipolysis. The main fate of plasma glycerol is the liver where it could be used as substrate for gluconeogenesis (that produces glucose to be released to plasma in order to be used by extrahepatic tissues) (323, 325) or be used for the synthesis of triacylglycerols. However, part of the glycerol formed by lipolysis could be re-utilized by the adipocytes through the action of the enzyme glycerol kinase (GK), which under normal conditions shows a low activity in the adipose tissue (255). However, under conditions of an increased GK activity in the adipose tissue, the increased amount of glycerol-3-phophate enhances the intracellular re-esterification of fatty acids and consequently the TAG synthesis, which could contribute to an increase in the adipose tissue weight. Indeed, in the present study, female and male animals fed CD and CD + R showed higher body weight (figure 80) and adiposity index (table 3) when compared to SD and SD + R animals respectively. Therefore, the measurement of GK activity in adipose tissue would be necessary to evaluate if glycerol from lipolysis in our animals was being re-esterified intracellularly for TAG synthesis in the adipocytes and could contribute to such changes in the adiposity index. Values are shown below.

As NEFA reach the liver, they could be either re-esterified with glycerol for synthesis of triacylglycerols or phospholipids or be β-oxidized and broken down into molecules of acetyl-CoA that are used to produce ATP for the hepatocyte. However, it is known that liver mitochondria not only generate energy for liver cells but also provide metabolic fuels for extra-hepatic tissues through fatty acid oxidation and consequent ketone bodies production. Therefore, surplus acetyl-

CoA (not used to generate energy to hepatocyte) is metabolized into ketone bodies (3-hydroxybutyrate and acetoacetate) in the mitochondria and released to plasma to be used as a source of energy for different extra-hepatic tissues (328). As reported above, plasma 3-hydroxybutyrate concentrations in animals fed SD + R was particularly higher than in SD in both females and males which also fits with their increased adipose tissue lipolytic activity (329). In summary, we found three lipolytic products in higher concentrations in plasma of animals fed SD + R compared to SD: NEFA, glycerol and the ketone body 3-hydroxybutyrate, and could conclude that in rats fed the standard diet the *Rubus* extract has enhanced adipose tissue lipolysis, contributing to their lower adipose tissue mass.

As commented, our findings showed for the first time that the dietary supplement of *Rubus* extract given to rats on standard diet increased adipose tissue lipolytic activity in both female and male rats. The massive increased 3-hydroxybutyrate plasma concentration in females and males fed SD + R indicated an important increase in lipolysis and β-oxidation (figure 83). It is possible that the female and male animals receiving supplement of *Rubus* extract were in a catabolic state despite of a normal caloric intake shown throughout 80 days of experiment (figure 77). Our findings agree with those reported by Park showing that a natural phenolic compound from *Rubus idaeus L.* (Raspberry) was able to increase both lipolysis and fatty acid oxidation in 3T3-L1 adipocytes *in vitro* (330). However, the response in rats given a CD supplemented with *Rubus* extract was not as evident as in those fed with SD. Although more studies are required, it is possible that the hypercaloric content of the CD diet together with high body and adipose tissue weights and high insulin resistance impaired the *Rubus* extract effect on lipolysis in females and males.

2.3 Liver lipid metabolism

As described above, the main fate of circulating NEFA and glycerol, products of lipolysis, is the liver. Therefore, we decided to evaluate the total liver lipid content and the amount of triacylglycerols (TAG) of female and male rats given SD and CD supplemented or not with *Rubus* extract, rich in anthocyanins, for 80 days.

Figure 86 shows total liver lipid content in females and males fed with the experimental diets. Females fed with SD + R showed higher concentrations of total lipids in liver than animals fed SD. And this difference coincides with the concentration of liver triacylglycerol in the same groups (figure 87). At the same time, in relation to liver triacylglycerol concentrations, females fed CD showed higher concentrations than SD at 80 days of treatment (figure 87).

Following the same trend that we found in females, males total liver lipids were higher in SD than in SD + R group (figure 86). Also, liver triacylglycerol concentrations like in females were higher in SD + R than in SD, and in males fed CD were higher than in those fed SD (figure 87). Differently from females, males fed with CD + R diet showed higher liver triacylglycerol concentrations than SD + R.

Therefore, when the animals received the cafeteria diet there was no influence of the *Rubus* extract supplement in liver total lipids of females and males whereas the *Rubus* extract increased liver lipids, mainly corresponding to triacylglycerols in those animals fed with standard diet. These changes agree with those described above where lipolysis is involved.

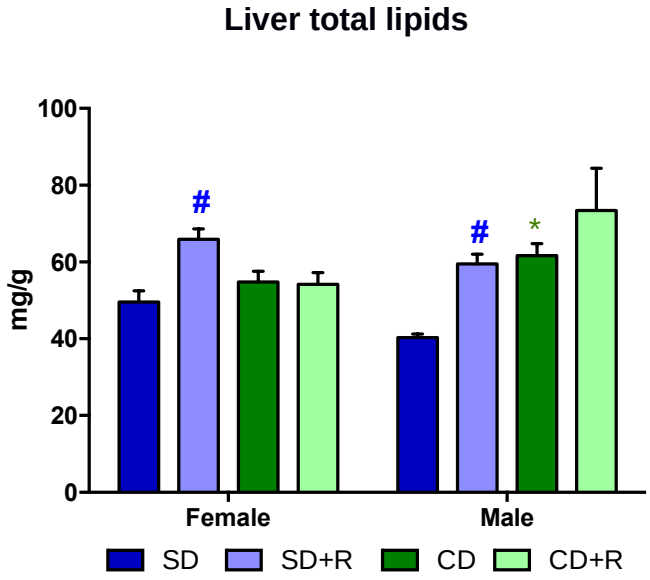

Figure 86: Effects of standard diet, standard diet supplemented with *Rubus* extract, cafeteria diet and cafeteria diet supplemented with *Rubus* extract in liver total lipids content of female (left) and male (right) rats for 80 days.

Significant differences by two-way ANOVA *****, SD *vs* CD; *, SD+R *vs* CD+R, #, SD *vs* SD+R, #, CD *vs* CD+R ($p < 0.05$). SD: standard diet, SD+R: standard diet plus *Rubus*, CD: cafeteria diet, CD+R: cafeteria diet + *Rubus*.

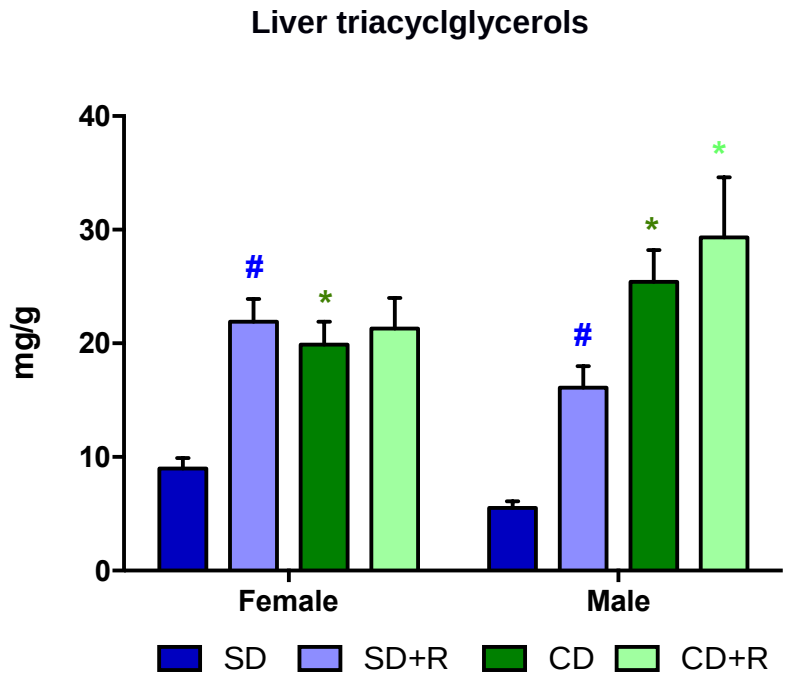

Figure 87: Effects of standard diet, standard diet supplemented with *Rubus* extract, cafeteria diet and cafeteria diet supplemented with *Rubus* extract in liver triacylglycerol content of female (left) and male (right) rats for 80 days.

Significant differences by two-way ANOVA *, SD *vs* CD; *, SD+R *vs* CD+R, #, SD *vs* SD+R, #, CD *vs* CD+R ($p < 0.05$). SD: standard diet, SD+R: standard diet plus *Rubus*, CD: cafeteria diet, CD+R: cafeteria diet + *Rubus*.

As described in section 2.2, when non-esterified fatty acids (NEFA) reach the liver they are converted either to their acyl-CoA form to be re-esterified to produce TAG and phospholipids or they β-oxidized inside the mitochondria. Our results suggest that both pathways are enhanced in animals fed a standard diet supplemented with *Rubus* extract.

The increased lipolytic activity shown by higher plasma NEFA, glycerol and 3-hydroxybutyrate in the female and male rats fed the *Rubus* supplemented diet together with the increased liver TAG content in these animals is consistent with a catabolic state in the adipose tissue despite of the normal energy intake (figure 77). In the present study, we found that the *Rubus* extract offered with standard diet is able to lead the animals to a similar state as observed during

fasting periods. In other words, our *Rubus* extract, rich in anthocyanins, increased plasma glycerol, NEFA, ketone bodies (3-hydroxybutyrate) and liver TAG as well as decreased insulin and glucose concentrations as shown below in section 2.4 in female and male rats fed with supplemented standard diet for 80 days of experiment. To have a better understanding of the health benefits referred to berries consumption we also analysed the glucose metabolism.

2.4 Glucose metabolism

In type 2 DM, it is described an important role of oxidative stress and chronic nonspecific inflammation. Thus, due to the described effect of hyperglycaemia-induced oxidative stress (331), in the present study, we also evaluated the effect of the *Rubus* extract, rich in anthocyanins, in glucose metabolism in rats fed standard and cafeteria diet.

As shown in figure 88 plasma glucose concentrations in female fed with SD + R were lower than those on SD. When SD + R females were compared to CD + R, glucose concentration was lower in animals fed with SD + R than in CD + R. Following the same trend, when we analysed plasma insulin concentrations in these females (figure 89), we found that SD + R showed lower plasma insulin concentrations when compared to CD + R and also SD + R showed lower plasma insulin concentrations than SD. However, females fed with CD showed higher plasma insulin concentration than SD. When we calculated HOMA values of females as index of insulin resistance, we found higher values in calculated HOMA of CD when compared to SD (figure 90). When the HOMA values of SD females were compared to SD + R, SD showed higher HOMA values than SD + R, indicating that *Rubus* supplement has caused an increased insulin sensitivity in these animals. Finally, when comparing SD + R and CD + R HOMA values, SD + R females showed lower values than CD + R. Therefore, all these changes indicated that the *Rubus* supplement reduces plasma insulin levels and increases

insulin sensitivity in female rats fed the standard diet. However, the cafeteria diet clearly enhanced insulin resistance and it exists the possibility that such decreased insulin responsiveness impaired the response to the *Rubus* extract supplement.

In male rats, the results concerning plasma glucose concentrations were similar to the ones found in females, i. e., lower plasma glucose concentrations in SD + R than in SD males and higher plasma glucose concentrations in CD + R when compared to CD male group (figure 88). In plasma insulin concentrations in males, the results were also similar to females (figure 89). SD + R male group showed lower plasma insulin concentrations than in males from SD group. When males fed with CD + R were compared to SD + R, CD + R males showed higher plasma insulin concentrations than SD + R (figure 89). Figure 90 shows the calculated HOMA values in males, and as it can be seen, they are similar to the results found in female animals. When comparing SD and CD groups, CD male animals showed higher HOMA values than SD animals. Also, CD + R males showed higher HOMA values than SD + R animals (figure 90), and no differences between CD + R in comparison to CD rats were found.

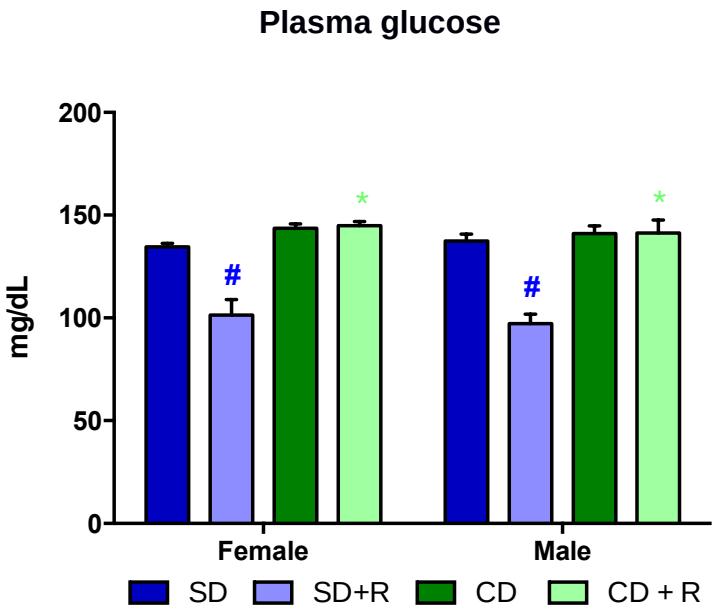

Figure 88: Effects of standard diet, standard diet supplemented with *Rubus* extract, cafeteria diet and cafeteria diet supplemented with *Rubus* extract in plasma glucose of female (left) and male (right) rats for 80 days.

Significant differences by two-way ANOVA *, SD+R *vs* CD+R, #, SD *vs* SD+R, #, CD *vs* CD+R ($p < 0.05$).
SD: standard diet, SD+R: standard diet plus *Rubus*, CD: cafeteria diet, CD+R: cafeteria diet + *Rubus*.

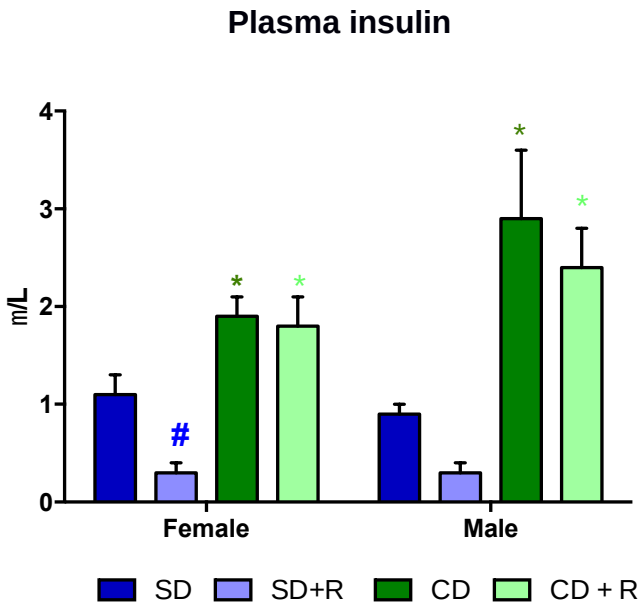

Figure 89: Effects of standard diet, standard diet supplemented with *Rubus* extract, cafeteria diet and cafeteria diet supplemented with *Rubus* extract in plasma insulin of female (left) and male (right) rats for 80 days.

Significant differences by two-way ANOVA *****, SD *vs* CD; *, SD+R *vs* CD+R, #, SD *vs* SD+R, #, CD *vs* CD+R ($p < 0.05$). SD: standard diet, SD+R: standard diet plus *Rubus*, CD: cafeteria diet, CD+R: cafeteria diet + *Rubus*.

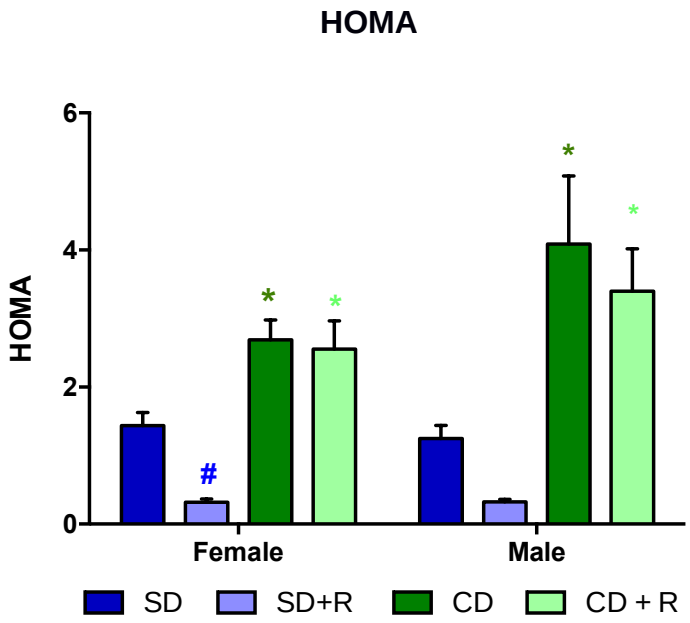

Figure 90: Effects of standard diet, standard diet supplemented with *Rubus* extract, cafeteria diet and cafeteria diet supplemented with *Rubus* extract in HOMA value of female (left) and male (right) rats for 80 days.

Significant differences by two-way ANOVA *, SD *vs* CD; *, SD+R *vs* CD+R, #, SD *vs* SD+R, #, CD *vs* CD+R ($p < 0.05$). SD: standard diet, SD+R: standard diet plus *Rubus*, CD: cafeteria diet, CD+R: cafeteria diet + *Rubus*.

These findings of reduced plasma glucose and insulin levels in *Rubus* supplemented rats fed the SD must be a consequence of increased insulin sensitivity induced by the *Rubus* itself which was reflected in the calculated HOMA values shown in figure 90. As commented, the effects of *Rubus* extract on reducing insulin resistance were not seen in animals fed the cafeteria diet. In the present study, the cafeteria diet consumption lead to an increased body and adipose tissue depots weight besides of a very high insulin resistance that was not improved by the *Rubus* extract consumption. In part, this result may be due to the fact that CD consumption provokes itself an intense insulin resistance, in agreement to previous results (65, 66). But on the other hand, it has been reported that the bioavailability of blackberry anthocyanins in rats is very low (317). Thus, it is possible that the positive effects described by other studies after

blackberry consumption by obese animals used higher anthocyanins doses than the one we used in the present study.

However, in animals fed the standard diet the *Rubus* extract used in the present study was able to produce an important increase in insulin sensitivity in female when compared to not supplemented animals on SD. This effect appeared not to be sufficient to decrease adipose tissue lipolysis, but probably the low levels of insulin present in these animals have been able to compensate for their enhanced insulin sensitivity allowing them to maintain an enhanced lipolytic activity.

The mechanisms by which anthocyanins show hypoglycaemic effects are still not clear. *In vitro* studies showed that anthocyanins could work as anti-diabetic drugs reducing glucose availability in gut (332, 333) such as acarbose whose target is the carbohydrate digestion (334). This drug reduces glucose absorption by intestinal absorptive cells and therefore prevents the increase of plasma glucose concentrations (334). Akkarachiyasit et al (335) evaluated the effects of certain anthocyanins in the activity of α-glucosidase and α-pancreatic amylase and suggested that anthocyanins are extremely efficient inhibitors of these enzymes that digest carbohydrates. Thus, it is possible that, in our study, in animals fed SD the anthocyanins reduced glucose availability for intestine absorption by decreasing carbohydrate digestion and that this effect could be supressed by the ingredients of our cafeteria diet. In fact, as shown in figure 90 no differences were found in insulin resistance (HOMA) between animals fed with supplemented and not supplemented CD. The higher amount of carbohydrates and/or lipids of the CD when compared to SD could modify the carbohydrate digestion limiting the inhibitory effect of the anthocyanins in animals fed CD diet. In agreement with this interpretation, previous reported studies have shown that the amount of anthocyanin absorbed also vary accordingly to the food matrix (336, 337).

Moreover, it is known that one of the diabetic complications is the production of reactive oxygen species (ROS) mainly by neurons and vascular cells but also by other tissues. One of the important targets of the ROS produced by these tissues are the pancreatic β-cells that are responsible to produce and release insulin to the blood torrent when hyperglycaemia occurs. Since pancreatic β-cells express a reduced number of antioxidants enzymes, such as catalase and superoxide dismutase, these cells become particularly sensitive to damage caused by ROS (oxidative stress) (338, 339). In fact it is described that β-pancreatic cells from type 2 diabetic mice when treated with antioxidants reduce the glucose-stimulated insulin secretion and supress apoptosis of β-pancreatic cells without changing the rate of β-cells proliferation, i. e. preserving pancreatic cell mass (281, 338, 339). Thus, it is possible that, in the present study, our *Rubus* extract rich in anthocyanins protected β-pancreatic cells of SD + R female rats from glucose-induced oxidative stress leading to a reduction of plasma glucose and insulin concentrations and HOMA value when compared to not supplemented rats (SD) (figures 88 – 90).

Furthermore, Scazzocchio et al. studied PPARγ gene expression in human omental and murine adipocytes and found that anthocyanin increased glucose uptake through glucose transporter type 4 (GLUT4) that was caused by increased PPARγ activity (340). Although GLUT4 is a glucose transporter dependent of insulin present mainly in skeletal muscle, adipose tissue and heart, such study found out that PPARγ activation is also able to increase GLUT4 in plasma membrane in absence of insulin and consequently reduce plasma glucose concentration (340). It is possible that in our study in animals fed the standard diet supplemented with *Rubus* extract the glucose uptake by tissues mediated by GLUT4 was increased and this contributed to their glucose reduction in plasma concentrations (figure 88). However, none of these mechanisms seemed to occur

when the *Rubus* extract was offered with a cafeteria diet in both female and male rats.

It is interesting to point out that, to our knowledge, the positive effects of *Rubus* extract on glucose-insulin relationship found in the present study in healthy rats fed the standard diet is the first time that it is described. In fact, the anti-hyperglycemic effect of blackberry extract has been reported only in diabetic rats (317). On the sight of the reduced HOMA values found in the present study, it could be proposed that the hypoglycemic effects of *Rubus* extract in animals fed SD could be related to an increased insulin sensitivity, though the exact mechanisms involved needs further investigations. Another interesting result that needs additional research is the fact that our CD completely blocked the *Rubus* extract effect observed in animals fed with SD.

2.5 Fatty acids profiles

In the present study we analysed the fatty acid profile of plasma, liver and lumbar adipose tissue of female and male rats fed SD, SD + R, CD and CD + R diets. The cafeteria diets supplemented or not with *Rubus* extract were prepared using not only a higher amount of fat than the standard diets with or without *Rubus* extract, but also a different source of fat (table 2). Thus, the fatty acid profile of the diets shown in figure 75 was different between the standard and cafeteria diets. However, no differences were found in the fatty acid profile between SD and SD + R likewise between CD and CD + R. The CD and CD + R diets showed higher amounts of the saturated palmitic acid (PA) and stearic acid (SA) and monounsaturated fatty acids, oleic acid (OA) and palmitoleic acid (POA) than in SD and SD + R diets. On the other hand, no differences were found in any of the polyunsaturated fatty acids present in the diets (figure 75). It is known that the fatty acids from the diet could be metabolized by the liver and the adipose tissue producing different fatty acid profiles. As shown in the experiment I (section 1.6),

the cafeteria diet was able to modify the fatty acid composition in tissues of rats fed the CD diet for 90 days.

Due to the effects of the *Rubus* extract in rats given a standard diet reported in the previous section which involved mainly an increase in adipose tissue lipolysis as well as an increase in insulin sensitivity, we wanted to investigate whether these results were also related with changes in the fatty acid profile. It is known that lipids are able to activate some kinases that could increase serine-phosphorylation of insulin signalling, like the insulin receptor substrate 1 (IRS1), which can decrease insulin-dependent GLUT4 translocation to the membrane and consequently glucose uptake (341). Additionally, fatty acids can modify inflammatory pathways that are closely related to insulin sensitivity. It is known that n-3 fatty acids are predominantly precursors of anti-inflammatory eicosanoids, whereas n-6 fatty acids are predominantly precursors of the inflammatory ones (342). Thus, in the present section we discuss the effects of the *Rubus* extract supplement on plasma, liver and lumbar adipose tissue fatty acid profiles of female and male rats fed standard and cafeteria diets.

2.5.1 Liver fatty acid profile

Since we found a higher concentration of TAG in liver of SD +R compared to SD in females and males (figure 87), we tried to find out which fatty acids were mainly responsible for such change. Figure 91 shows liver fatty acid profile of females. As it was expected due to the composition of the diets, the concentrations of the saturated fatty acid palmitic acid (PA) and the monounsaturated oleic acid (OA) were higher in females fed with CD than SD, and also, we found a higher concentration of OA in CD + R than in SD + R. However lower linoleic acid (LA) concentrations were found in CD + R when compared to SD + R (figure 91). When SD female rats were compared to SD + R, PA and LA were higher in animals fed with supplemented standard diet when compared to non-supplemented standard animals. As shown in figure 92, in

males, liver fatty acid profile was equal to female liver fatty acid profile except for the higher concentrations of arachidonic acid (AA) in males fed SD + R compared to SD and lower AA in males fed CD + R compared to those fed SD + R.

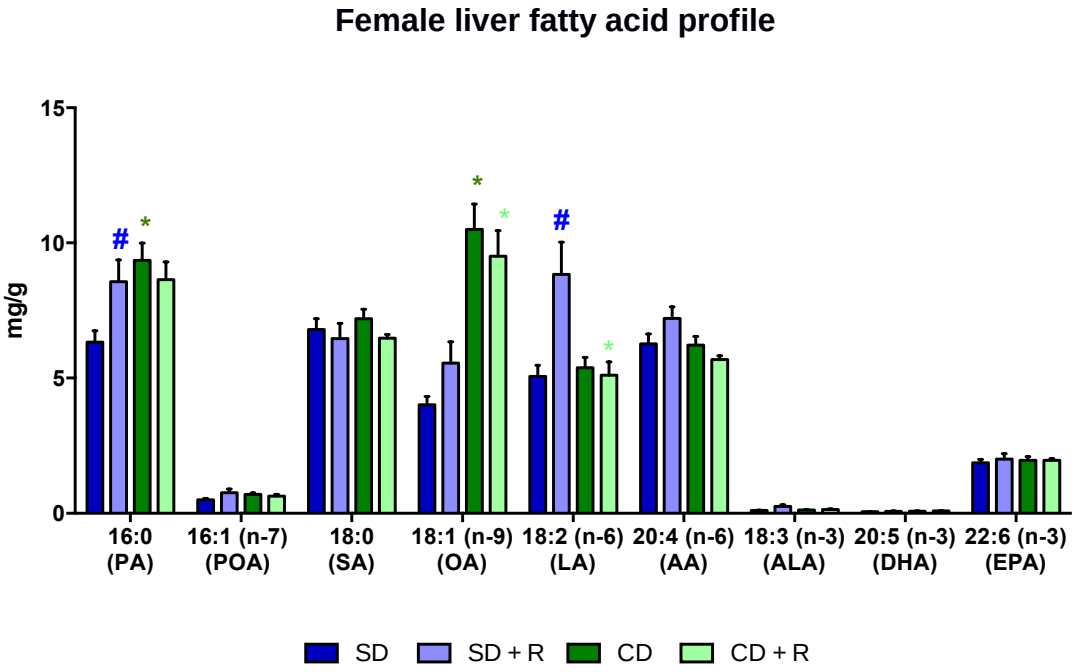

Figure 91: Effects of standard diet, standard diet supplemented with *Rubus* extract, cafeteria diet and cafeteria diet supplemented with *Rubus* extract for 80 days in female liver fatty acid profile.

Significant differences by two-way ANOVA *****, SD *vs* CD; *, SD+R *vs* CD+R, #, SD *vs* SD+R, ($p < 0.05$).
SD: standard diet, SD+R: standard diet plus *Rubus*, CD: cafeteria diet, CD+R: cafeteria diet + *Rubus*.

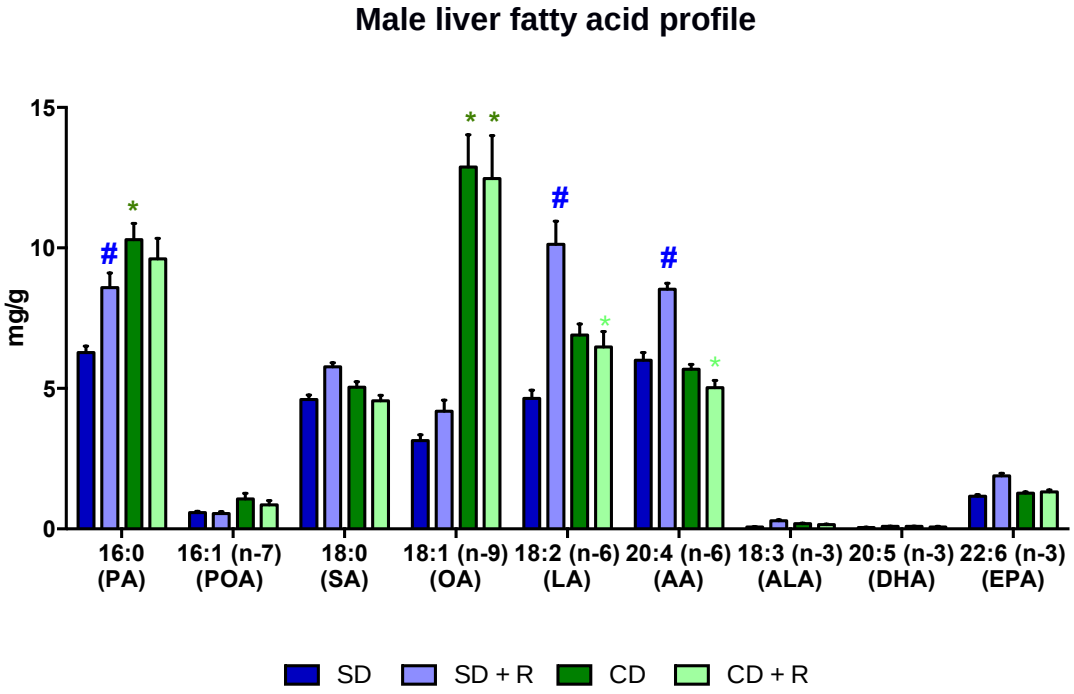

Figure 92: Effects of standard diet, standard diet supplemented with *Rubus* extract, cafeteria diet and cafeteria diet supplemented with *Rubus* extract for 80 days in male liver fatty acid profile.

Significant differences by two-way ANOVA *, SD *vs* CD; *, SD+R *vs* CD+R, #, SD *vs* SD+R, ($p < 0.05$). SD: standard diet, SD+R: standard diet plus *Rubus*, CD: cafeteria diet, CD+R: cafeteria diet + *Rubus*.

As commented above, female and male animals fed cafeteria diet showed higher concentrations of specific fatty acids in liver (figure 92) when compared to standard diet which corresponds to the higher fatty acid concentrations found in the cafeteria diets when compared to standard diets (figure 75). However, previous result of *Rubus* extract effects in plasma and liver of SD + R animals (sections 2.2 and 2.3) showed a higher lipolytic activity in animals fed SD + R compared to CD + R. These results suggest the possibility that the *Rubus* extract could have modified intrinsic liver fatty acid metabolism in addition to those fatty acids reaching the liver as result of the high lipolytic activity in SD + R animals.

There are many different types of fatty acids that can be synthesized by a variety of cell types from the fatty acids obtained through the diet. The length and

saturation level of the aliphatic chains are able to modify the fatty acid function in the cell as a result of desaturase and elongase enzyme activities (343). Since it is known that polyunsaturated fatty acids are synthetized from their corresponding precursors, we calculated the quotient product/precursor of different fatty acids to obtain an index of the different desaturases and elongases in liver that play a role in their conversion: 20:4 n-6/20:3 n-6 (as an index of Δ^5 desaturase), 18:3 n-6/18:2 n-6 (as an index of Δ^6 desaturase), 18:1 n-9/18:0 (as an index of Δ^9 desaturase) 20:3 n-6/18:3 n-6 (as an index of elongase 5), 18:0/16:0 (as an index of elongase 6) and 16:1 n-7/16:0 (as an index of desaturase Scd1).

Liver Δ^5 desaturase activity index

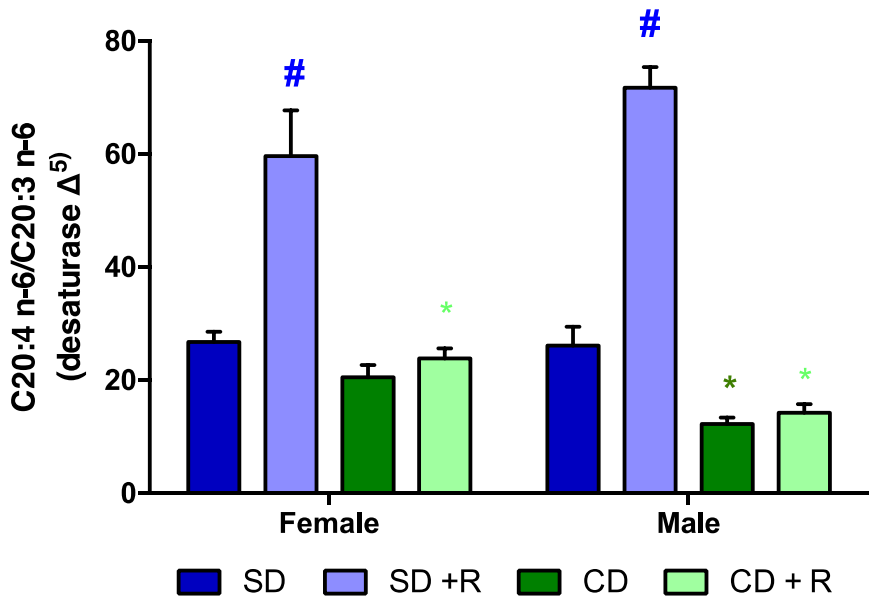

Figure 93: Effects of standard diet, standard diet supplemented with *Rubus* extract, cafeteria diet and cafeteria diet supplemented with *Rubus* extract for 80 days Δ^5 desaturase activity index in liver of female and male rats.

Significant differences by two-way ANOVA *, SD *vs* CD; *, SD+R *vs* CD+R, #, SD *vs* SD+R, ($p < 0.05$).
SD: standard diet, SD+R: standard diet plus *Rubus*, CD: cafeteria diet, CD+R: cafeteria diet + *Rubus*.

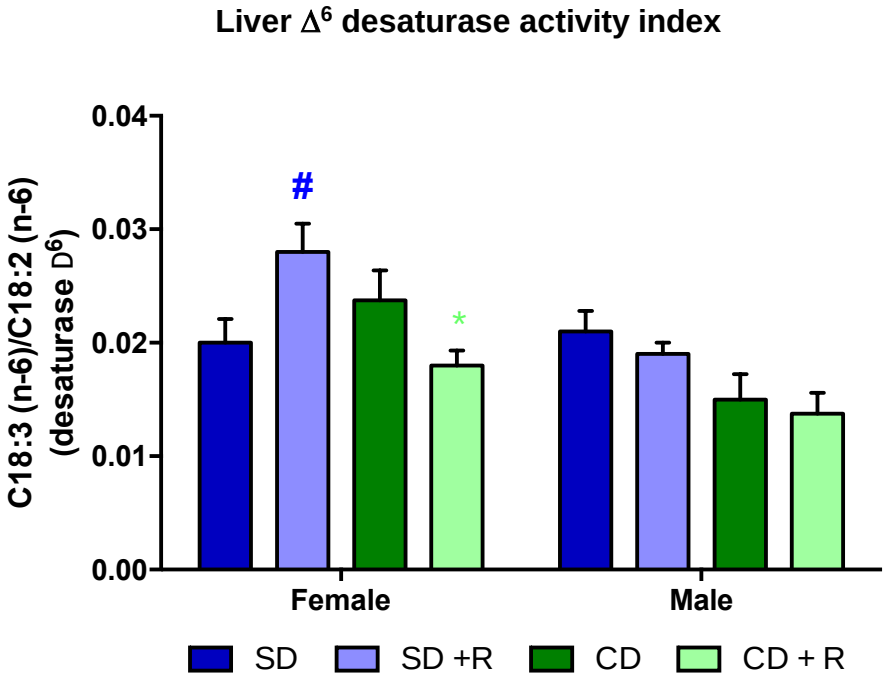

Figure 94: Effects of standard diet, standard diet supplemented with *Rubus* extract, cafeteria diet and cafeteria diet supplemented with *Rubus* extract for 80 days Δ^6 desaturase activity index in liver of female and male rats.

Significant differences by two-way ANOVA *, SD+R *vs* CD+R, #, SD *vs* SD+R, ($p < 0.05$). SD: standard diet, SD+R: standard diet plus *Rubus*, CD: cafeteria diet, CD+R: cafeteria diet + *Rubus*.

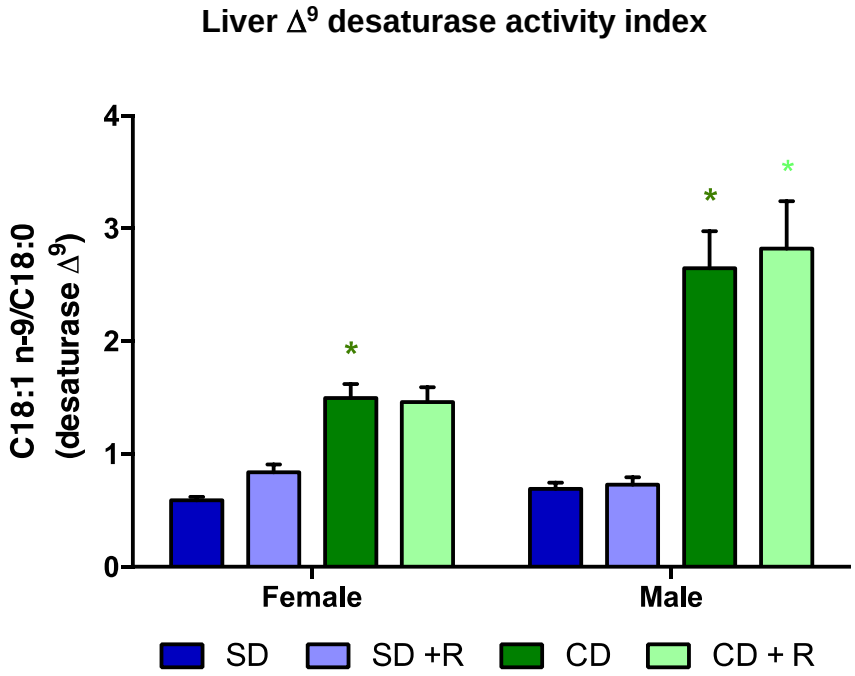

Figure 95: Effects of standard diet, standard diet supplemented with *Rubus* extract, cafeteria diet and cafeteria diet supplemented with *Rubus* extract for 80 days Δ^9 desaturase activity index in liver of female and male rats.

Significant differences by two-way ANOVA *****, SD *vs* CD; *, SD+R *vs* CD+R, ($p < 0.05$). SD: standard diet, SD+R: standard diet plus *Rubus*, CD: cafeteria diet, CD+R: cafeteria diet + *Rubus*.

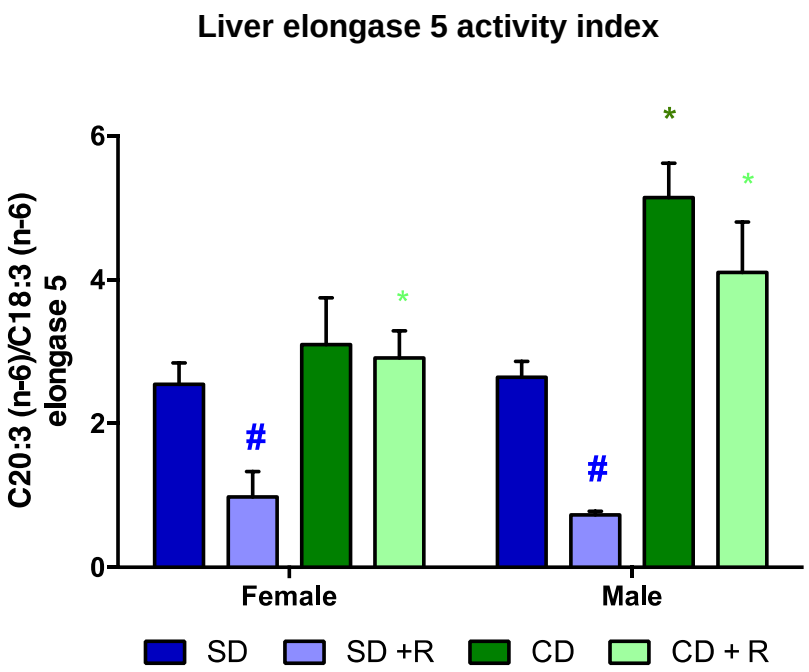

Figure 96: Effects of standard diet, standard diet supplemented with *Rubus* extract, cafeteria diet and cafeteria diet supplemented with *Rubus* extract for 80 days elongase 5 activity index in liver of female and male rats.

Significant differences by two-way ANOVA *****, SD *vs* CD; *, SD+R *vs* CD+R, #, SD *vs* SD+R, ($p < 0.05$).
SD: standard diet, SD+R: standard diet plus *Rubus*, CD: cafeteria diet, CD+R: cafeteria diet + *Rubus*.

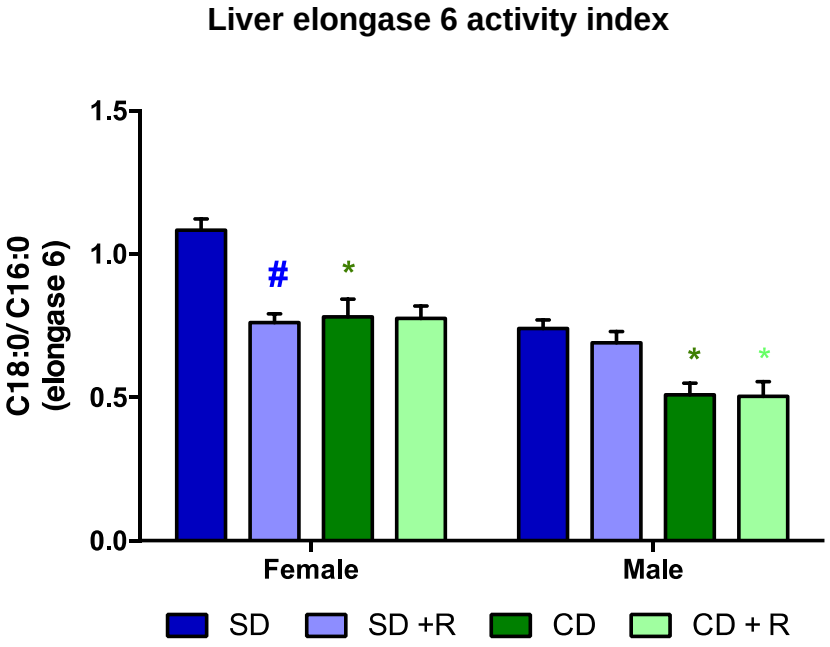

Figure 97: Effects of standard diet, standard diet supplemented with *Rubus* extract, cafeteria diet and cafeteria diet supplemented with *Rubus* extract for 80 days elongase 6 activity index in liver of female and male rats.

Significant differences by two-way ANOVA *****, SD *vs* CD; *, SD+R *vs* CD+R, #, SD *vs* SD+R, ($p < 0.05$).
SD: standard diet, SD+R: standard diet plus *Rubus*, CD: cafeteria diet, CD+R: cafeteria diet + *Rubus*.

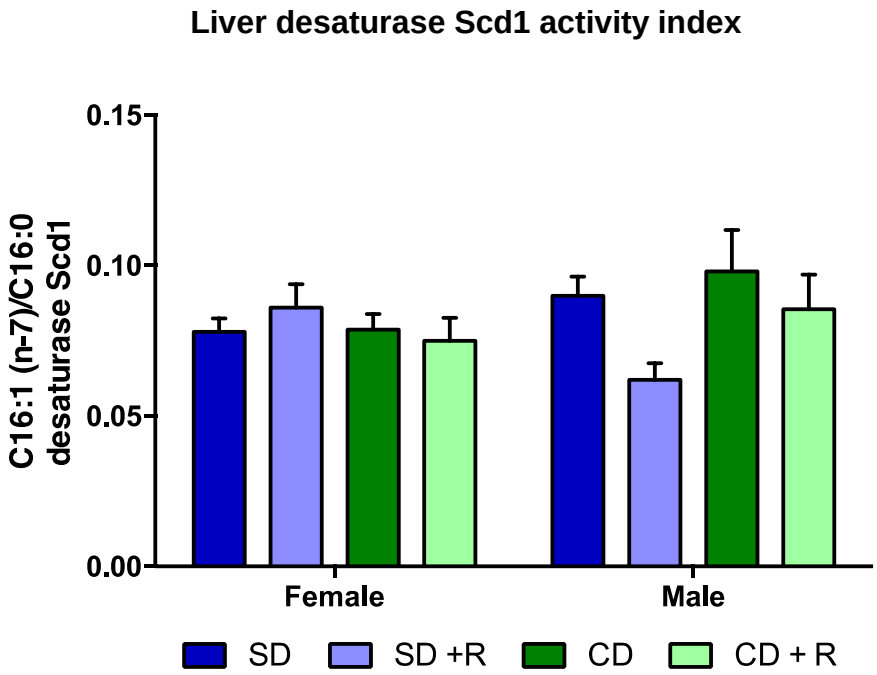

Figure 98: Effects of standard diet, standard diet supplemented with *Rubus* extract, cafeteria diet and cafeteria diet supplemented with *Rubus* extract for 80 days desaturase Scd1 activity index in liver of female and male rats.

No significant differences were found by two-way ANOVA. SD: standard diet, SD+R: standard diet plus *Rubus*, CD: cafeteria diet, CD+R: cafeteria diet + *Rubus*.

The results concerning Δ^5 and Δ^6 desaturase activity indexes in liver allow to have an idea of the activity of the enzymes involved in the endogenous biosynthesis of n-6 and n-3 long chain polyunsaturated fatty acids from their corresponding precursors, LA and LNA respectively. The calculated activity index of these enzymes in females was higher in those fed with SD + R when compared to SD (figures 93 and 94). Between both supplemented groups, females fed with CD + R showed higher Δ^5 desaturase activity index than SD + R, whereas Δ^6 desaturase activity index showed a lower value in CD + R than in SD + R females (figures 93 and 94). However, elongase 5 and elongase 6 activity indexes were lower in SD + R than in SD (figures 96 and 97). Thus, elongase 5 activity index

in females was higher in CD + R than in SD + R (figure 96). Figure 97 shows that elongase 6 activity index was lower in CD than in SD females.

On the other hand, CD female animals showed higher Δ^9 desaturase activity index than SD (figure 95). This enzyme is involved in endogenous biosynthesis of monounsaturated fatty acids that are frequently in higher amounts in tissues, and also it is usually associated with lipogenesis (266-269). No differences were seen between the groups in calculated Scd1 desaturase activity index in females (figure 98).

In males, Δ^5 desaturase activity index in liver was similar to the values calculated for females. Animals fed CD showed statistically lower Δ^5 desaturase activity index in liver than SD (figure 93). Males from SD + R showed higher Δ^5 desaturase activity index when compared to SD as found in females. Also, supplemented male animals showed lower Δ^5 desaturase activity index in CD + R than in SD + R (figure 93). Differently from females, Δ^6 desaturase activity index in liver did not show significant differences among the groups (figure 94). Even though, elongase 5 activity index in liver of males fed with CD was higher than in those fed with SD. Likewise found in females, males fed SD + R showed lower values of liver elongase 5 activity index than in SD and higher CD + R values than in SD + R (figure 96). Elongase 6 activity index in males was partially the same as calculated for females: lower values in CD compared to SD but also lower values in CD + R when compared to SD + R. In other words, the cafeteria groups showed lower elongase 6 activity index than the standard ones when supplemented or not with *Rubus* extract (figure 97).

In the case of Δ^9 desaturase activity index in males, the results were again similar to females: higher values in CD when compared to SD and also higher values in CD + R compared to SD + R (figure 95). Likewise, in females no differences were seen in calculated Scd1 desaturase activity index in males (figure 98).

The higher amount of LA and AA acid found in liver together with the higher Δ^5 and Δ^6 desaturase and reduced elongases activity indexes suggest that there was an increase in the long chain polyunsaturated fatty acid biosynthesis from their corresponding essential fatty acids in the liver of female and male animals fed SD + R diet when compared to SD. However, this effect seems not to occur in animals fed with cafeteria diet supplemented with *Rubus* extract compared to the animals fed with not supplemented cafeteria diet. The regulation of polyunsaturated fatty acid biosynthesis is still not completely understood. Nevertheless, it is known that part of the fatty acid biosynthesis regulation occurs due to a competition of fatty acid substrates, but it is also described that dietary nutrients are able to regulate fatty acid biosynthesis (344).

On the other hand, it is known that a high Δ^9 desaturase activity index is related to increased lipogenesis (266-269). In the present study we found a higher Δ^9 desaturase activity index in female and males fed with CD when compared to SD suggesting a higher lipogenic activity found in liver of animals fed with CD. Additionally, in males fed CD + R the higher Δ^9 desaturase activity index compared to SD + R shows that the *Rubus* extract was not able to reduce lipogenesis in these animals, the effect of the cafeteria diet remains regardless of the presence of *Rubus* extract in the diet. Our findings are in agreement with those reported by López et al. (345) concerning the higher Δ^9 desaturase (stearoyl CoA) gene expression in liver of animals fed cafeteria diet compared to the liver of control diet in rats. The authors found higher expression of genes involved in lipid metabolism in diet-induced obese animals when compared to animals fed with standard diet.

2.5.2 Plasma fatty acid profiles

As differences were found in liver fatty acid profiles, we were also interested to know in which way plasma fatty acids were affected by the different studied diets.

As shown in figure 99, in females, a higher OA concentration was found in CD animals when compared to SD. However, LA and AA were lower in plasma of CD than in SD animals (figure 99). Also, females fed SD + R showed a higher AA concentration than in SD. When comparing SD + R and CD + R groups, CD + R showed higher OA concentration than SD + R, but lower AA concentration in CD + R than in SD + R (figure 99).

Males showed a different plasma fatty acid profile than females. As shown in figure 100, male CD rats showed higher concentrations of PA and OA when compared to SD. Male animals from SD + R group showed lower concentrations of OA and AA when compared to SD animals (figure 100). Additionally, CD + R males showed higher PA, OA and LA than SD + R but lower AA concentrations in CD + R than in plasma of SD + R (figure 100).

Since plasma level of fatty acids are the result of those derived from the corresponding diets and those released from adipose tissue, all of them addressed to liver for their metabolization, we do not think that there is sense to analyze their indexes of desaturases and elongases. In any case, as already commented, the effects of *Rubus* supplement in females was small in what plasma fatty acids is concerned whereas in males fed the standard diet the supplement produced a significant decline in both OA and LA. We do not have enough data to interpret this finding, which would require additional study.

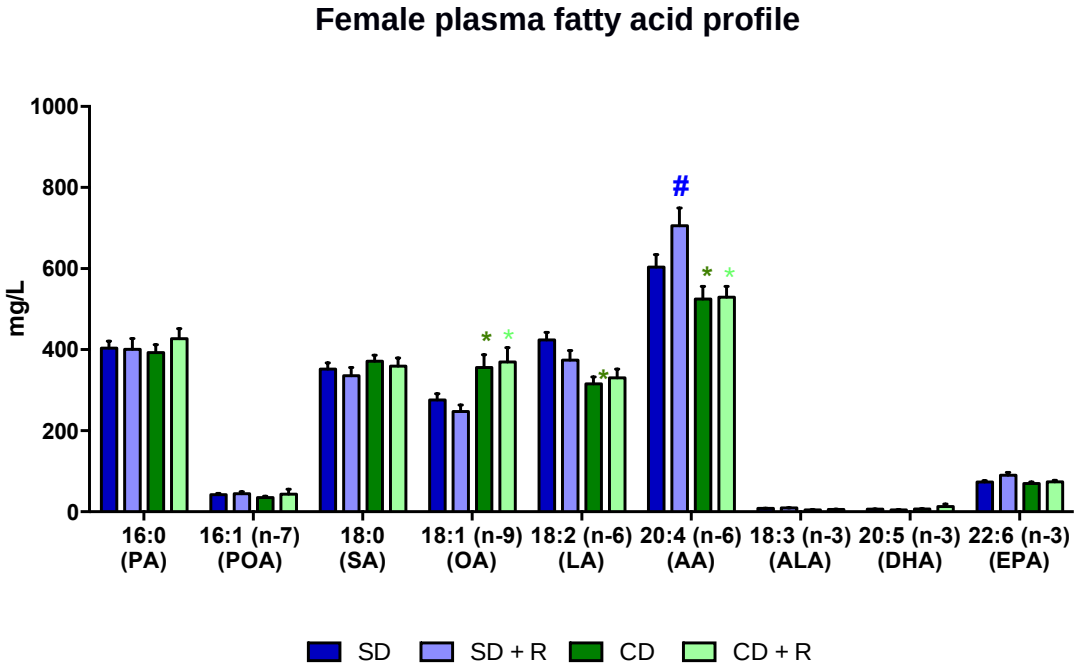

Figure 99: Effects of standard diet, standard diet supplemented with *Rubus* extract, cafeteria diet and cafeteria diet supplemented with *Rubus* extract for 80 days in female plasma fatty acid profile.

Significant differences by two-way ANOVA *****, SD *vs* CD; *, SD+R *vs* CD+R, #, SD *vs* SD+R, ($p < 0.05$).
SD: standard diet, SD+R: standard diet plus *Rubus*, CD: cafeteria diet, CD+R: cafeteria diet + *Rubus*.

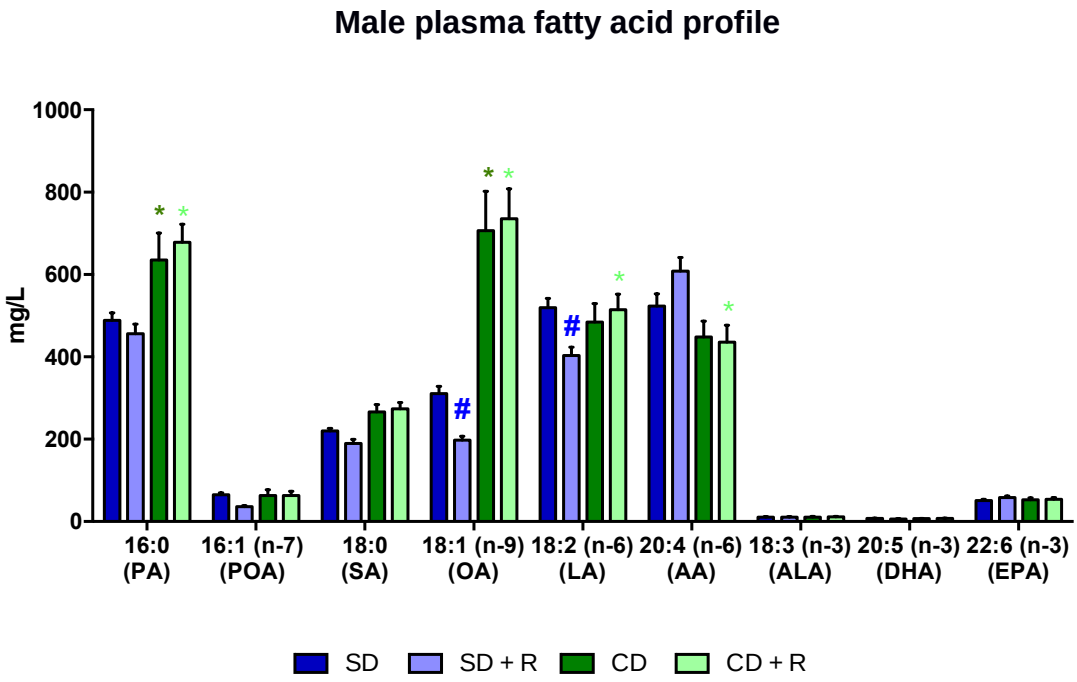

Figure 100: Effects of standard diet, standard diet supplemented with *Rubus* extract, cafeteria diet and cafeteria diet supplemented with *Rubus* extract for 80 days in male plasma fatty acid profile.

Significant differences by two-way ANOVA *, SD *vs* CD; *, SD+R *vs* CD+R, #, SD *vs* SD+R, ($p < 0.05$). SD: standard diet, SD+R: standard diet plus *Rubus*, CD: cafeteria diet, CD+R: cafeteria diet + *Rubus*.

2.5.3 Lumbar adipose tissue

To have a better perspective of the effects of *Rubus* supplement given together with standard and cafeteria diet in lipid metabolism, we have also analyzed the fatty acid profile in lumbar adipose tissue of these animals. As described in section 2.2 we found higher plasma NEFA and glycerol concentrations in both female and male rats fed the standard diet and being supplemented with *Rubus,* indicating an enhanced adipose tissue lipolytic activity. In order to complete this study, we decided to investigate the adipose tissue fatty acid profiles. Figure 101 shows lumbar adipose tissue fatty acid profile in females. Similar to their concentrations in the diets, higher concentrations of the saturated and monounsaturated fatty acids, PA, SA, OA

were found in CD when compared to SD. However, even though LA concentration among the diets were similar, LA concentration in lumbar adipose tissue was lower in CD and CD+R than in SD and SD+R groups. Comparing SD and SD + R, PA concentration was lower in SD + R than in SD. Between the supplemented groups, a higher PA, SA and OA in CD + R than in SD + R but lower LA in CD + R than in SD + R females were seen.

In males, lumbar adipose tissue fatty acid profile was similar to females. The differences found in PA and OA in females were also found in males (figure 102). However, SA concentration in male lumbar adipose tissue was only higher in CD + R compared to SD + R. In the case of LA, a lower concentration was found in CD compared to SD in males, like in females but male animals fed with SD + R showed higher LA concentration than SD. Additionally, CD + R males showed lower LA concentration than SD + R (figure 102).

The effects of the cafeteria diets in lumbar adipose tissue fatty acid profile of female and male rats seem to occur regardless of the *Rubus* extract presence in the diet as it was found in liver (section 2.5.1). As described above, lumbar adipose tissue fatty acid profile of animals fed with CD + R were not affected by the presence of *Rubus* extract since no differences between CD and CD + R were found. Moreover, with the exception LA concentration the differences in most fatty acid concentrations in lumbar adipose tissue found between SD *vs.* CD and SD + R *vs.* CD + R are in agreement with the differences in the fatty acid profile of the diets that the animals received for 80 days of experiment (figure 75), indicating a direct uptake of dietary fatty acids in adipose tissue.

Since linoleic acid (LA) is an essential fatty acid and therefore it cannot be synthesized by the liver, it was intriguing the fact that a higher LA concentration was found in lumbar adipose tissue of both standard groups (SD and SD + R) when compared to the cafeteria groups (CD and CD + R) in female and male rats (figures 101 and 102). Even though the energy intake was higher in animals fed

with the cafeteria diets than in those fed with standard diets (figure 77), the absolute amount of food intake of animals fed with CD and CD + R was lower than in SD and SD + R (figure 76). After a week on a hypercaloric diet, rats reduce the amount of food intake in order to maintain their energy balance. However, in the present study, the reduction of cafeteria diets intake did not reduce the energy intake of these animals to the same amounts of the standard groups as shown in figures 76 and 77. Thus, it is possible that animals fed with SD and SD + R consumed a greater amount of LA (figures 78 and 79) due to their higher food intake than the CD and CD + R animals which lead the standard groups to a higher lumbar adipose tissue accumulation of LA than the cafeteria groups.

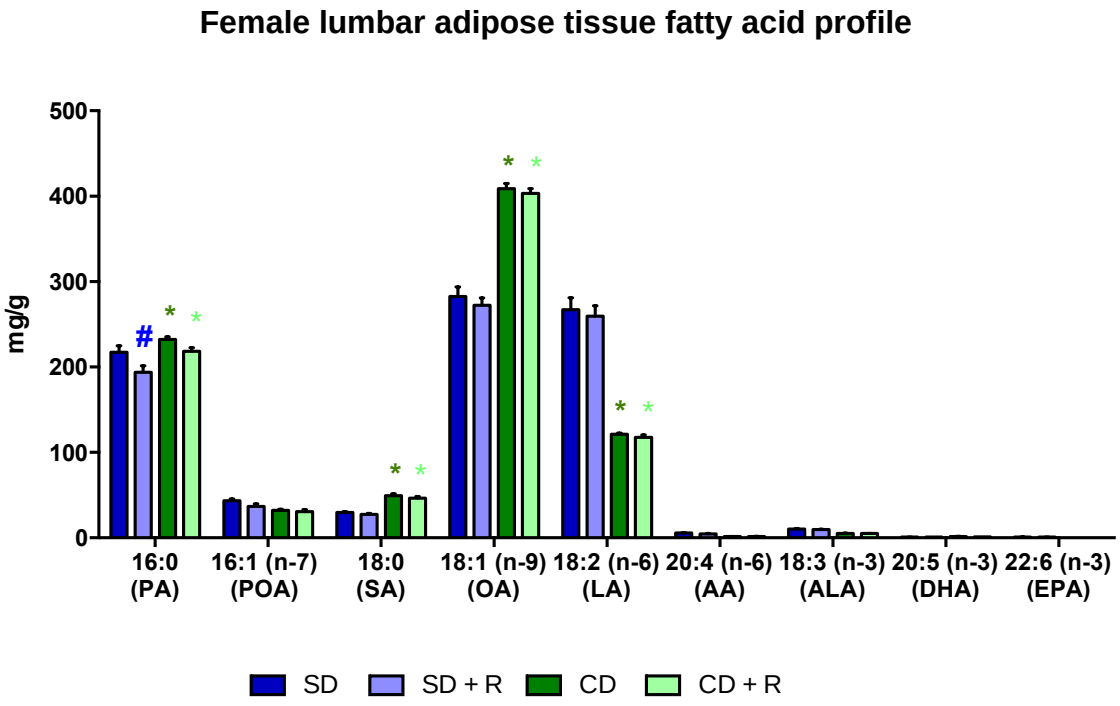

Figure 101: Effects of standard diet, standard diet supplemented with *Rubus* extract, cafeteria diet and cafeteria diet supplemented with *Rubus* extract for 80 days in lumbar adipose tissue profile of female rats.

Significant differences by two-way ANOVA *, SD *vs* CD; *, SD+R *vs* CD+R; #, SD *vs* SD+R, ($p < 0.05$). SD: standard diet, SD+R: standard diet plus *Rubus*, CD: cafeteria diet, CD+R: cafeteria diet + *Rubus*.

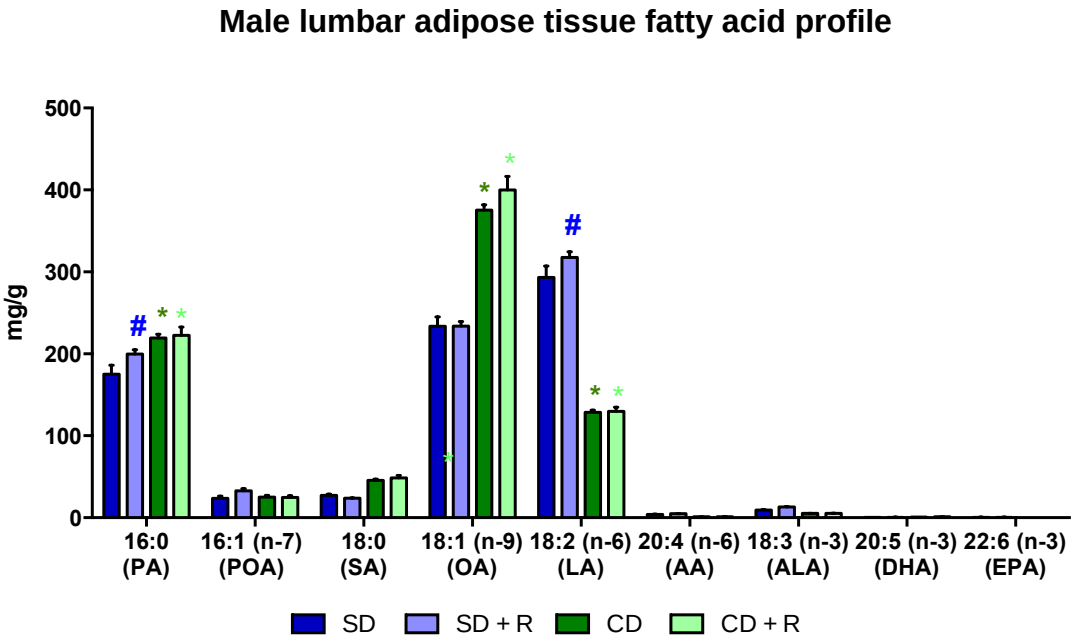

Figure 102: Effects of standard diet, standard diet supplemented with *Rubus* extract, cafeteria diet and cafeteria diet supplemented with *Rubus* extract for 80 days in lumbar adipose tissue profile of male rats.

Significant differences by two-way ANOVA *****, SD *vs* CD; *, SD+R *vs* CD+R; #, SD *vs* SD+R, ($p < 0.05$). SD: standard diet, SD+R: standard diet plus *Rubus*, CD: cafeteria diet, CD+R: cafeteria diet + *Rubus*.

In order to have a deeper understanding of the effects of the diets in the adipose tissue metabolism, we also calculated desaturase and elongase activity indexes in lumbar adipose tissues of females and males fed with standard and cafeteria diets supplemented or not with *Rubus* extract. We calculated the quotient product/precursor of different fatty acids to obtain an index of the different desaturases and elongases as it was done in liver: 20:4 n-6/20:3 n-6 (as an index of Δ^5 desaturase), 18:3 n-6/18:2 n-6 (as an index of Δ^6 desaturase), 20:3 n-6/18:3 n-6 (as an index of elongase 5), 18:0/16:0 (as an index of elongase 6), 18:1 n-9/18:0 (as an index of Δ^9 desaturase) and 16:1 n-7/16:0 (as an index of desaturase Scd1). These indexes are shown in figures 103, 104, 105, 106, 107 and 108. Among them, the most consistent change appeared to be the Δ^5

desaturase index (figure 103), that was clearly lower in both female and male rats fed the CD supplemented or not with *Rubus* as compared to those fed the SD. Such change could be well related to the lower LA concentration seen in those same animals commented above.

To summarize, in lumbar adipose tissue we found less differences in fatty acid profile than the ones observed in liver probably due to the fact that the adipose tissue reflects more directly fatty acid intake whereas the liver modify most of the fatty acids that reach it from the diet or from those released from the adipose tissue.

Lumbar adipose tissue Δ^5 desaturase activity index

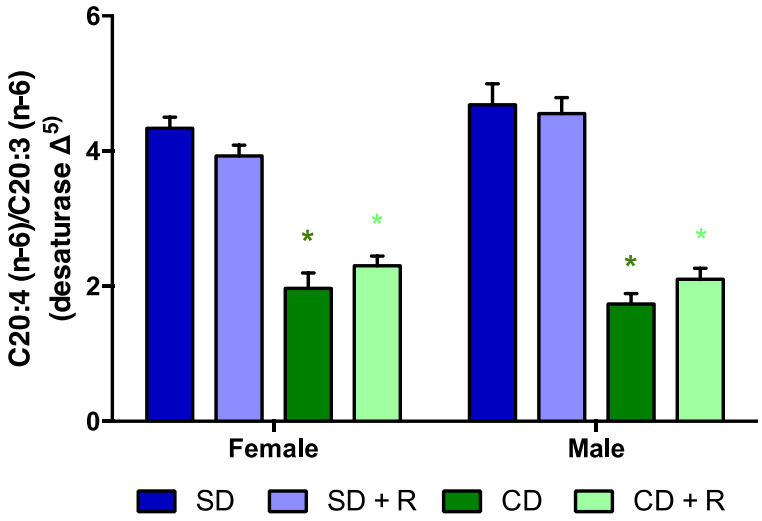

Figure 103: Effects of standard diet, standard diet supplemented with *Rubus* extract, cafeteria diet and cafeteria diet supplemented with *Rubus* extract for 80 days in Δ^5 desaturase activity index in lumbar adipose tissue of female and male rats.

Significant differences by two-way ANOVA *, SD *vs* CD; *, SD+R *vs* CD+R, ($p < 0.05$). SD: standard diet, SD+R: standard diet plus *Rubus*, CD: cafeteria diet, CD+R: cafeteria diet + *Rubus*.

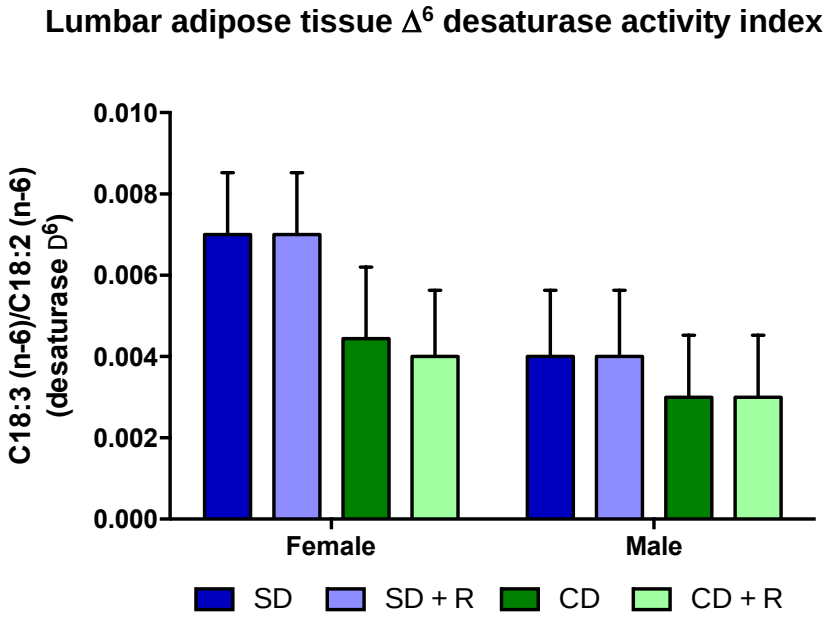

Figure 104: Effects of standard diet, standard diet supplemented with *Rubus* extract, cafeteria diet and cafeteria diet supplemented with *Rubus* extract for 80 days in Δ^6 desaturase activity index in lumbar adipose tissue of female and male rats.

No significant differences were found by two-way ANOVA. SD: standard diet, SD+R: standard diet plus *Rubus*, CD: cafeteria diet, CD+R: cafeteria diet + *Rubus*.

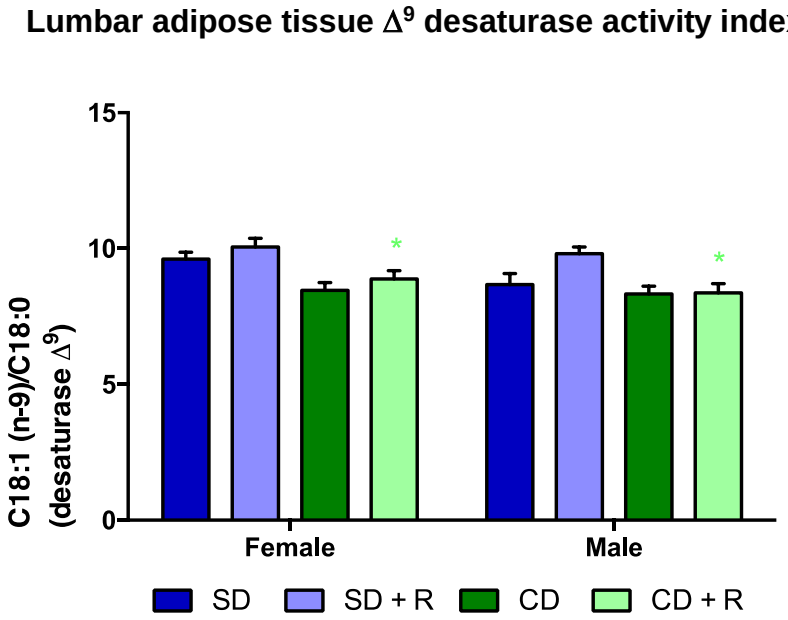

Figure 105: Effects of standard diet, standard diet supplemented with *Rubus* extract, cafeteria diet and cafeteria diet supplemented with *Rubus* extract for 80 days in Δ^9 desaturase activity index in lumbar adipose tissue of female and male rats.

Significant differences by two-way ANOVA *, SD+R *vs* CD+R, ($p < 0.05$). SD: standard diet, SD+R: standard diet plus *Rubus*, CD: cafeteria diet, CD+R: cafeteria diet + *Rubus*.

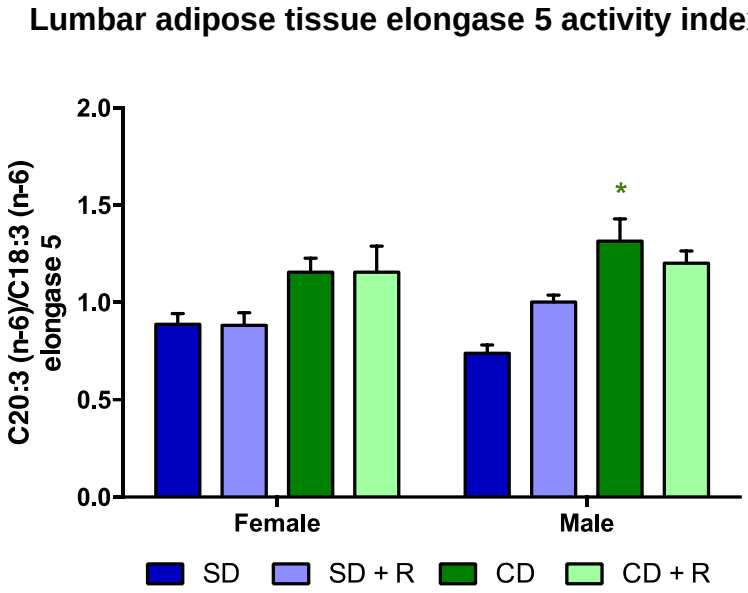

Figure 106: Effects of standard diet, standard diet supplemented with *Rubus* extract, cafeteria diet and cafeteria diet supplemented with *Rubus* extract for 80 days in elongase 5 activity index in lumbar adipose tissue of female and male rats.

Significant differences by two-way ANOVA *, SD *vs* CD, ($p < 0.05$). SD: standard diet, SD+R: standard diet plus *Rubus*, CD: cafeteria diet, CD+R: cafeteria diet + *Rubus*.

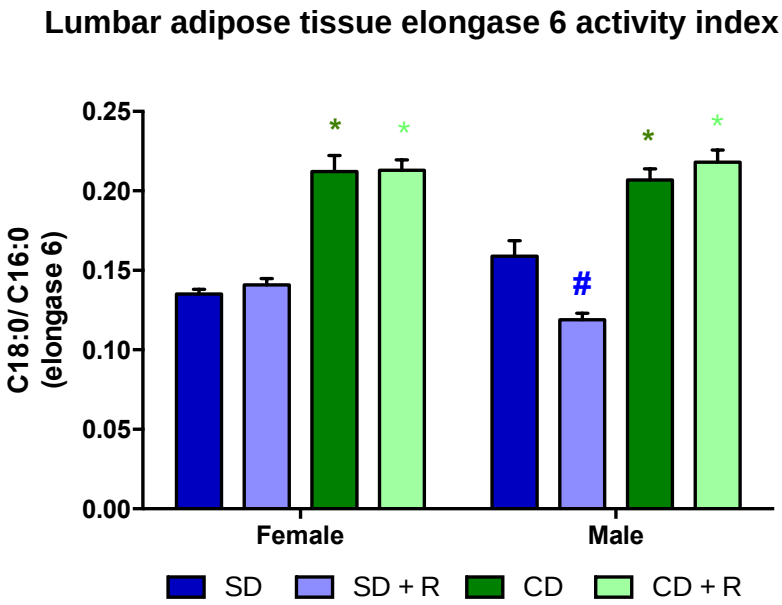

Figure 107: Effects of standard diet, standard diet supplemented with *Rubus* extract, cafeteria diet and cafeteria diet supplemented with *Rubus* extract for 80 days in elongase 6 activity index in lumbar adipose tissue of female and male rats.

Significant differences by two-way ANOVA *****, SD *vs* CD; *, SD+R *vs* CD+R; #, SD *vs* SD+R, ($p < 0.05$).
SD: standard diet, SD+R: standard diet plus *Rubus*, CD: cafeteria diet, CD+R: cafeteria diet + *Rubus*.

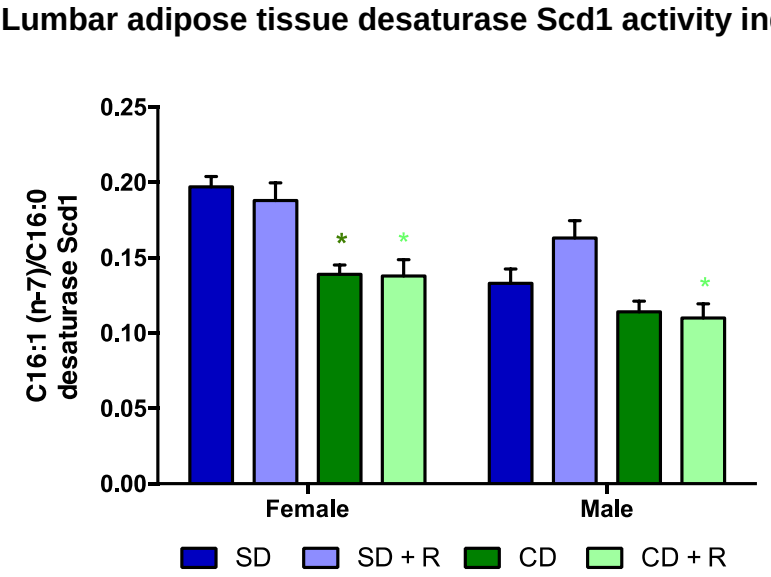

Figure 108: Effects of standard diet, standard diet supplemented with *Rubus* extract, cafeteria diet and cafeteria diet supplemented with *Rubus* extract for 80 days in elongase 6 activity index in lumbar adipose tissue of female and male rats.

Significant differences by two-way ANOVA *, SD *vs* CD; *, SD+R *vs* CD+R, ($p < 0.05$). SD: standard diet, SD+R: standard diet plus *Rubus*, CD: cafeteria diet, CD+R: cafeteria diet + *Rubus*.

Taken together all these results show that *Rubus* extract offered as supplement of the standard diet led to an increase of Δ^5 and Δ^6 desaturases activity indexes in liver of females and males, when compared to not supplemented animals fed with SD. As described above (sections 2.2 and 2.3), SD + R compared to the SD group of female animals showed higher adipose tissue lipolytic activity which can be connected to the high levels of Δ^5 and Δ^6 desaturase activity indexes in the liver. Also, a high Δ^5 and Δ^6 desaturase activities are related to the high levels of polyunsaturated fatty acids (LA and AA) found in liver of females and males animals fed with SD + R at day 80 of treatment (figures 93 and 94). Furthermore, it is known that desaturase Δ^9 activity index is positively correlated with lipogenesis and adipogenesis (266-269). In fact, in the present study, Δ^9 activity index was higher in animals fed with CD when compared

to SD in liver of females and males (figure 95). It is interesting to point out that the presence of *Rubus* extract in the cafeteria diet did not reduced desaturase Δ^9 activity index in liver, particularly in males (figure 95).

On the face of these results, we have to recognized that the effects of *Rubus* extract offered together with standard and cafeteria diet were not completely consistent. The changes in fatty acid profile in liver, plasma and lumbar adipose tissue and the changes in enzymatic activity indexes were greater in females than in males from SD + R groups when compared to SD groups. In relation to the effects of the cafeteria diet supplemented with *Rubus* extract concerning the fatty acids profile, the results found were not particularly different from those found in the experiment I between SD and CD groups (section 1.6). Thus, it seems that the cafeteria diet impairs the *Rubus* extract effect in fatty acid metabolism. However, in the present study, we are not able to identify if these effects were a direct effect of *Rubus* extract or a secondary effect of the reduced insulin levels or reduced insulin resistance found in these groups (section 2.4). The mechanisms by which *Rubus* extract affects lipid metabolism in a gender dependent manner remains unclear and requires further investigation.

CONCLUSIONES

1. Los pesos de los diferentes tejidos adiposos y de hígados resultaron más altos en las ratas hembra alimentadas con dieta de cafetería que con dieta control (estándar) durante 90 días. Este efecto desaparece cuando tras esa alimentación las ratas hembra son alimentadas con dieta control durante 110 días. Sin embargo, en los machos alimentados con dieta de cafetería no se observa ese incremento de los pesos de tejido adiposo o de hígado. Esa diferente respuesta a la dieta de cafetería en función del género podría ser consecuencia de la conocida disminución del efecto estimulador de los adreno-receptores en tejido adiposo de las ratas hembra tras la ingesta de dieta de cafetería, lo cual no ocurre en los machos.
2. La respuesta de los parámetros metabólicos (niveles plasmáticos de colesterol, triacilgliceroles y ácidos grasos libres, así como los triacilgliceroles y colesterol en hígado) a la alimentación con dieta de cafetería resultó ser mayor en machos que en hembras. Esta diferente respuesta en función del género de las ratas podría ser consecuencia de la mayor masa del tejido adiposo en los machos que en las hembras, lo cual habría producido una mayor respuesta lipolítica a la dieta de cafetería en los primeros.
3. El mayor efecto de la dieta de cafetería sobre el peso del tejido adiposo en hembras frente a los machos podría relacionarse con el mayor efecto de dicha dieta sobre la actividad gliceroquinasa en hembras en comparación con su efecto en los machos.,. La actividad basal de esta enzima en tejido adiposo es baja y su incremento facilita que el glicerol intracelular sea reutilizado para la síntesis de triacilgliceroles, contribuyendo al mayor incremento de masa del tejido adiposo que hemos encontrado en las hembras alimentadas con dieta de cafetería.
4. El efecto de la dieta de cafetería incrementando la actividad de la lipoproteína lipasa en algunos tejidos adiposos resultó ser también mayor en las ratas hembra, pero no en los machos. Dicho efecto debe contribuir también al mayor

efecto de la dieta de cafetería sobre la masa del tejido adiposo en las hembras, y no en los machos.

5. La dieta de cafetería cambió de forma evidente el perfil de ácidos grasos en plasma, hígado y tejido adiposo, tanto en hembras como en machos, con incrementos en la concentración de ácidos grasos saturados y monoinsaturados. Cuando los ácidos grasos fueron utilizados para calcular los distintos índices de desaturasas y elongasas en plasma, hígado y tejido adiposo, resultó que la ingesta con dieta de cafetería tenía efectos específicos sobre ellos, la mayoría de los cuales desaparecieron tras 110 día a dieta control.
6. Los tests de tolerancia a la glucosa oral mostraron un disminuido índice de sensibilidad insulínica en las ratas hembra que fueron alimentadas con dieta de cafetería, mientras que no se observaron cambios en los machos. Este dato es también acorde con la mayor respuesta a la dieta de cafetería que habíamos encontrado en tejido adiposo de hembras en relación a los machos, con un mayor incremento en los niveles de leptina en plasma de las ratas hembras alimentadas con dieta de cafetería, mientras que no es así en los machos.

7. El suplemento de la dieta con extracto de *Rubus* a las ratas alimentadas con dieta control incrementó la actividad lipolítica del tejido adiposo tanto en hembras como en machos. Este resultado, junto al incremento de la concentración de 3-hidroxibutirato en el plasma de estos animales, indica que tanto las hembras como los machos que recibieron el suplemento con extracto de *Rubus* y dieta control durante 80 días, presentaban un estado catabólico, a pesar de tener una ingesta calórica normal.
8. La administración de una dieta de cafetería a machos y a hembras durante 80 días, les produjo un aumento de la adiposidad. Por otro lado, la respuesta al suplemento de extracto de *Rubus* en las ratas alimentadas con dieta de cafetería no fue tan evidente como en las ratas alimentadas con dieta control. Proponemos que el contenido hipercalórico de la dieta de cafetería junto a los altos pesos corporales y de tejido adiposo que encontramos en estos grupos impiden la respuesta lipolítica del extracto de *Rubus* tanto en ratas hembra como en machos.
9. El efecto hipoglucémico del extracto de *Rubus* observado en los animales alimentados con dieta control podría relacionarse con un incremento en la sensibilidad insulínica, aunque el mecanismo implicado requiere de posterior investigación. Por otro lado, cuando el extracto de *Rubus* se administró junto a la dieta de cafetería, el efecto que habíamos observado con la dieta control desapareció completamente.
10. El suplemento de la dieta control con extracto de *Rubus* también produjo un incremento de ácidos grasos poliinsaturados (LA y AA) en el hígado de las hembras y de los machos a los 80 días de tratamiento, lo cual podría relacionarse con los altos índices de actividades desaturasas Δ^5 y Δ^6 - encontrados en el hígado de estos animales. Estos cambios fueron mayores en hembras que en machos.
11. A pesar del efecto de la dieta de cafetería sobre los perfiles de ácidos grasos de los animales que la ingirieron, parece que en estas condiciones

desaparece el efecto del extracto de *Rubus* sobre el metabolismo de los ácidos grasos.

12. De forma global, el suplemento con extracto de *Rubus* en ratas alimentadas con dieta control (estándar) incrementa la sensibilidad insulínica y la lipolisis del tejido adiposo, modificando también el metabolismo de los ácidos grasos en los animales. Sin embargo, cuando dicho suplemento se administra junto a la dieta de cafetería, no se observa dicho efecto. Proponemos que esa falta de efecto en las ratas alimentadas con dieta de cafetería podría ser consecuencia de la alta resistencia insulínica de estos animales.

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APPENDIX

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Journal of Food & Nutritional Disorders

Research Article A SCITECHNOL JOURNAL

Blackberry (*Rubus* sp. var. Loch Ness) Extract Reduces Obesity Induced by a Cafeteria Diet and Affects the Lipophilic Metabolomic Profile in Rats

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Abstract

Blackberries (*Rubus sp. var. Loch Ness*) contain large amounts of anthocyanins and flavonols, which have several health benefits. The present study was designed to determine the effects of a methanolic blackberry extract in rats fed a cafeteria diet. Weaned female rats were assigned to one of three dietary groups: standard pellet diet (SD), cafeteria diet (CD) and cafeteria diet supplemented with *Rubus* extract (CRD) for 90 days. Plasma metabolites and insulin were analyzed with commercial kits and fatty acid profile was measured by gas chromatography whereas other aliquots were subjected to metabolomics fingerprinting analysis using ultra high efficiency liquid chromatography. Lipoprotein lipase (LPL) activity was determined in fat depots by a radiochemical method. In comparison to the SD group, rats of the CD and CRD groups had increased plasma myristic, palmitic and oleic acids and those of the CD group had increased liver and different adipose tissue weights; the area under the curve of glucose and insulin after oral glucose load and inguinal adipose tissue LPL activity were also increased. Any of these variables were lower in rats of the CRD group, which also showed increased plasma triacylglycerols. However, both the CD and CRD decreased the insulin sensitivity index (ISI). The metabolomic variables showed that most of the acyl-carnitines were up-regulated whereas most of the phosphatidylcholines and lysophosphatidylcholines were down-regulated when comparing rats of the CD group versus those of SD, while when comparing CRD versus CD, oleic acid and lysophosphatidylethanolamines as well as phosphatidylserine and lysophosphatidylserine were up-regulated.

In conclusion, besides evidencing the obesogenic and metabolic effects of a cafeteria diet in female rats, results show that such effects are reduced when the same diet is supplemented with this *Rubus* extract, although it did not modify the decreased ISI values.

Keywords: Blackberry fruit extract; Adipose tissue; Insulin sensitivity; Lipoprotein lipase; Metabolomic profile; Rats

Introduction

Anthocyanins have several health benefits such as preventing cholesterol-induced atherosclerosis [1], inhibiting platelet aggregation [2] and having antiinflammatory [3] and anticarcinogenic [4] activities. Blackberry has a high content of phenolic compounds, which have been shown to inhibit oxidation of human LDL and lecithin liposomes [5] in vitro. Furthermore, blackberry anthocyanins suppress cancer cell growth by modifying cancer cell signaling pathways [6,7] and have been shown to improve body weight and body composition and to reduce obesity in mice [8]. Nevertheless, the health benefits of blackberry have not been sufficiently explored [9].

Rodents fed a high-fat diet rapidly develop insulin resistance and impaired activation of the insulin-signaling pathway [10-13]. However, high-fat feeding has been considered a radical dietary intervention, whereas a cafeteria diet, which is a highly palatable hypercaloric diet with a more balanced caloric composition, better resembles a Western diet [14]. The cafeteria diet – composed of high-fat and high-sugar supermarket products – results in obesity, glucose intolerance and insulin resistance in rats [15,16] and hamsters [17], and reduced insulin clearance in mice [18], and has been considered a robust model of metabolic syndrome in humans [19].

Studies carried out in animal models generally focus on a series of metabolic markers or parameters, previously defined as the best or most studied indicators of a given disease. However, little is known about other metabolites that are not considered in these studies. The development of metabolomic techniques is enabling these gaps in knowledge to be corrected; the huge amount of data provided by these tools combined with multivariate analysis can reveal those factors (metabolites) where the diseased condition differs from the normal (healthy) condition [20]. The benefits of a holistic approach using metabolomics may result in identification of new metabolic markers to predict the development of some diseases [21] or in drawing unexpected conclusions when correlations appear.

On the basis of the small amount of information available about the metabolic effects of blackberry, we aimed to determine the effects of a blackberry extract on some metabolic variables and on the lipophilic metabolomics profile in rats fed a cafeteria diet. In order to determine the actual response of the rats to the cafeteria diet, another group of animals fed the standard diet was studied in parallel. Since different sex responses to both high-fat diet [22] and cafeteria diet [23] in terms of adiposity and lipid handling have previously been reported, the present study was carried out exclusively in adult female rats. Multivariate analysis of lipophilic metabolomic profiles was used to integrate all the data.

The results show the expected increase in fat depots and in adipose tissue lipoprotein lipase activity, as well as the low insulin sensitivity index and changes in the metabolomics profile in the rats fed the cafeteria diet. They also show that the dietary supplement with

blackberry extract can reduce the impact of the cafeteria diet on all these variables.

Materials and Methods

Preparation of the Rubus extract

Blackberries (*Rubus* sp. var. Lochness) were kindly provided by Agricola El Bosque (Lucena del Puerto, Huelva, Spain). *Rubus* extracts were obtained by lyophilization and extraction with 80% methanol in water as previously described [24]. For determination of total anthocyanin, the extract was diluted 1:9 (v/v) in methanol, and anthocyanin content was determined quantitatively by the pH differential method previously described [25] with minor modifications. The concentration of anthocyanins was 5.42 g of cyanidin-3-glucoside per 100 g of *Rubus* extract.

Flavonoid content was measured by the aluminum chloride assay [26] using catechin (Sigma Chemical Co., St. Louis, MO) as standard; (-)epicatechin was the predominant flavonoid as described in [27] with a content of 499 ± 8 mg of epicatechin per 100 g of *Rubus* extract.

Animals and experimental design

Female Sprague Dawley rats were obtained from the animal quarter of University San Pablo CEU, Madrid, Spain. The experimental protocol was approved by the Animal Research Committee of the University San Pablo CEU. The rats were weaned at 21 days of age, placed in collective cages (5 per cage) under controlled conditions (22 ± 2°C, 55 ± 10% relative humidity and constant cycle light/dark of 12 h with continuous ventilation). Rats were given a standard pellet diet (Harlan Global Diet 2014, Madison, WI) for 5 days, after which they were randomly assigned to one of three dietary treatment groups: the standard diet group (SD) was maintained on the pellet diet, the cafeteria diet group (CD) was given the cafeteria diet, and the cafeteria plus *Rubus* diet group (CRD) was given the cafeteria diet supplemented with the *Rubus* extract. The cafeteria diets were based in those previously reported by others [28-30] and prepared as a homogeneous paste by mechanically (Sammic, Guipúzcoa, Spain) blending the components. Composition and fatty acid profile of the diets are shown in Table 1 and 2 respectively.

Ingredient (g)	Standard diet (SD)	Cafeteria diet (CD)	Cafeteria plus *Rubus* diet (CRD)
Pellets[1]	100	23	23
Condensed milk[2]	-	35	35
Sugar[3]	-	7	7
Muffins[4]	-	6	6
Croissants[5]	-	6	6
Powder milk[6]	-	15	15
Butter[7]	-	8	8
Rubus Fruticosus extract	-	-	13
Water	-	13	-
Caloric content (kcal)	310	474	474

[1]Harlan, Global Diet 2014, Madison, MI; [2]Nestlé, Barcelona; [3]Azucarera, Madrid; [4]Panrico SA, Barcelona; [5]Bimbo, Barcelona; [6]Central Lechera Asturiana, Asturias; [7]El Pozo, Murcia. Percentual macronutrient components of dry diets, according to the suppliers were: 15.8% protein, 4.4% fat and 69.7% carbohydrate for the Standard diet, and 13.0% protein, 23.1% fat and 63.9% carbohydrate for Cafeteria diet.

Table 1: Composition of the diets per 100 g

Fatty acid	Standard diet (SD)	Cafeteria diet (CD)	Cafeteria plus *Rubus* diet (CRD)
14:00	0.07 ± 0.03^a	4.80 ± 0.10^b	4.81 ± 0.02^b
16:00	4.26 ± 0.39^a	34.9 ± 0.6^b	32.7 ± 0.8^c
18:00	0.69 ± 0.09^a	15.96 ± 0.27^b	14.10 ± 0.17^c
Sum of saturated fatty acid	5.33 ± 0.53^a	56.67 ± 1.00^b	52.55 ± 0.89^c
18:1(n-9)	6.03 ± 0.60^a	46.1 ± 0.7^b	41.2 ± 0.7^c
18:2(n-6)	15.7 ± 1.2^a	17.4 ± 0.3^a	16.7 ± 0.7^a
18:3(n-6)	0.02 ± 0.00^a	0.09 ± 0.00^b	0.08 ± 0.00^b
22:5(n-6)	0.24 ± 0.08^a	0.16 ± 0.03^a	0.23 ± 0.03^a
20:4(n-6)	n.d.	n.d.	n.d.
Sum of n-6 acids	16.0 ± 1.3^a	17.9 ± 0.2^a	17.3 ± 0.8^a
18:3(n-3)	1.03 ± 0.10^a	1.34 ± 0.02^b	1.26 ± 0.02^b
20:5(n-3)	0.02 ± 0.00^a	0.13 ± 0.02^b	0.12 ± 0.00^b
22:5(n-3)	0.11 ± 0.08^a	0.20 ± 0.01^a	0.18 ± 0.02^a
Sum of n-3 acids	1.16 ± 0.17^a	1.74 ± 0.03^b	1.60 ± 0.03^b

Values correspond to mean ± SEM of 3 samples; n.d: not detectable. For the fatty acid profile analysis nonadecenoic acid (19:1) was added as internal standard to aliquots of each diet and they were analyzed in their lipid extracts by gas chromatography. Different lowercase letters mean statistical significant difference between the groups ($p < 0.05$).

Table 2: Concentration of fatty acids in the experimental diets (mg/g)

The CD and CRD were stored at -20°C until use. Rats had free access to the assigned diet and tap water. After 80 days on the experimental diets, rats were subjected to an oral glucose tolerance test (OGTT) that was performed as follows. Tests were conducted between 11:00 and 13:00 after a 3 h fast. After tail blood was collected (time 0), rats received an oral load of 2 g glucose/kg body weight, and blood was collected at 7.5, 15, 30 and 60 min into tubes containing 1 g Na_2EDTA/L. Plasma was separated by centrifugation at 1,500 g for 15 min at 4°C and stored at -80°C until analyzed for glucose and insulin. The insulin sensitivity index (ISI) was calculated as previously described [31] using the following equation: ISI = 10,000/√(FPG x FPI x mean G x mean I) where FPG is fasting plasma glucose (in mg/dL), FPI is fasting plasma insulin (in µL/mL) , and mean G and mean I are the mean glucose and mean insulin concentrations in the same units determined during the OGTT. One week after the OGTT, rats were sacrificed using a guillotine while under CO_2 anesthesia and trunk blood collected into ice-chilled tubes containing 1 g Na_2EDTA/L. Plasma was separated from fresh blood and stored as described above.

Liver and different fat depots were rapidly dissected and placed into liquid nitrogen for weighing, and fat depots were stored at -80ºC until analysis.

Processing of the metabolic variables

Plasma glucose, triacylglycerols (TAG) and cholesterol (Spinreact Reactives, Spain) and non-esterified fatty acids (NEFA) (Wako Chemicals, Germany) were determined with commercial kits by enzymatic methods and insulin was analyzed by ELISA (Mercodia, Sweden). For the analysis of the fatty acids profile, nonadecenoic acid (19:1) (Sigma Chemical Co.) was added as the internal standard to fresh aliquots of each diet and of frozen plasma, which were used for lipid extraction and purification [32]. The final lipid extract was evaporated to dryness under vacuum and the residue resuspended in methanol/toluene and subjected to methanolysis in the presence of acetyl chloride at 80ºC for 2.5 h as previously described [33]. Fatty acid methyl esters were separated and quantified on a Perkin-Elmer gas chromatograph (Autosystem) with a flame ionization detector and a 20 m Omegawax capillary column (internal diameter 0.25 mm). Nitrogen was used as carrier gas, and the fatty acid methyl esters were compared with purified standards (Sigma Chemical Co.). Quantification of the fatty acids in the sample was performed as a function of the corresponding peak areas compared to that of the internal standard. Lipoprotein lipase (LPL) activity was assayed in inguinal and lumbar fat depots in acetone/diethyl ether extracts by the conversion of triolein, [carboxyl-14C] (Perkin Elmer, Boston, MA) to [1-14C]-oleic acid as previously described [34].

Metabolomic analysis

LC-MS grade organic solvents and reagents for the metabolomics analysis were purchased from Fluka Analytical (Sigma – AldrichChemie GmbH, Steinheim, Germany).

Plasma samples were thawed in ice. To remove proteins from the samples, 3 volumes of ice-cold methanol/ethanol 1:1 (v/v) were added to each plasma aliquot and incubated in ice for 5 min. After centrifugation at 16,000 rpm and 4°C for 20 min, supernatants were filtered through a 0.22 µm nylon filter. Quality control (QC) was determined [35] in samples that were prepared independently by following the same protocol by pooling equal volumes from each plasma sample.

The metabolomic fingerprinting analysis of plasma was performed using ultra-high efficiency liquid chromatography (UHPLC) (Agilent 1290 Infinity LC System) in 0.5 µL of extracted plasma samples that were injected to a reverse-phase Zorbax Extend C18 column (2.1 × 50 mm, 1.8 µm, Agilent Technologies) at 60ºC. The composition of the mobile phases was: A - water with 0.1% (v/v) formic acid, and B - acetonitrile with 0.1% (v/v) formic acid. The chromatographic gradient using a constant flow rate of 0.6 mL/min was started at 5% phase B for the first minute, increasing to 80% from 1-7 min, then to 100% from 7-11.5 min, holding at 100% for 0.5 min, finally returning to 5% of phase B from 12 until 15 min (system re-equilibration). Samples were analyzed in positive ESI(+) and negative ESI(-) ionization modes in separate runs of MS, MS/MS analysis, respectively operated in full scan mode from 50-1000 m/z for positive and 50-1100 m/z for negative mode. Capillary voltage was set to 3 kV for positive and negative ionization mode; fragmentor voltage was set to 175 V for positive and 250 V for negative ionization mode; the drying gas flow rate was 12 L/min at 250ºC and gas nebulizer 52 psi. Samples were injected in randomized order in two runs (for positive and negative ion mode). At the beginning of each run, a batch of 10 injections of QC samples was used to condition the column.

For the metabolomics study, MassHunter Workstation Software LC/MS Data Acquisition version B.05.00 (Agilent Technologies) was used for control, acquisition and processing of all data obtained with UHPLC–QTOF/MS. The resulting data file was cleaned of background noise and unrelated ions by the Molecular Feature Extraction (MFE) tool. Alignment and data filtering were performed by Mass Profiler Professional (MPP, version B.12.1, Agilent Technologies) software. Accurate masses of features were searched for possible structure against the online databases such as CEU mass mediator (http://ceumass.eps.uspceu.es/mediator), METLIN (http://metlin.scripps.edu), HMDB (http://hmdb.ca), KEGG (http://genome.jp/kegg) and LipidMaps (http://lipidmaps.org). The identity of compounds was confirmed by LC-MS/MS by using a QTOF (6550 system, Agilent Technologies) with the same chromatographic conditions as used in the primary analysis. Ions were targeted for collision-induced dissociation (CID) fragmentation on the fly, based on the previously determined accurate mass and retention time. Comparison of the structure of the proposed compound with the obtained fragments as well as comparison with the retention time and isotopic distribution of commercially available standards was used to yield to final confirmation of the identity of metabolites.

Statistics and data processing

Statistical analysis for the metabolic variables was carried out using GraphPad Prism 5.O. (GraphPad Software Inc. La Jolla, 115 CA). After checking a normal distribution of the data using the Kolmogorov-Smirnov test, one-way analysis of variance (ANOVA) was used to compare different diets. Bartlett's test was used to prove homogeneity of the variance. When treatment effects were significantly different (p <0.05), Newman-Keuls simultaneous tests were used to establish statistical differences between individual dietary interventions.

For data provided by the metabolomics study, normality was verified by evaluation of the Kolomgorow-Smirnov-Lillefors test and variance ratio by the Levene's test. Differences between experimental groups were performed by one way ANOVA (equal or unequal variance) or non-parametric Kruskal-Wallis test. The levels of statistical significance were set at p<0.05. Statistical analysis were performed using Matlab R2010a (Mathworks) software. The multivariate analysis, statistical calculations and plottings were obtained with SIMCA P+ 12.0 (Umetrics, Umea, Sweden).

Results

Metabolic changes

As shown in Figure 1a, daily food intake by rats of the three groups was similar throughout all the experiment, although due to the higher caloric content of the cafeteria diet the daily energy intake of rats in groups CD and CRD was higher than those in the SD group (Figure 1b).

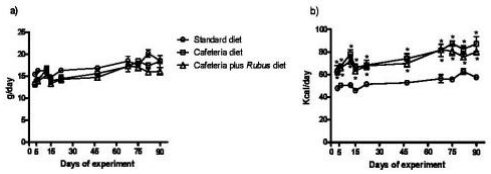

Figure 1: Mean ± SEM of 5 rats/group for daily food (a) and energy intake (b) at different days of experiment in rats fed standard diet (SD), cafeteria diet (CD) or cafeteria plus *Rubus* extract diet (CRD). Asterisks indicate statistical significant difference of the CD or CRD groups versus the SD group whereas no difference was found between these two groups for energy intake nor between the three groups for food intake.

As well as having higher caloric content, cafeteria diets (i.e. both CD and CRD) contain more saturated and monounsaturated fatty acids than the standard diet (SD), whereas their content of n-6 polyunsaturated fatty acids (PUFA) mainly corresponding to linoleic acid (18:2 n-6), is similar and that of n-3 fatty acids mainly corresponding to α-linolenic acid (18-2 n-3), is slightly higher (Table 2). The different fatty acid composition of the diets affected the level of specific fatty acids in plasma: myristic (14:0), palmitic (16:0) and oleic (18:1, n-9) acids were higher in both the CD and CRD groups than in the SD group, whereas no significant differences were found in the plasma concentrations of either stearic acid (18:0) or any of the PUFA between groups (Table 3).

Fatty acid	SD	CD	CRD
14:00	10.9 ± 3.8a	20.6 ± 1.2ab	28.2 ± 4.4b
16:00	280 ± 21a	448 ± 36b	434 ± 47b
18:00	311 ± 10a	368 ± 14a	337 ± 30a
18:1(n-9)	142 ± 13 a	318 ± 73b	402 ± 59b
18:2(n-6)	311 ± 23.2a	322 ± 37a	403 ± 50a
18:3(n-6)	7.07 ± 0.75 a	5.61 ± 0.47 a	7.23 ± 0.60 a
20:4(n-6)	573 ± 49a	548 ± 46a	501 ± 11a
22:5(n-6)	20.5 ± 2.6a	17.5 ± 1.3a	16.3 ± 1.1a
18:3(n-3)	5.53 ± 0.63a	4.99 ± 1.18a	7.90 ± 1.88a
20:5(n-3)	4.89 ± 0.99a	4.22 ± 0.73a	6.89 ± 1.22a
22:5(n-3)	8.88 ± 0.33a	11.7 ± 1.2a	12.0 ± 1.6a
22:6(n-3)	71.5 ± 4.0a	73.4 ± 4.8a	64.8 ± 2.2a

Mean ± SEM of 5 rats per group. For the fatty acid profile analysis nonadecenoic acid (19:1) was added as internal standard to aliquots of frozen plasma and they were analyzed in their lipid extracts by gas chromatography Different lowercase letters mean statistical significant difference ($p<0.05$) between the groups.

Table 3: Fatty acid concentration in plasma (mg/L) of female rats that were fed with standard diet (SD), cafeteria diet (CD) or cafeteria diet supplemented with *Rubus* extract (CRD) for 90 days, when they were sacrificed by decapitation after a 3 h fast.

Body, liver and different fat depot weights and plasma metabolic variables of the rats are shown in Table 4.

	SD	CD	CRD
Body weight (g)	293 ± 10a	356 ± 27a	301 ± 21a
Liver weight (g)	7.99 ± 0.27a	9.05 ± 0.35b	7.87± 0.39a
Weight of total adipose tissue (g)	9.17 ± 0.44a	27.91 ± 5.05b	20.07 ± 3.09b
Weight of inguinal adipose tissue (g)	1.03 ± 0.25a	3.67 ± 0.61b	2.81 ± 0.64ab
Weight of perirenal adipose tissue (g)	0.69 ± 0.06a	2.19 ± 0.22b	1.46 ± 0.23b
Weight of mesenteric adipose tissue (g)	3.59 ± 0.19a	3.93 ± 0.49b	3.05 ± 0.65ab
Weight of lumbar adipose tissue (g)	2.23 ± 0.17a	6.12 ± 0.81b	5.85 ± 0.99b
Weight of periuterine-periovaric fat (g)	3.59 ± 0.19a	7.29 ± 0.92b	6.89 ± 1.34b
Basal plasma glucose (mg/dL)	104 ± 6a	110 ± 3a	113 ± 4a
Basal plasma insulin (μU/mL)	17.5 ± 3.0a	15.0 ± 1.8a	25.8 ± 4.1a
Area under the curve (AUC) for glucose in OGTT[1]	7992 ± 243a	8819 ± 144b	8753 ± 262b
Area under the curve (AUC) for insulin in OGTT[1]	94.1 ± 15.5a	171 ± 26b	138 ± 10ab
Insulin sensitivity index (ISI)	106 ± 22a	58.2 ± 6.6b	63.7 ± 8.8b
Triacylglycerols (mg/dL)	22.2 ± 4.1a	57.6 ± 16.3ab	68.9 ± 12.0b
Non-esterified fatty acids (NEFA) (μmoles/L)	314 ± 20a	405 ± 33a	369 ± 27a
Cholesterol (mg/dL)	65.2 ± 5.2a	76.8 ± 8.1a	57.6 ± 4.1a

Mean ± SEM of 5 rats per group. 1At 80 days on the diets rats were subjected to an oral glucose tolerance test (OGTT) with 2 g of glucose/kg body weight and blood collected at 0, 7.5, 15, 30 and 60 min. 2The insulin sensitivity index was calculated as ISI= 10,000/√(FPG x FPI x mean G x mean I), where FPG is fasting plasma glucose (mg/dL), FPI is fasting plasma insulin (μU/mL) and mean G and I are the concentrations of glucose and insulin during the OGTT. Different lowercase letters mean statistical significant difference ($p<0.05$) between the groups.

Table 4: Body, liver and different fat depots weights and plasma metabolic variables in female rats that were fed with standard diet (SD), cafeteria diet (CD) or cafeteria diet supplemented with *Rubus* extract (CRD) for 90 days, when they were sacrificed by decapitation after a 3 h fast.

The consumption of either CD or CRD did not increase body weight, whereas liver weight was higher in the CD group than in SD ($p<0.05$), the effect disappearing in the CRD group. All the adipose tissue depots studied (inguinal, perirenal, mesenteric and lumbar adipose

tissue) showed a significantly higher weight in the rats on CD than in those on SD, and this effect appeared lower in those rats fed the CRD – in the case of inguinal and mesenteric adipose tissues, the difference compared to the SD was no longer significant (p >0.05). Basal plasma glucose and insulin levels did not differ between the groups, whereas the area under the curve (AUC) for both glucose and insulin after the oral glucose load (OGTT) was higher in rats fed the CD than the SD. The *Rubus* supplement (CRD) did not modify the augmented AUC for glucose seen in the CD rats but it decreased the AUC for insulin to values that were not significantly different from either of the other two groups. Values of plasma glucose and insulin, both basal and after the oral glucose load, were used to determine insulin sensitivity index (ISI). The ISI values calculated were lower in the two groups fed the CD than in those on SD, with no difference observed between those receiving or not receiving the *Rubus* supplement (Table 4). Plasma triacylglycerols were higher in those rats fed the CD or the CRD than on those on the SD although the difference was only significant in the case of the CRD group. However, neither NEFA nor cholesterol concentrations differed among the three groups.

LPL activity was measured in both inguinal and lumbar adipose tissue. As shown in Table 5, the LPL activity of inguinal adipose tissue was higher in rats on the CD group than in those on SD; once again this variable decreased in rats on the CRD group to values that no longer differed from the SD group. A similar trend was found in LPL activity of the lumbar adipose tissue, although the differences among the groups did not reach statistical significance due to the high standard error values.

	Diet		
	SD	**CD**	**CRD**
Inguinal LPL	277 ± 51a	553 ± 80b	367 ± 47ab
Lumbar LPL	543 ± 101a	899 ± 192a	562 ± 96a
Mean ± SEM of 5 rats per group Different lowercase letters mean statistical significant difference (p<0.05) between the groups.			

Table 5: Lipoprotein lipase (LPL) activity (pkats/mg protein) in inguinal and lumbar adipose tissue of female rats that were fed standard diet (SD), cafeteria diet (CD) or cafeteria diet supplemented with *Rubus* extract (CRD) or 90 days, when they were sacrificed by decapitation after a 3 h fast.

Metabolomic variables

The common statistical approach used in metabolomics data analysis is based on univariate and multivariate analysis (MVA). To evaluate the quality of controls (QC), samples were first tested by unsupervised principal components analysis (PCA-X). QCs were clustering together (data not shown), reflecting the system's stability and performance, and the repeatability of the sample treatment procedure [36]. The coefficient of variation (% CV) of QC samples was calculated and values are shown in Table 6.

Compound	**Mass**	**RT**	**CV in QC**	**Changes (%) and p-value**		
				CD vs. CRD*	**CD vs. SD****	**CRD vs. SD*****
Oleic Acid	282.2558	5.26	8.14	-4.23 NS	+48.34 <0.05	+42.06 NS
Tetradecanoylcarnitine (C14:0)	371.3042	4.63	12.82	-10.89 NS	+55.65 <0.05	+38.71 NS
Palmitoylcarnitine (C16:0)	399.3356	5.13	9.58	-11.12 NS	+55.35 <0.05	+38.07 NS
Stearoylcarnitine (C18:0)	427.3652	5.59	7.67	-9.00 NS	+129.81 <0.05	+109.12 <0.05
Linoleylcarnitine (C18:2)	423.3349	4.96	11.38	-11.63 NS	-34.35 <0.05	-41.98 <0.05
PC(O-14:0)	467.3007	4.83	7.41	+10.70 NS	+45.80 NS	+61.40 <0.05
PC(21:0(CHO))	593.3752	5.9	8.01	+8.71 NS	-44.06 <0.05	-39.19 <0.05
PC(36:4)	781.5606	9.62	8.43	+2.33 NS	-22.11 <0.05	-20.30 <0.05
PC(40:3)	839.6161	9.62	8.54	+1.91 NS	-25.16 <0.05	-23.73 <0.05
PC(42:7)	859.6035	10.3	28.46	-11.53 NS	-37.54 <0.05	-44.74 <0.05
LysoPC(14:0)	467.3018	4.95	6.79	+10.43 NS	+46.11 NS	+61.34 <0.05
LysoPC(17:0)	509.3482	5.65	11.33	+22.20 NS	+57.92 NS	+92.98 <0.05
LysoPC(18:0)	523.3664	6.17	3.9	-7.44 NS	+13.84 <0.05	+5.38 NS
LysoPC(20:0)	551.3947	6.78	5.64	-5.54 NS	-22.37 NS	-26.67 <0.05
LysoPC(20:1)	549.3789	6.29	6.03	+9.52 NS	-24.55 <0.05	-17.37 NS
LysoPC(20:2)	547.3637	5.9	6.62	+0.28 NS	-43.71 <0.05	-43.55 <0.05
LysoPC(22:4)	571.3601	6.29	14.92	+9.47 NS	-24.54 <0.05	-17.40 NS
LysoPC(22:5)	569.3443	5.9	13.42	+5.63 NS	-36.65 <0.05	-33.08 <0.05
LysoPC(24:0)	607.4573	8.06	9.65	+15.56 NS	-34.12 <0.05	-23.87 NS

LysoPE(P-16:0)	453.287	4.95	7.25	+25.93 NS	+22.08 NS	+53.73 <0.05
LysoPE(18:1)	479.3057	5.14	7.44	+12.94 NS	-32.57 <0.05	-23.84 <0.05
LysoPE(20:1)	507.3359	5.72	7.58	+19.36 NS	+7.85 NS	+28.73 <0.05
LysoPS(21:0)	567.3614	5.72	8.04	+18.69 NS	+10.84 NS	+31.57 <0.05
LysoPA(16:0)	410.2452	5.56	8.5	+2.70 NS	-12.29 <0.05	-9.92 NS
PS(O-20:0/19:1)	817.6276	10.56	19.72	+26.79 NS	+1.68 NS	+28.92 <0.05
CerP(d18:1/18:0)	505.3545	6.17	9.74	-16.03 NS	+29.29 <0.05	+8.57 NS

*regulation in CRD, **CD and ***CRD group. (+) (-) indicate direction of changes: up-regulation or down-regulation. p value set as p<0.05. RT, Retention time, CV, Coefficient of variation, QC, Quality control. PC- Phosphatidylcholines, LysoPC – Lysophosphatidylcholines, LysoPE – Lysophosphatidylethanolamines, LysoPS – Lysophosphatidylserine, LysoPA - Lysophosphatidic Acid, PS – Glycerophosphoserine, CerP - Ceramide-phosphate. Number of rats per group = 5.

Table 6: Most relevant metabolomics changes in plasma, related to specified groups of the rats that were fed with standard diet (SD), cafeteria diet (CD) or cafeteria died supplemented by Rubus extract.

In fact, the experimental variables can only be considered significantly different when the percentage of change between groups is higher than the %CV for the corresponding QC.

Figure 2 (panels A and C), shows a trend to SD group clustering independently from CD and CDR groups only in the negative MS polarity when a not-supervised principal components analysis (PCA-X) of the results was employed.

In the supervised models, a better separation among the three groups (SD, CD and CDR) is seen (Figure 2, panels B and D), where the variance explained was R2=0.77 (+) ESI, R2=0, 85 () ESI and variance predicted Q2=0.14 (+) ESI, Q2=0.52 (-) ESI. Differences between the groups became more obvious when they were compared as pairs (CD vs. CRD, CD vs. SD and CRD vs. SD) as it is shown in Table 6.

When comparing rats of the CD group vs. those of SD (Table 6), it was found that oleic acid and most of the acylcarnitine derivatives, especially stearoylcarnitine (C18:0) were up-regulated in the CD group. Supplementation with *Rubus* extract to the CD group (CRD) provides a clear metabolic change compared to both CD and SD groups. Although, no significant changes between CD vs. CRD groups were found, a clear tendency to down-regulate acylcarnitine derivatives in the CRD group was observed. Interestingly, a remarkable impact of the *Rubus* supplement to cafeteria diet (CRD) as compared to the standard diet (CRD vs. SD) is evidenced, since the significant effect of cafeteria diet (CD) vs. SD decreases when the former is supplemented with the *Rubus* extract (CRD).

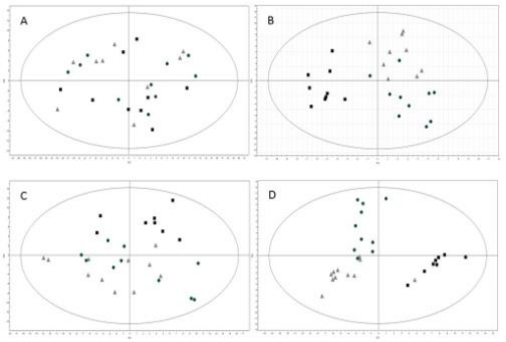

Figure 2: Panel A unsupervised principal component analysis (PCA) for the data in electrospray inonization (ESI)(+) and Panel C for the data in ESI(-). Panel B Partial Least Squares-Discriminat Analysis (PLS-DA) plot of analyzed samples in ESI(+) and Panel D in ESI(-). Legend: Standard diet (SD) (), Cafeteria diet (CD) (), Cafeteria + Rubus diet (CRD) (). N= 5 rats/group.

In general, most of the glycerophospholipids including phosphatidylcholines (PC), lysophosphatidylcholines (lysoPC) and lysophosphatidylethanolamines (lysoPE) were significantly down-regulated in both CD and CRD vs. SD, without any significant differences between CD and CRD (Table 6). Some of the lysophosphatidylcholines like lysoPC(14:0) and lysoPC(17:0) with saturated fatty acid chain were up-regulated in the CD group as compared to SD, although the differences were not significant. However, when the same variables were compared between CRD and SD, the differences were statistically significant. It's worth mentioning CerP, another compound that was elevated in CD in comparison to SD, the difference disappearing when comparing CRD and SD.

Discussion

In this study it was found that a hypercaloric diet containing a high proportion of saturated fatty acids to young female rats over 90 days increased the mass of fat depots and plasma triacylglycerol concentrations and decreased the insulin sensitivity index studied after an oral glucose load. Further, the plasma profile of fatty acids in these animals (again compared to standard diet controls) showed significant increments in saturated fatty acids without a change in the level of PUFA, and they had increased fat depots. These findings show that the dietary model of energy-dense palatable food applied here (i.e. the so-called cafeteria diet) to young female rats corresponds to a diet-induced obesity that mimics metabolic syndrome in humans, in agreement with a previous proposal [19]. The detected increase in adipose tissue LPL activity in the rats fed the CD is consistent with the increase in the sizes of fat depots, and indicates that their hypertriacylglycerolemia did not result from reduced clearance of circulating triacylglycerols by extrahepatic tissues, but more likely from an increased production by the liver. Treatment of animals fed the CD with an extract of *Rubus* (CRD) had a small effect on plasma lipid components, probably as a consequence of their low insulin

sensitivity, which was not modified by this treatment. However, some consistent decline in liver and inguinal and mesenteric adipose tissue mass could be detected in rats fed the CRD. Such a change in adipose tissue mass is consistent with the decline in LPL activity found in these animals, but the mechanism involved will require additional investigation.

In order to understand further the metabolic changes caused by the treatments, the highly sensitive and reproducible LC-QTOF-MS tool for metabolomic analysis has been employed to study plasma aliquots. The multivariate analysis carried out considering all data from the lipophilic metabolic profiles demonstrated a clear separation of the three groups (Figure 2) and indicated which metabolites were responsible for the separation, with statistical significance (Table 6). The main findings from the metabolomic fingerprinting in the case of the cafeteria diet are related to phospholipids, mainly zwitterionic glycerphospholipids, the related lysophospholipids as well as long-chain acylcarnitines. The carnitine ester profiles (tetradecanoylcarnitine 14:0; palmitoylcarnitine 16:0 and stearoylcarnitine 18:0) tended to be higher in the CD group than in the SD, however, linoleylcarnitine 18:2 is observed to be down-regulated. Acylcarnitines are ester derivatives of carnitine, the homeostasis of which is maintained by dietary intake, a modest rate of endogenous synthesis from lysine and methionine and by renal reabsorption. The carnitine system, including free carnitine and acylcarnitines, is essential for the transport of long-chain fatty acids from cytoplasm into mitochondria for their subsequent oxidation [37]. Interestingly, our findings fit to the one mass spectrometry based metabolic profiling reported by Koves, et al. [38] showing that long-chain acylcarnitines (16:0, 18:0 and 18:1) are increased in diet-induced obese rats. Higher levels of long-chain saturated and monounsaturated acylcarnitine species, analyzed by tandem mass spectrometry (MS/MS), in obese and insulin-resistant subjects compared to lean controls have also been shown in human obesity [39]. It may therefore be possible that, in the circumstances of our rats on the cafeteria diet, an inefficient tissue fatty acid beta-oxidation, due in part to a relatively low tricarboxylic acid cycle capacity, generates acylcarnitine molecules that activate proinflammatory pathways implicated in insulin resistance, as hypothesized for type 2 diabetic women [40].

A specific accumulation of saturated acylcarnitines was found in plasma of rats fed the cafeteria diet, indicating inefficient beta-oxidation [41,42], which together with the abundance of these fatty acids in the diet would contribute to their higher concentrations in plasma.

The greatest difference in the metabolomic profiles found here among the three studied groups (i.e. SD, CD and CRD groups) was in the glycerophospholipids which biological properties are dependent to differences of their structure and fatty acids composition (chain length, position, degrees of saturation and double bond location). The main group of identified glycerophospholipids, as lysoPC with the shorter chain fatty acids (14:0, 17:0, 18:0) and lysoPE(P-16:0), lysoPE(20:1)), showed up-regulation in rats fed the CD vs SD, whereas those with the long chain fatty acids, mainly polyunsaturated, were down-regulated in these same rats (i.e. in the CD group). It appears that *Rubus* supplementation reduce these changes in rats fed with the CD diet, making their comparison with the SD much less significant.

In general, a reduction in plasma lysoPC species in the rats fed the CD was found (Table 6). Our data are similar to others', which demonstrated that plasma lysoPC concentrations are reduced in mice fed a high fat diet [43] and in human obese subjects [44]. The mechanism responsible for the reduction in circulating lysoPC in these conditions is unknown, but could be related to an increase in either its breakdown or its clearance from the circulation by metabolically active tissues, as previously proposed for newly diagnosed type 2 diabetic subjects [45].

Furthermore, recently lysoPC has also been shown to have a role in the metabolism of glucose. It has been reported that lysoPC activates glucose uptake by adipocytes and that after acute lysoPC administration to mouse models of diabetes, there is an improvement in their glycemia [46]. In agreement with this involvement of plasma lysoPC in the glucose homeostasis, our rats fed the CD, with lower lysoPC levels than the SD group, showed a glucose metabolism that was somehow affected as revealed by their higher AUC for both insulin and glucose in the OGTT, and the lower ISI values as compared to those in rats fed the SD.

Knowledge about lysophosphatidylethanolamines (lysoPE) is not as wide but, it seems that they can be synthesized in a similar way to lysoPC [47]. In agreement with this view, our study shows that changes in lysoPE levels in CD and SD rats were similar, being up-regulated as in case of lysoPE(P-16:0) and lysoPE(20:1) or down-regulated as lysoPE(18:1). Some of the changes induced by the CD observed on the metabolomic parametres (Table 6) disappear after the *Rubus* supplement, for example, LysoPC18:0, LysoPC20:1, LysoPC22:4, LysoPC22:5 LysoPC24:0, LysoPA16:0, CerP18:1 CerP18:0. Although their physiological meaning is not well known, the changes in these compounds appear as putative indicators of the metabolic alteration caused by a hipercaloric and fat rich diet. Interestingly, the *Rubus* supplement revert these changes although the mechanism of action remains to be elucidated.

The addition of *Rubus* extract to the CD produced some interesting results. The supplement decreased some fat depot accumulation in the rats fed the CD, although did not modify their low insulin sensitivity index. However, the intake of the *Rubus* extracts increased plasma TAG levels over the values found in rats of the SD group. This hypertriacyglycerolemic effect of the *Rubus* supplement can't be related to any change in the LPL activity found in these animals when compared to those fed the SD, despite of the known effect of this enzyme controlling the clearance of circulating TAG [48,49]. The effect could be related to an increased production of TAG by the liver although the mechanism involved has not been established yet.

In view of the current results, it can be proposed that these effects of the *Rubus* extract supplement could contribute to reducing the impact on glucose metabolism in rats given the cafeteria diet as shown by the reduction in the AUCs for insulin without any changes in glucose levels observed in the OGTT.

As the main components of the *Rubus* extract are the flavonols and anthocyanins, it is reasonable to assume that the effects observed are due to these compounds, although small amounts of other phytochemicals are also present and may also be involved in the effects on health. In order to obtain reproducible results, extracts need to be obtained from the same plant material which has been standardized in terms of their contents of specific flavonols and anthocyanins, since the contents of these phytochemicals are known to fluctuate depending on environmental conditions [50]. Furthermore, absorption is subject to microfloral activity [9] so this also needs to be considered. Alternatively, studies using more purified extracts may yield more information about the active compounds responsible for these effects.

In conclusion, the study reported here not only demonstrates the obesogenic and metabolic effects of a cafeteria diet in female rats, but also shows that some of those effects are reduced when the same diet is supplemented with a *Rubus* extract. According to the current findings, it appears that such positive effects occur even without a significant change in insulin sensitivity, but additional research is needed to establish whether it also appears in rats fed the standard diet and to establish the mechanism involved.

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ORIGINAL PAPER

Supplementing Diet with Blackberry Extract Causes a Catabolic Response with Increments in Insulin Sensitivity in Rats

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Abstract Blackberry (Rubus sp.) fruit has a high content of anthocyanins, but its health benefits have not been sufficiently explored in healthy individuals. Thus, the aim of the study was to determine the effects of blackberry extract on lipid and glucose variables in female and male rats. Sprague Dawley rats were given a standard pellet (SD) or cafeteria (CD) diet supplemented (SD+R and CD+R) or not with Rubus extract for 80 days. Female rats given SD+R had lower body and liver weights than SD females; both sexes given SD+R showed lower plasma glucose and insulin, higher plasma NEFA, glycerol and 3-hydroxybutyrate, and higher liver concentration of triacylglycerols than SD rats. The homeostasis model of insulin resistance (HOMA) was lower in SD+R rats than in SD rats, but higher in CD rats. No effects of Rubus extract were observed in CD rats. In conclusion, Rubus extract, in rats given SD, decreased glycemia and increased insulin sensitivity. It also increased lipid breakdown in adipose tissue. The effects were greater in females than in males. No effect was seen in rats given CD, probably as a result of their high insulin resistance.

Keywords Blackberry fruit extract - Plasma glucose - Insulin sensitivity - Adipose tissue - Cafeteria diet

Introduction

Blackberry (Rubus sp.) fruit has a high content of anthocyanins and ellagitannis and other phenolic compounds that contribute to its high antioxidant capacity [1]. However, health benefits of blackberry have not been sufficiently explored [2], even though its antioxidant activity is high [3, 4] and has been ranked second after bilberry (Vaccinium myrtillus) [5]. Blackberry extract has been shown to have anti-hyperglycemic effects in diabetic rats [6] and biotransformed blueberry juice has been shown to have anti-obesity and anti-diabetic effects in KKA^{Y} mice [7]. Furthermore, the anti-hyperglycemic effect of different berry extracts on diabetic rats have been shown to be quite variable [6]. Although some studies have addressed the health benefits and metabolic effects of phenolic compounds present in blackberry, studies of blackberry extracts in healthy individuals are lacking. For that reason our aim was to determine the effects of a blackberry extract on lipid and glucose metabolic variables in rats given a standard diet and to compare them with its effect in other rats given a cafeteria diet, which is considered to be a model of metabolic syndrome in humans [8].

Materials and Methods

Blackberry Extract

Blackberries (Rubus sp. var. Loch Ness) were kindly provided by Agricola El Bosque (Lucena del Puerto, Huelva, Spain). Rubus extracts were obtained by lyophilization and extraction with 80 % methanol in 0.1 % HCl [9]. The total anthocyanin and flavonoid contents were determined [10, 11].

Animals and Diets

Female and male Sprague Dawley rats of 70 to 75 days of age were obtained from the animal quarter of University San Pablo CEU, Madrid, Spain. The experimental protocol was approved by the Animal Research Committee of the University San Pablo CEU (reference 101112). The rats were maintained in shared cages (5 per cage) under controlled conditions

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(22±2 °C, 55±10 % relative humidity and 12-hourly cycling of light and dark). Forty male and 40 female rats were selected, matched for body weight and assigned at random (10 male and 10 female per group) to one of four dietary treatment groups: the standard diet group (SD), the standard diet supplemented with Rubus extract (SD+R), the cafeteria diet group (CD) and the cafeteria diet plus Rubus extract (CD+R). The diets were prepared by blending the components shown in Table 1. The diets were stored at −20 °C until use. Rats had free access to the assigned diet and tap water. After 80 days on the corresponding diet, rats were decapitated under CO_2 anesthesia after a 3 h fast. Trunk blood from the neck wound was collected into ice-chilled receptacles containing Na_2EDTA. Plasma was separated from fresh blood by centrifugation at 1,500 x g for 30 min at 4 °C and stored at -80 °C until analysis. Liver and different fat depots were rapidly dissected and placed into liquid nitrogen for weighing and liver was kept at -80 °C until analysis.

Analytical Methods

Metabolites were determined enzymatically using commercially available kits as follows: plasma glucose, triacylglycerols (TAG) and cholesterol with kits from Spinreact Reactives (Girona, Spain), plasma glycerol with a kit from Sigma Chemical Co. (St. Louis, MO) and non-esterified fatty acids (NEFA) with a kit from Wako Chemicals (Germany). Insulin was analyzed by an enzyme-linked immunoassay (Mercodia, Sweden). 3-Hydroxybutyrate was analyzed [12] in deproteinized plasma [13]. Liver lipids were extracted and purified [14] for gravimetric determination followed by TAG and cholesterol analysis [15].

The homeostasis model assessment of insulin resistance (HOMA) values was calculated [16] using the following equation: HOMA=(FPG×FPI)/2,430, where FPG is fasting plasma glucose (expressed in mg/dl) and FPI is fasting plasma insulin (expressed as μU/ml).

Statistical Analysis

The values are quoted as mean±standard error of the mean. The effect of the diet (D), Rubus extract supplement (R) and interaction (D X R) were tested using two-way ANOVA. Contrast between means was assessed by the Bonferroni correction. Student's t-test was performed for comparisons between sexes. The statistical analyses were performed with SPSS 20.0 for Windows (SPSS, Chicago, Il.).

Results and Discussion

During the 80 days of the study the energy intake was higher in rats fed the cafeteria diet (CD) than in those fed the standard diet (SD), but there was no difference in food intake between rats that received the Rubus extract supplement in their diet and those that did not (data not shown). At the end of the study (day 80) male and female rats that fed on the CD had increased body, liver and fat pad weights compared to those given the standard diet (SD) (Table 2). Supplementing diets with Rubus extract did not modify these variables in males on the cafeteria diet (CD+R) whereas in those given SD+R liver weight was lower. In female rats on the cafeteria diet, the Rubus supplement (CD+R) did not modify these variables except for a small decrease in inguinal adipose tissue, whereas in the females fed SD+R it decreased body and liver weights without a significant change in any of the fat pads studied (Table 2).

Table 3 shows that in male rats on the CD there was no difference in plasma concentrations of glucose and cholesterol

Table 1 Composition of the diets

Composition (g/100 g)	Diet			
	Standard (SD)	Standard plus Rubus (SD+R)	Cafeteria (CD)	Cafeteria plus Rubus (CD+R)
Pellets[a]	58	58	19	19
Condensed milk			35	35
Sucrose			7	7
Muffins			6	6
Croissants			6	6
Powdered milk			15	15
Butter			8	8
Agar	2	2		
Rubus extract[b]		4		4
Water	40	36	4	
Calories(kcal)	108	108	403	403

[a] From Harlan, Global Diet 2014, Madison, MI. [b] The amount of Rubus extract added to 100 g of diet corresponded to 217 mg anthocyanins and 12.3 mg flavonoid

Table 2 Body, liver and adipose tissue (AT) of rats that were fed standard diet, standard diet supplemented with Rubus extract, cafeteria diet or cafeteria diet supplemented with Rubus extract for 80 days

	Standard diet (SD)		Cafeteria diet (CD)		ANOVA (p-values)		
	No-Rubus (SD)	Rubus (SD+R)	No-Rubus (CD)	Rubus (CD+R)	D	R	D x R
Males							
body weight (g)	483±17	489±7	580±29*	562±17*	0.000	0.749	0.532
liver (g)	14.3±0.4	11.2±0.3$^{\#}$	17.5±1.6*	18.2±0.9*	0.000	0.211	0.063
Adipose tissue							
lumbar (g)	2.9±0.4	4.2±0.5	11.8±2.2*	8.9±1.1*	0.000	0.562	0.116
inguinal (g)	2.8±0.3	2.9±0.3	8.8±1.6*	8.1±1.1*	0.000	0.752	0.703
mesenteric (g)	1.7±0.3	3.4±0.5	6.9±1.4*	5.5±0.5	0.000	0.846	0.053
epididymal (g)	2.1±0.1	3.4±0.3	5.2±0.8*	4.3±0.3	0.000	0.675	0.022
adiposity index(%)	2.1±0.2	3.1±0.2	5.8±0.7*	5.1±0.4*	0.000	0.737	0.064
Females							
body weight (g)	338±8^{c}	294±7$^{\#c}$	379±10^{*c}	381±17^{*c}	0.000	0.062	0.043
liver (g)	8.8±0.3^{c}	6.1±0.1$^{\#c}$	10.0±0.3^{*c}	9.7±0.5^{*c}	0.000	0.000	0.001
Adipose tissue							
lumbar (g)	3.0±0.3	2.2±0.3^{b}	6.1±0.6^{*a}	6.2±0.6^{*a}	0.000	0.451	0.297
inguinal (g)	2.6±0.4	1.5±0.2^{b}	5.9±0.9*	3.9±0.8$^{\#*b}$	0.000	0.018	0.454
mesenteric (g)	1.9±0.2	1.3±0.2^{c}	3.3±0.4^{*a}	2.8±0.4^{*c}	0.000	0.088	0.796
adiposity index(%)	3.2±0.3^{b}	2.9±0.4	5.9±0.5*	5.4±0.4*	0.000	0.360	0.861

Mean± SEM of 10 animals per group. Significant differences were tested by two-way ANOVA ($p<0.05$): *, SD vs. CD; #, No-Rubus vs. Rubus; D, diet effect; R, Rubus effect; D x R, diet and Rubus interaction. Comparisons between females and males by the Student's t test are shown by superscript letters: a, $p<0.05$; b, $p<0.01$; c, $p<0.001$

compared to those on the SD, but plasma concentrations of 3-hydroxybutyrate, NEFA, glycerol, TAG and insulin were all higher than in the male rats fed the SD. In male rats fed the CD+R, the supplement did not modify plasma concentrations of glucose, insulin, cholesterol, glycerol and TAG, but decreased 3-hydroxybutyrate; in those on the standard diet the supplement (SD+R) significantly increased plasma concentrations of NEFA, glycerol, and 3-hydroxybutyrate, and it decreased the concentrations of glucose without modifying the concentration of insulin. In fact, when the two later variables were used to calculate the HOMA value (Fig. 1), it was much higher in male rats on the CD than those on the SD; furthermore, whereas the Rubus supplement did not modify the HOMA value in male rats on CD+R, it decreased the value in the male rats on the SD+R. In female rats, plasma concentrations of glucose, cholesterol, TAG, NEFA and 3-hydroxybutyrate did not differ between the two diets whereas plasma glycerol and insulin concentrations were higher on the CD (Table 3). In female rats on the CD the addition of the supplement (CD+R) did not modify plasma cholesterol, glucose, glycerol, TAG or 3-hydroxybutyrate concentrations. In contrast, in female rats on the SD, addition of the supplement (SD+R) did not modify plasma cholesterol and TAG concentrations but did increase NEFA, glycerol and 3-hydroxybutyrate concentrations and decrease both glucose and insulin. Again, the calculated HOMA value was higher in females on CD than on SD with no effect attributable to the Rubus supplement (CD+R), but it was lower in the female rats on the SD+R than in those on the unsupplemented SD (Fig. 1).

The findings we report here show for the first time that dietary supplements of Rubus extract given to rats on a standard diet increased adipose tissue lipolytic activity and decreased both glycemia and insulinemia to values that clearly indicate increased insulin sensitivity reflected in their lower HOMA values. Moreover, these changes appeared greater in female than in male rats. The response to dietary Rubus extract supplement was not evident when rats were given a cafeteria diet, which was hypercaloric and resulted in increased body weight and adipose tissue depot weights.

The decrease in plasma glucose levels in rats fed the standard diet supplemented with Rubus extract is consistent with the increased insulin sensitivity found in these animals and agrees with the reported anti-hyperglycemic effect of blackberry extract in diabetic rats [6]. However, to our knowledge, the current study is the first one to report a hypoglycemic effect of blackberry extract in healthy animals. On the basis of the reduced HOMA values found in these animals, it could be proposed that the hypoglycemic effect of the Rubus extract in the rats fed the SD is related to the increased insulin sensitivity in these animals, although the actual mechanism

Table 3 Concentrations of glucose, insulin and lipid components in plasma of rats that were fed standard diet, standard diet supplemented with Rubus extract, cafeteria diet or cafeteria diet supplemented with Rubus extract for 80 days

	Standard diet (SD)		Cafeteria diet (CD)		ANOVA (p-values)		
	No-Rubus (SD)	Rubus (SD+R)	No-Rubus (CD)	Rubus (CD+R)	D	R	DxR
Males							
Plasma							
glucose (mg/dL)	137±4	97±5 #	141±4	141±6*	0.000	0.000	0.000
insulin (µg/L)	0.9±0.1	0.3±0.0	2.9±0.7*	2.4±0.4*	0.000	0.186	0.921
non esterified fatty acids (NEFA, µM)	342±33	686±43#	508±42*	414±21*	0.147	0.001	0.000
glycerol (µM)	158±12	218±16#	226±12*	196±12	0.089	0.261	0.001
3-hydroxybutyrate (µM)	371±49	3577±153#	857±149*	502±74#*	0.000	0.000	0.000
cholesterol (mg/dL)	84±4	74±4	79±6	75±4	0.638	0.128	0.000
Triacylglycerols (mg/dL)	85±7	67±5	130±18*	141±13*	0.000	0.747	0.213
Liver							
total lipids (mg/g)	40.3±0.9	59.5±2.5#	61.7±3.1*	73.4±11.0	0.005	0.012	0.528
triaclyglycerols(mg/g)	5.5±0.6	16.1±1.9#	25.4±2.8*	29.3±5.3*	0.000	0.036	0.329
Females							
Plasma							
glucose (mg/dL)	134±2	101±8#	144±2	145±2*	0.000	0.001	0.000
insulin (µg/ L)	1.1±0.2	0.3±0.0#	1.9±0.2*	1.8±0.3*	0.000	0.021	0.090
non esterified fatty acids (NEFA, µM)	474±34[a]	1050±62#[c]	542±44	414±21*	0.000	0.000	0.000
glycerol (µM)	164±11	332±30#[b]	248±25*	288±22	0.395	0.000	0.009
3-hydroxybutyrate (µM)	517±59	2343±171#[c]	850±93	686±110*	0.000	0.000	0.000
cholesterol (mg/dL)	81±3	84±7	73±4	77±5	0.144	0.546	0.928
Triacylglycerols (mg/dL)	66±4.0[a]	74±5	76±7[b]	91±13[b]	0.109	0.171	0.646
Liver							
total lipids(mg/g)	49.6±2.9	65.9±2.7#	54.8±2.8	54.2±3.0	0.267	0.010	0.006
triacylglycerols(mg/g)	9.0±0.9	21.9±2.0#	19.9±2.0*	21.3±2.7	0.024	0.002	0.011

Mean±SEM of 10 animals per group. Significant differences were tested by two-way ANOVA ($p<0.05$): *, SD vs. CD; #, No-Rubus vs. Rubus; D, diet effect; R, Rubus effect; D x R, diet and Rubus interaction. Comparisons between females and males by the Student's t-test are shown by superscript letters: a, $p<0.05$; b, $p<0.01$; c, $p<0.001$

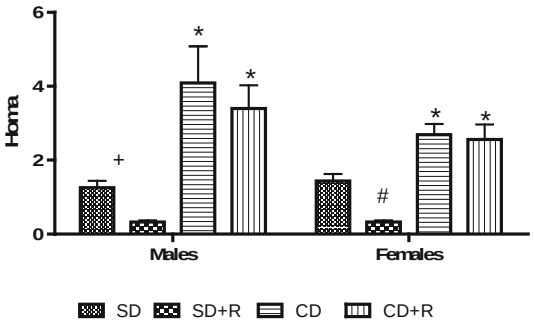

Fig. 1 The homeostasis model assessment of insulin resistance (HOMA) of male and female rats that were fed standard diet (SD), standard diet supplemented with Rubus extract (SD+R), cafeteria diet (CD) or cafeteria diet supplemented with Rubus extract (CD+R) for 80 days, when they were sacrifies by deacpitation after a 3 h fast. Mean ± standard error of 10 rats per group. Statistical comparisons with ANOVA ($p<0.05$): *, SD vs. CD; #, SD+R vs. SD; +, Student´s t-test SD vs. SD+R $p<0.05$

involved needs additional investigation. In fact, the hypoglycemic and increase in insulin sensitivity effects of the Rubus extract completely disappeared when rats were fed the CD, which by itself had an intense insulin resistant effect.

The purported health benefits of berries have been attributed to their bioactive phenolic compounds, such as anthocyanins [17, 18], but the bioavailability of blackberry anthocyanins has been found to be very low in rats [19]. It is therefore proposed that higher doses of Rubus extract would be necessary in rats to decrease the insulin resistance caused by the cafeteria diet. In fact, preparations different to those used here but containing higher doses of anthocyanins reduced insulin resistance [20] or had anti-diabetic effects in obese rats [21].

The increased lipolytic activity of adipose tissue in rats treated with the Rubus extract given the SD is shown by the increased plasma concentrations of the two products of lipolysis, NEFA and glycerol, the main fate of which is the liver.

As also shown in Table 3, male rats on CD had higher liver concentrations of total lipids and of TAG compared to male rats on SD. While the Rubus extract supplement did not result in any measured differences in the livers of males on the CD+R compared to unsupplemented CD, there were distinct changes resulting from supplementation of the SD: male SD+R rats had higher values for liver total lipids and TAG concentrations. Rubus extract supplements did not modify any of the studied variables in the livers of female rats given CD+R (compared to CD). However, in female rats given the standard diet, the supplement (SD+R) increased liver total lipid and TAG concentrations and despite these changes, the concentrations of cholesterol in liver showed no differences among the studied groups (data not shown).

Fatty acids reaching the liver are converted into their acyl-CoA form to be either re-esterified for the synthesis of TAG and phospholipids or to enter the mitochondria for β-oxidation. The results here suggest that both routes were active in the rats on the supplemented standard diet. The re-esterification route is supported by the increased lipid and TAG concentrations found in the livers, while liver cholesterol concentrations remained unchanged. Increased liver synthesis of TAG usually results in an increase in its re-export to the blood as very low-density lipoprotein (VLDL). Based on the lack of an increase in plasma TAG, this process does not appear to have occurred. This could be because a more prolonged treatment time is required to detect such an effect or that the clearance of TAG-rich lipoproteins is also increased in these animals. Further investigation is required to resolve this question. The second destination of NEFA reaching the liver is the mitochondria where they are oxidized, and the enormous increase in plasma concentrations of the ketone body, 3-hydroxybutyrate, found in rats of both sexes on the supplemented standard diet is indicative of greatly increased β-oxidation. Taken with the increased lipolytic activity, this finding is consistent with these animals (supplemented standard diet) being in a (relatively) catabolic state despite having a normal caloric intake. In fact, the metabolic condition of these animals mimics that seen under fasting conditions when plasma increases in NEFA, glycerol and ketone bodies occur alongside decreased glucose and insulin concentrations and increased liver TAG [22].

Differences in the responses to the Rubus extract supplement by the two sexes deserve some comment, the effect being decreased body and liver weights and increased lipolytic response in females compared to males. Different influences of sex hormones on adipose tissue metabolism have been clearly defined [23–25]. Furthermore, different responses to dietary agents by the sexes in terms of adiposity and lipid handling have previously been reported [26], and we have also previously found a sex-dependent nutritional programming effect on neonates caused by fish oil intake during pregnancy [27]. It follows that the sensitivity of each sex to any nutritional factor varies according to the nature of the intervention and additional investigation is required to determine the precise mechanism involved in such a differential response.

In conclusion, dietary supplement with Rubus extract in rats given a standard diet decreased plasma glucose and increased insulin sensitivity, and also increased adipose tissue lipolysis, as shown by higher concentrations of plasma glycerol and NEFA as well as by a more rapid arrival of fatty acids at the liver causing an increase in liver TAG concentrations and an increased concentration of 3-hydroxybutyrate in plasma. This response is sex-dependent, being greater in females than in males. However, the same supplementation in rats given a cafeteria diet to cause substantial insulin resistance, failed to produce the same effects.

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Conflicts of Interest All authors listed have contributed sufficiently to the project to be included as authors. I also confirm that each author has seen and approved the contents of the submitted manuscript, and none of the authors had any financial or personal conflicts of interest.

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ORIGINAL CONTRIBUTION

Influence of cafeteria diet and fish oil in pregnancy and lactation on pups' body weight and fatty acid profiles in rats

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Abstract
Purpose The aim was to determine the effects of cafeteria diet (CD) and fish oil supplements given to pregnant and lactating rats on the birth weight and fatty acid profiles of their offspring.
Methods Female rats were given standard diet (STD) or CD for 22 days before pregnancy. After mating, some animals remained on STD or CD; for some CD rats, the diet was supplemented with 8.78 % fish oil (CD-FO). After 12 days, half the CD-FO group returned to CD (CD-FO12) and the others remained on CD-FO.
Results At birth, body weights of pups of the three CD groups were lower than STD, maintained until 21 days in the CD-FO group only. At the end of lactation, dams of the CD groups had increased plasma triacylglycerols (TAG), non-esterified fatty acids, and glycerol concentrations, whereas most n-6 long-chain polyunsaturated fatty acids (LCPUFA) were decreased, the effect being greatest in the CD-FO group, where most n-3 LCPUFA were increased and indices of Δ^5 and Δ^6 desaturase activities decreased. The 21-day-old pups of the CD group had increased plasma TAG, not present in the CD-FO group, which had increased 3-hydroxybutyrate concentrations. In both 2- and 21-day-old CD pups, plasma concentrations of ARA were lower than STD, and even lower in the two CD-FO groups.
Conclusions The effect of CD and CD-FO decreasing pups body weight could be related to decreased concentrations of ARA, caused by the inhibition of the Δ^5 and Δ^6 desaturases in the pathway of n-6 LCPUFA biosynthesis.

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Keywords Cafeteria diet · Dietary fish oil supplement · Postnatal development · High-fat diet · Index of desaturases · Arachidonic acid

Introduction

Obesity is a global public health problem that affects both sexes at all ages. Women of reproductive age are particularly at risk; obesity in pregnancy has several adverse consequences. Women who are overweight or obese at conception are more likely to develop hypertension and diabetic or thrombotic complications [1, 2] and have increased rates of delivery complications, postpartum hemorrhage, and cesarean section [1, 3, 4]. Fetal overgrowth is typical in pregnancies of overweight and obese women [5–7], but an association between obesity in pregnancy and intrauterine growth restriction has been also reported [8, 9].

The influence of high-calorie-processed foods on fetal development in experimental models remains to be characterized [10]. A "cafeteria diet," which is a highly palatable and semi-purified diet containing high fat and sugars, has been used in rodents to study the effects of maternal obesity and its consequences for the offspring [11, 12]. Previous studies using this diet during pregnancy in rats have shown that it consistently causes an increase in both maternal body weight and fat depots in offspring, but that the body weight of the offspring at birth and at weaning [13, 14] either decreases or remains unaffected [11, 15].

Altering the fat composition of the diet of a pregnant or suckling mother could affect the availability of certain fatty acids to the fetus or to the suckled newborn. The possibility exists therefore that the amount of essential fatty acid reaching the fetus or the newborn could be reduced,

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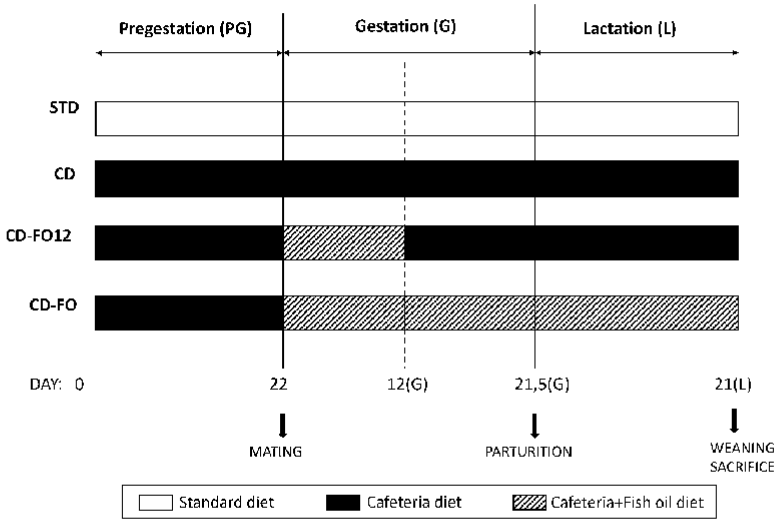
Fig. 1 Experimental design



resulting in a compromised growth pattern. On the other hand, fish oil is rich in n-3 long-chain polyunsaturated fatty acids (LCPUFA) such as eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), and these fatty acids appear to be important for normal neuronal [16] and visual function [17]. Depletion of dietary DHA is associated with adverse neurological outcomes in animals [18], and it has been suggested that increasing the consumption of fish oil could prevent certain diseases [19, 20]. These considerations and the fact that fish oil supplementation during pregnancy increases fetal n-3 LCPUFA content [21–23] have prompted the advice that women should supplement their diets with fish oil during pregnancy and lactation [24–26]. However, in both women [27–29] and rats [30], it has been shown that n-3 LCPUFA supplementation is associated with low arachidonic acid (ARA, 20:4n-6) status, worrying because it has been proposed that ARA during early life has a growth-promoting effect [31, 32].

The main objective of the current study was to use the cafeteria diet during pregnancy and lactation to determine the potential association between the altered fatty acid status it causes [33] and decreased body weight in newborn rats. This approach was extended by supplementing the cafeteria diet with fish oil during either the first 12 days of their 21.5-day pregnancy or throughout pregnancy and lactation. The results show that the cafeteria diet caused a decline in the plasma concentrations of ARA in newborn and weaning pups, an effect which was exaggerated by the inclusion of fish oil in the diets, and that such changes may be associated with their lower body weights.

Materials and methods

Animals and diets

Female Sprague–Dawley rats aged 40–45 days and weighing 165–175 g were obtained from the animal quarters of University San Pablo CEU, Madrid, Spain. The experimental protocol was approved by the Animal Research Committee of the University San Pablo CEU (reference 220113). The rats were initially fed a standard pellet diet (Harlan Global Diet 2014, Madison, WI) and maintained in shared cages (5 per cage) under controlled conditions (22 ± 2 °C, 55 ± 10 % relative humidity and 12-hourly cycling of light and dark) for 5 days. The study design is shown in Fig. 1. Rats were randomly assigned to one of two dietary treatment groups: the standard diet group (STD) and the cafeteria diet group (CD). After 22 days on their respective diet, rats were mated with males fed the standard diet, and the day spermatozoa first appeared in vaginal smears was considered day 0 of pregnancy. At this time, pregnant rats were placed in individual cages, and the CD group was subdivided into two subgroups: one subgroup was maintained on its original (CD) diet, and the diet of the other subgroup was supplemented with 8.78 % of fish oil (the CD-FO group). On the 12th day of pregnancy, tail blood from all the rats was collected in Na_2EDTA and plasma-separated by centrifugation at 1500× *g* for 30 min at 4 °C and stored at − 80 °C until analysis. At this time, half of the group on the diet supplemented with fish oil was returned to the cafeteria diet without the fish oil supplement (CD-FO12 group), and the other half was maintained on the fish oil-supplemented diet for the remainder of pregnancy and

Table 1 Composition of the Diets

	Diet		
	Standard (STD)	Cafeteria (CD)	Cafeteria plus Fish Oil (CD-FO)
Components (g/100 g)			
Pellets[a]	100	25.2	25.2
Condensed milk (Nestlé, Barcelona)	–	11.0	11.0
Sucrose (Azucarera, Madrid)	–	17.2	17.2
Muffins (Panrico SA, Barcelona)	–	6.58	6.58
Croissants (Bimbo, Barcelona)	–	6.58	6.58
Powdered milk (Central Lechera Asturiana, Asturias)	–	24.7	24.7
Lard (El Pozo, Murcia)	–	8.78	–
Fish oil (Fagron Iberica, Barcelona)	–	–	8.78
Composition (g/100 g)			
Water content	8.67	11.4	10.6
Fat content	3.90	17.1	16.9
Total fiber	15.1	3.38	3.10
Protein content	14.4	10.6	11.2
Ashes	3.79	2.47	2.70
Caloric content[b] (kJ/100 g)	1339	1863	1863
Energy derived from fat (%)	11.5	36.9	35.8
Energy derived from protein (%)	18.9	8.63	10.6
Energy derived from carbohydrates (%)	69.6	54.5	53.6

[a] From Harlan, Global Diet 2014, Madison, MI

[b] According to manufacturers

Table 2 Fatty acid (FA) composition of the experimental diets

Fatty acids (μmol/g)	Diet		
	Standard (STD)	Cafeteria (CD)	Cafeteria plus Fish Oil (CD-FO)
MA, 14:0	N.D.^{A}	40.7 ± 1.23^{B}	49.4 ± 1.6^{C}
PAM, 16:0	28.2 ± 1.6^{A}	185 ± 26^{B}	151 ± 3^{B}
STA, 18:0	3.62 ± 0.13^{A}	73.5 ± 2.3^{B}	36.8 ± 1.0^{C}
PAO, 16:1n-7	0.611 ± 0.042^{A}	15.1 ± 0.4^{B}	31.8 ± 0.6^{C}
OLA, 18:1n-9	33.4 ± 1.3^{A}	232 ± 6^{B}	156 ± 2^{C}
LNA, 18:2n-6	83.3 ± 3.1^{A}	77.9 ± 0.7^{A}	63.4 ± 2.2^{B}
DGLA, 20:3n-6	N.D.	N.D.	N.D.
GLA, 18:3n-6	N.D.^{A}	N.D.^{A}	0.69 ± 0.03^{B}
ARA, 20:4n-6	N.D.^{A}	0.835 ± 0.024^{B}	1.58 ± 0.06^{C}
DPAn-6, 22:5n-6	N.D.^{A}	N.D.^{A}	0.543 ± 0.008^{B}
ALA, 18:3n-3	5.67 ± 0.22^{A}	6.35 ± 0.07^{B}	7.49 ± 0.18^{C}
EPA, 20:5n-3	N.D.^{A}	0.712 ± 0.040^{B}	20.2 ± 0.3^{C}
DPAn-3, 22:5n-3	N.D.^{A}	N.D.^{A}	3.32 ± 0.08^{B}
DHA, 22:6n-3	N.D.^{A}	N.D.^{A}	22.5 ± 0.2^{B}

Values correspond to mean of triplicate samples ± SEM. Statistical comparisons between the diets are shown by upper-case superscript letters (different letters indicate $P < 0.05$)

during lactation (CD-FO). The diets were prepared as a homogeneous paste by mechanically blending the components shown in Table 1. Table 1 also shows the gross composition of the diets, with the CD diets containing more water and less protein, fiber, and ash, but having higher fat and energetic contents. The fatty acid composition of the diets shows (Table 2) that the CD diets contain more fatty acids than the STD diet, with the greatest difference

corresponding to changes in the amounts of saturated and monounsaturated fatty acids. The CD-FO diet has higher amounts of MA, PAO, GLA, ARA, DPA, ALA, EPA, DPA and DHA but less LNA than the others. The diets were stored at −20 °C until use. Rats had free access to the assigned diet and tap water. On day 2 after delivery, litters were reduced to eight pups per dam; the extra pups were decapitated, and trunk blood from the neck wound was collected into ice-chilled receptacles containing Na_2EDTA. Milk yield was estimated from pups' weight and weight gain on day 15–16 of lactation as previously described [34]. At weaning (day 21 of age), dams and pups were decapitated under CO_2 anesthesia, and trunk blood from the neck wound was collected into ice-chilled receptacles containing Na_2EDTA. Dams' livers and lumbar fat pads were rapidly dissected and placed into liquid nitrogen for weighing and kept at −80 °C until analysis. Plasma from dams and pups was separated by centrifugation at 1500×*g* for 30 min at 4 °C and stored at −80 °C until analysis.

Analytical methods

Plasma triacylglycerols (TAG), glycerol (Spinreact Reactives, Spain), and non-esterified fatty acids (FFA) (Wako Chemicals, Germany) were determined enzymatically using commercial kits. Plasma 3-hydroxybutyrate was also measured [35]. Aliquots of diets were used for the gravimetric analysis of lipids after lipid extraction and purification [36], proteins, water, and ash by following the official methods of AOAC International (method 995.04 for proteins, 934.01 for water, 991.42 for fiber, and 942.05 for ash) [36].

For the quantitative analysis of the fatty acids profile, nonadecenoic acid (19:1n-9) (Sigma Chemical Co.) was added as the internal standard to fresh aliquots of each diet, frozen liver, frozen adipose tissue, and plasma that were used for lipid extraction and purification [37]. The final lipid extract was evaporated to dryness under vacuum and the residue suspended in methanol–toluene and subjected to methanolysis in the presence of acetyl chloride at 80 °C for 2.5 h as previously described [38]. Fatty acid methyl esters were separated and quantified on a Perkin-Elmer gas chromatograph (Autosystem) with a flame ionization detector and a 30-m Omegawax capillary column (internal diameter 0.25 mm). Nitrogen was used as carrier gas, and the fatty acid methyl esters were compared with purified standards (Sigma Chemical Co.). Quantification of the fatty acids in the sample was performed as a function of the corresponding peak areas relative to the internal standard.

Statistical analysis

Results are expressed as mean ± standard error. Statistical analysis was carried out using GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, CA). One-way analysis of variance (ANOVA) was used to compare the changes due to the different diets. When treatment effects were significantly different ($P < 0.05$), values were analyzed by the Newman–Keuls test. Correlations were carried out by the Pearson test.

Results

Animal measurements

As shown in Table 3, during the pre-gestational period, the daily dietary energy intake was higher in CD than STD rats. During pregnancy (at day 10 of gestation) the dietary energy intake was also higher in CD than in STD rats, but the difference disappeared in the fish oil groups (CD-FO12 and CD-FO). During lactation (at day 16 of postpartum), there was an increase in the daily energy intake in all the groups, with no significant differences between the groups.

Table 4 shows the changes in body weight throughout the experiment. At the onset of the experiment, there was no difference between the groups. Rats given the cafeteria diet (all three CD groups) for 22 days before gestation showed a higher body weight than those fed the standard diet (STD group). However, at day 12 of pregnancy or at day 21 postpartum, this difference disappeared regardless of whether or how long their diets were supplemented with fish oil. The weight of lumbar adipose tissue at this later time was higher in rats of the CD group than those of the STD group; fish oil supplementation during the first 12 days of pregnancy (CD-FO12) did not modify this finding in the CD rats, but supplementation for the whole of pregnancy and lactation (CD-FO) removed the difference, and the group was not statistically different from the STD

Table 3 Average daily maternal intakes of diet during the pre-gestational (12 days before the start of gestation), gestational (day 10 of gestation) and lactational periods (day 16 of lactation)

Food intake (kJ/day)	STD	CD	CD-FO12	CD-FO
Pre-gestation	227 ± 4^{Aa}	334 ± 8^{Ba}	–	–
n	6	19	–	–
Gestation	302 ± 1^{Aa}	398 ± 11^{Bb}	336 ± 12^{Aa}	330 ± 8^{Aa}
n	6	8	5	6
Lactation	733 ± 72^{b}	828 ± 48^{c}	678 ± 112^{b}	841 ± 109^{b}
n	6	8	5	5

Values correspond to mean ± SEM. Statistical comparisons between the groups are shown by upper-case superscript letters, and between different periods within the same group by lower-case superscript letters (different letters indicate $P < 0.05$)

Table 4 Body weight at different time points of the experiment, lumbar and perirenal adipose tissue weights of dams at day 21 of lactation, number and weight of pups and milk yield

	STD	CD	CD-FO12	CD-FO	*n*
Body weight					
Day 0 of experiment	172 ± 2	177 ± 3	178 ± 3	176 ± 2	5–8
Day 22 of pre-gestation	223 ± 2^{A}	246 ± 5^{B}	242 ± 3^{B}	243 ± 5^{B}	5–8
Day 12 of pregnancy	284 ± 5	292 ± 7	308.4 ± 6.3	304.2 ± 7.4	5–8
Day 21 of postpartum	281 ± 8	262 ± 7	292 ± 10	266 ± 7	5–8
Lumbar adipose tissue weight (day 21 of postpartum)	0.793 ± 0.079^{A}	1.83 ± 0.31^{B}	2.12 ± 0.52^{B}	1.64 ± 0.19^{AB}	5–8
Perirenal adipose tissue weight (day 21 of postpartum)	1.08 ± 0.22	1.49 ± 0.25	2.18 ± 0.57	1.27 ± 0.24	5–8
Number of pups born per dam	11.5 ± 1.3	13.4 ± 0.4	13.3 ± 1.2	12.7 ± 1.5	5–8
Pups body weight at birth	6.92 ± 0.17^{A}	6.17 ± 0.20^{B}	5.78 ± 0.17^{B}	5.87 ± 0.19^{B}	32–45
Pups body weight (21 days old)	49.7 ± 0.9^{A}	48.2 ± 1.0^{A}	44.0 ± 1.8^{AB}	43.1 ± 1.2^{B}	18–31
Milk yield at 15–16 days of lactation	4.22 ± 0.24	4.59 ± 0.28	3.28 ± 0.82	4.00 ± 0.48	5–8

Values correspond to mean ± SEM (n = 5–8 dams). Statistical comparisons between the groups are shown by upper-case superscript letters (different letters indicate $P < 0.05$)

group. The weight of perirenal adipose tissue follows a comparable trend among the groups as lumbar adipose tissue although their differences were not statistically different.

There was no difference in the number of pups born to any group, but the body weights of pups at birth was lower in those of CD, CD-FO12, and CD-FO compared to those of the STD group. By the time of weaning (day 21 of age), body weight values in pups of the CD or the CD-FO12 groups no longer differed from those of the STD group, whereas body weight in those of the CD-FO remained lower than those of both the STD and CD groups. Milk yield, measured at day 15–16 of lactation, did not differ between the groups.

Metabolic variables in dams at the end of lactation

As shown in Table 5, at the end of lactation (day 21 postpartum), plasma concentrations of TAG, FFA, and glycerol in rats of the CD group were higher than in those of the STD group. Values for TAG and glycerol were also higher than STD in the CD-FO12 and CD-FO groups, but FFA values for the CD-FO12 group were different from neither the STD nor the CD group.

At the same time the plasma fatty acid profile in the dams was determined, and the results are summarized in Table 5. For the saturated and monounsaturated fatty acids, only myristic acid (MA, 14:0), which was higher in the CD, CD-FO, and CD-FO12 groups than in the STD group, and oleic acid (OLA, 18:1n-9), which was higher in the same three groups but not significantly so in the CD-FO group, showed any differences. Values for palmitic (PAM, 16:0), stearic (STA, 18:0), or palmitoleic (PAO, 16:1n-7) acids showed no differences.

There were no differences in the plasma concentrations of any of the n-6 PUFA between either the CD or the CD-FO12 groups and the STD group. However, in the CD-FO group, compared to the three other groups, lower concentrations of γ-linolenic (GLN, 18:3n-6), dihomo-γ-linolenic (DGLA, 20:3n-6), arachidonic (ARA, 20:4n-6), and n-6 docosapentaenoic (DPAn-6, 22:5n-6) acids were observed.

Concentrations of the n-3 PUFA in plasma showed no difference among the groups for α-linoleic acid (ALA), but values of EPA and DHA in the CD-FO group were higher than in any of the other groups. Values of n-3 docosapentanoic acid (DPAn-3, 22:5n-3) were higher in the CD-FO12 than in the STD group, and those of DHA (22:6n-3) were higher in the CD-FO12 and CD-FO groups than in either CD or STD groups, which had similar values.

To understand some of the observed changes, the product/precursor ratios, which can give estimates of elongase-5 and Δ^5, Δ^6, and Δ^9 desaturase activities, were calculated. These results are also shown in Table 5. When compared to values in the STD group, the index for the n-6 Δ^5 desaturase (ARA 20:4n-6/DGLA 20:3n-6) was lower in plasma of the CD group, whether or not it was supplemented with FO. The index of n-6 Δ^6 desaturase (GLN, 18:3 n-6/LA, 18:2 n-6), however, was lower only in rats of the CD-FO12 group. The index of Δ^9 desaturase (OLA, 18:1 n-9/STA, 18:0) was higher in the CD-FO group than in any of the others and the index of elongase (DGLA, 20:3 n-6/GLN, 18:3 n-6) was higher in the CD and CD-FO12 groups than in either STD or CD-FO. The liver represents the main organ contributing to fatty acids metabolism. Therefore, it was decided to determine the liver fatty acid profile in the dams at the end of lactation (day 21), and the values are shown in Table 6. The concentrations of both

Table 5 Plasma concentrations of triacylglycerols (TAG), non-esterified fatty acids (FFA), glycerol, and fatty acids in dams at day 21 of postpartum

	STD	CD	CD-FO12	CD-FO
TAG (μmol/L)	0.513 ± 0.051^{A}	0.872 ± 0.099^{B}	0.998 ± 0.128^{B}	0.879 ± 0.062^{B}
FFA (μmol/L)	183 ± 31^{A}	321 ± 35^{B}	262 ± 12^{AB}	304 ± 30^{B}
Glycerol (μmol/L)	150 ± 29^{A}	266 ± 21^{B}	303 ± 52^{B}	377 ± 40^{B}
Fatty acids (μmol/L)				
MA, 14:0	63.6 ± 10.8^{A}	210 ± 22^{B}	196 ± 23^{B}	315 ± 30^{C}
PAM, 16:0	1515 ± 83	2282 ± 290	2384 ± 165	2035 ± 98
STA, 18:0	908 ± 49	1353 ± 207	1366 ± 90	806 ± 21
PAO, 16:1n-7	167 ± 36	229 ± 23	213 ± 16	267 ± 18
OLA, 18:1n-9	1081 ± 105^{A}	1958 ± 216^{B}	2068 ± 186^{B}	1534 ± 162^{AB}
LNA, 18:2n-6	1676 ± 92	1683 ± 253	1847 ± 144	1294 ± 31
DGLA, 20:3n-6	71.9 ± 7.2^{A}	97.9 ± 17.6^{A}	101 ± 6^{A}	31.9 ± 1.1^{B}
GLN, 18:3n-6	73.4 ± 4.5^{A}	67.4 ± 52.0^{AB}	52.0 ± 3.5^{AB}	49.2 ± 6.3^{B}
ARA, 20:4n-6	1048 ± 67^{A}	944 ± 103^{A}	846 ± 47^{A}	320 ± 18^{B}
DPAn-6, 22:5n-6	49.1 ± 5.0^{A}	53.3 ± 15.8^{A}	19.8 ± 5.0^{AB}	6.04 ± 4.16^{B}
ALA, 18:3n-3	29.5 ± 9.8	22.9 ± 4.3	29.2 ± 8.7	30.8 ± 7.5
EPA, 20:5n-3	34.7 ± 4.6^{A}	45.0 ± 9.1^{A}	78.9 ± 30.4^{A}	724 ± 31^{B}
DPAn-3, 22:5n-3	24.3 ± 7.0^{A}	50.6 ± 13.1^{AB}	87.3 ± 6.9^{B}	44.9 ± 17.5^{AB}
DHA, 22:6n-3	49.7 ± 5.2^{A}	59.8 ± 16.0^{A}	128 ± 6^{B}	345 ± 33^{C}
Product/precursor indices				
Δ^5 *d* ARA/DGLA	15.8 ± 2.6^{A}	10.8 ± 1.2^{B}	8.39 ± 0.47^{B}	10.0 ± 0.4^{B}
Δ^6 *d* GLN/LNA	0.0483 ± 0.0040^{A}	0.0400 ± 0.0027^{AB}	0.0280 ± 0.0037^{B}	0.0383 ± 0.0040^{AB}
Δ^9 *d* OLA/STA	1.19 ± 0.09^{A}	1.51 ± 0.08^{A}	1.51 ± 0.08^{A}	1.90 ± 0.19^{B}
Elongase DGLA/GLN	0.909 ± 0.087^{A}	1.43 ± 0.12^{B}	1.98 ± 0.17^{C}	0.749 ± 0.162^{A}

Rats were given either standard diet (STD), cafeteria diet during pre-gestation, gestation and lactation (CD) cafeteria diet during pre-gestation, cafeteria diet supplemented with fish oil during the first 12 days of pregnancy and cafeteria diet during the remaining pregnancy and lactation (CD-FO12), or cafeteria diet during pre-gestation and cafeteria diet supplemented with fish oil during pregnancy and lactation (CD-FO)

Values correspond to mean ± SEM ($n = 5$–8). Statistical comparisons between the groups are shown by upper-case superscript letters (different letters indicate $P < 0.05$)

d Index of desaturase

MA and PAM, although not that of STA, were higher in rats given the CD irrespective of whether they had received the fish oil supplement. Of the monounsaturated fatty acids, both palmitoleic (PAO, 16:1n-7) and OLA appeared much higher in the three groups of CD than in the STD group. Among the n-6 PUFA, it appears that the concentration of LNA was higher in rats of the CD-FO group than in any of the others, whereas the concentration of ARA was highest in the STD group and became progressively lower in rats of the CD, CD-FO12, and CD-FO groups. All the n-3 PUFA that were analyzed were higher in rats of the CD-FO group than in any of the other groups, with no differences between these other groups. The indices for Δ^5 and Δ^6 desaturases were lower in the CD-FO group than in any of the other groups although values for Δ5 desaturase in the CD-FO group were not statistically different from the CD-FO12 group. When compared to the STD group, the index of the Δ^9 desaturase appeared higher in any of the CD groups, whereas the index of elongase showed the opposite trend.

The fatty acid profile in adipose tissue could add additional information about the changes in the metabolic fate of the different fatty acids. It can be seen in Table 7 that the concentration of MA in adipose tissue of rats from the CD, CD-FO12, and CD-FO was higher than from the STD group as was STA in the CD and CD-FO12 groups compared to the other two groups; no differences between the groups was detected for the other saturated fatty acids. OLA concentration was higher in the CD, CD-FO12, and CD-FO groups than in the STD group, and all the n-6 fatty acids appeared lower in the same three groups than in STD. The n-3 fatty acids showed a more complicated pattern: α-linolenic (ALA, 18:3n-3) was lower in CD, CD-FO12, and CD-FO groups than in the STD one, EPA was highest in the CD-FO group and both DPAn-3 and DHA were higher in both CD-FO12 and CD-FO groups than in either

Table 6 Concentration of specific fatty acids in liver of rats at day 21 of lactation that were given either standard diet (STD), cafeteria diet during pre-gestation, gestation and lactation (CD), cafeteria diet during pre-gestation, cafeteria diet supplemented with fish oil during the first 12 days of pregnancy and cafeteria diet during the remaining pregnancy and lactation (CD-FO12), or cafeteria diet during pre-gestation and cafeteria diet supplemented with fish oil during pregnancy and lactation (CD-FO)

	STD	CD	CD-FO12	CD-FO
Fatty acids (μmol/g)				
MA, 14:0	0.713 ± 0.026^{A}	6.90 ± 1.16^{B}	7.76 ± 1.84^{B}	11.7 ± 2.3^{B}
PAM, 16:0	20.3 ± 5.1^{A}	101 ± 16^{B}	118 ± 25^{B}	128 ± 23^{B}
STA, 18:0	21.7 ± 1.0	26.6 ± 1.5	28.6 ± 2.4	28.2 ± 2.2
PAO, 16:1n-7	2.48 ± 0.31^{A}	14.2 ± 2.7^{B}	15.6 ± 4.0^{B}	13.1 ± 2.6^{B}
OLA, 18:1n-9	16.4 ± 0.8^{A}	90.1 ± 14.1^{B}	109 ± 26^{B}	99.6 ± 20.1^{B}
LNA, 18:2n-6	13.5 ± 1.2^{A}	14.1 ± 0.6^{A}	16.3 ± 1.7^{A}	32.2 ± 7.3^{B}
DGLA, 20:3n-6	1.90 ± 0.19^{A}	1.64 ± 0.10^{A}	1.66 ± 0.08^{A}	0.886 ± 0.135^{B}
GLN, 18:3n-6	0.751 ± 0.092	0.907 ± 0.129	1.04 ± 0.18	0.851 ± 0.140
ARA, 20:4n-6	15.9 ± 0.9^{A}	13.2 ± 0.7^{B}	11.6 ± 0.6^{B}	4.67 ± 0.32^{C}
DPAn-6, 22:5n-6	2.00 ± 0.17^{A}	1.26 ± 0.11^{B}	0.950 ± 0.102^{BC}	0.605 ± 0.116^{C}
ALA, 18:3n-3	0.161 ± 0.042^{A}	0.167 ± 0.012^{A}	0.211 ± 0.055^{A}	1.70 ± 0.49^{B}
EPA, 20:5n-3	0.424 ± 0.070^{A}	0.628 ± 0.068^{A}	0.712 ± 0.073^{A}	10.4 ± 2.2^{B}
DPAn-3, 22:5n-3	0.827 ± 0.063^{A}	0.628 ± 0.038^{A}	0.660 ± 0.051^{A}	9.02 ± 2.61^{B}
DHA, 22:6n-3	2.56 ± 0.20^{A}	2.04 ± 0.16^{A}	3.10 ± 0.21^{A}	19.2 ± 3.5^{B}
Product/precursor indices				
Δ^5 *d* ARA/DGLA	8.76 ± 1.11^{A}	8.34 ± 0.76^{A}	7.03 ± 0.40^{AB}	5.55 ± 0.39^{B}
Δ^6 *d* GLN/LNA	0.0560 ± 0.0060^{A}	0.0625 ± 0.0077^{A}	0.0675 ± 0.0118^{A}	0.0283 ± 0.0031^{B}
Δ^9 *d* OLA/STA	0.755 ± 0.033^{A}	3.26 ± 0.40^{B}	3.65 ± 0.68^{B}	3.35 ± 0.46^{B}
Elongase DGLA/GLN	2.84 ± 0.22^{A}	1.85 ± 0.08^{B}	1.97 ± 0.43^{B}	1.21 ± 0.14^{B}

Values correspond to mean ± SEM (n = 5-8). Statistical comparisons between the groups are shown by upper-case superscript letters (different letters indicate $P < 0.05$)

d Index of desaturase

STD or CD groups. In adipose tissue, when compared to the STD group, the index for n-6 Δ^5 desaturase was lower in rats fed the CD diet with or without the FO supplement, whereas the index for n-6 Δ^6 desaturase was higher in the CD-FO12 group. The index of Δ^9 desaturase was higher in the CD and CD-FO groups than in the STD group. No differences among the groups were found for the elongase index.

Plasma metabolites and fatty acid profile in offspring plasma at day 2

Some of the pups were killed at day 2 after birth, and as shown in Table 8, neither plasma TAG, FFA, glycerol nor 3-hydroxybutyrate differed among the groups. Their plasma fatty acid profile was also analyzed, as also shown in Table 8. With the exception of MA, there were no observed differences in the saturated fatty acids. The monounsaturated fatty acids both showed differences with PAO higher in the CD-FO group than in the other three groups and OLA higher in the CD group than in the STD group only. Two of the n-6 PUFA (ARA and DPAn-6) were lower in CD pups than in STD, and they were even lower in the CD-FO12 and CD-FO groups. Both of the major n-3 LCPUFA present in fish oil (EPA and DHA) were progressively higher in pups of the CD-FO12 and CD-FO groups than in either the STD or CD groups. There were no differences among the four groups in the index for n-6 Δ^6 desaturase, but the index for Δ^5 desaturase was lower in the CD, CD-FO12, and CD-FO groups than in the STD, the greatest difference appearing in the CD-FO group. The indices of both Δ^9 desaturase and elongase were higher in the CD groups, with or without fish oil supplementation, than in the STD group.

Plasma metabolites and fatty acid profile in offspring plasma at weaning

Similar variables were measured in the remaining pups at the time of weaning (21 days old) and are shown in Table 9. Plasma TAG, FFA, glycerol, and 3-hydroxybutyrate were higher in the CD group than in the STD one. In the fish oil-supplemented groups, both TAG and glycerol concentrations decreased (relative to unsupplemented CD) to values that were not different to those of the STD group, but both FFA and 3-hydroxybutyrate remained significantly higher than in the STD group.

The plasma fatty acid profile showed that PAM, STA, PAO, and OLA (but not MA) were higher in the CD group than in the STD group, although some of these differences disappeared in pups of the fish oil-supplemented groups (see Table 9).

Table 7 Concentration of specific fatty acids in lumbar adipose tissue of rats at day 21 of lactation that were given either standard diet (STD), cafeteria diet during pre-gestation, gestation and lactation (CD), cafeteria diet during pre-gestation, cafeteria diet supplemented with fish oil during the first 12 days of pregnancy and cafeteria diet during the remaining pregnancy and lactation (CD-FO12), or cafeteria diet during pre-gestation and cafeteria diet supplemented with fish oil during pregnancy and lactation (CD-FO)

	STD	CD	CD-FO12	CD-FO
Fatty acids (μmol/g)				
MA, 14:0	54.6 ± 3.6^{A}	112 ± 3^{B}	125 ± 9^{C}	140 ± 6^{C}
PAM, 16:0	815 ± 65	853 ± 21	850 ± 58	860 ± 37
STA, 18:0	128 ± 9^{A}	159 ± 5^{B}	159 ± 2^{B}	141 ± 4^{A}
PAO, 16:1n-7	146 ± 19	129 ± 9	130 ± 22	171 ± 7
OLA, 18:1n-9	957 ± 76^{A}	1397 ± 30^{B}	1301 ± 45^{B}	1281 ± 37^{B}
LNA, 18:2n-6	881 ± 57^{A}	421 ± 11^{B}	432 ± 11^{B}	422 ± 18^{B}
DGLA, 20:3n-6	4.60 ± 0.16^{A}	3.02 ± 0.16^{B}	3.01 ± 0.11^{B}	2.82 ± 0.18^{B}
GLN, 18:3n-6	4.76 ± 0.32^{A}	2.20 ± 0.50^{B}	2.53 ± 0.93^{B}	2.42 ± 0.51^{B}
ARA, 20:4n-6	15.6 ± 1.9^{A}	6.30 ± 0.46^{B}	5.71 ± 0.23^{B}	6.54 ± 0.55^{B}
DPAn-6, 22:5n-6	6.93 ± 1.20^{A}	2.33 ± 0.57^{B}	1.68 ± 0.56^{B}	1.60 ± 0.56^{B}
ALA, 18:3n-3	28.3 ± 3.1^{A}	16.0 ± 0.9^{B}	19.4 ± 2.0^{B}	21.2 ± 1.8^{B}
EPA, 20:5n-3	2.00 ± 0.21^{A}	3.04 ± 0.22^{A}	4.90 ± 1.14^{A}	12.8 ± 1.6^{B}
DPAn-3, 22:5n-3	4.18 ± 1.06^{AB}	3.24 ± 0.55^{A}	6.33 ± 0.84^{B}	15.0 ± 1.6^{C}
DHA, 22:6n-3	3.29 ± 0.38^{A}	0.304 ± 0.199^{A}	10.2 ± 2.2^{B}	31.3 ± 3.7^{C}
Product/precursor indices				
Δ^5 *d* ARA/DGLA	3.39 ± 0.39^{A}	2.10 ± 0.12^{B}	1.91 ± 0.13^{B}	2.31 ± 0.12^{B}
Δ^6 *d* GLN/LNA	0.00540 ± 0.00030^{A}	0.00694 ± 0.00031^{AB}	0.00791 ± 0.00128^{B}	0.00692 ± 0.00055^{AB}
Δ^9 *d* OLA/STA	7.48 ± 0.17^{A}	8.86 ± 0.36^{B}	8.19 ± 0.30^{AB}	9.06 ± 0.33^{B}
Elongase DGLA/GLN	0.99 ± 0.09	0.99 ± 0.07	0.96 ± 0.18	0.95 ± 0.06

Values correspond to mean ± SEM ($n = 5$–8). Statistical comparisons between the groups are shown by upper-case superscript letters (different letters indicate $P < 0.05$)

d Index of desaturase

Several changes in the concentrations of the n-6 PUFA were observed: DGLA was lower in the CD-FO group than in the other groups; GLN was higher in the CD and CD-FO12 groups than in the others; both ARA and DPAn-6 were lower in CD than in STD pups, and values further decreased in the CD-FO12 group and even further in the CD-FO group. An opposite trend was seen for the n-3 LCPUFA where EPA, DPAn-3, and DHA were higher in the CD-FO pups than in any of the other groups.

The indices of Δ^5 desaturase progressively declined in pups of the CD, CD-FO12, and CD-FO groups as compared to those of the STD group, whereas the index for Δ^6 desaturase was significantly lower only in the CD-FO group than in any of the others. The index for Δ^9 desaturase and the index for the elongase were higher in the three CD groups as compared to the STD group.

Association of plasma PUFA concentrations and body weights in pups

In order to study any potential correlation between plasma PUFA concentrations and body weights of pups at 2 or 21 days of age, regression analysis between pairs of variables was carried out. As shown in Table 10 it appears that in 2-day-old pups, there was a significant linear correlation between body weight and both LA and ARA, the latter being the most significant. At 21 days old, there were significant linear correlations between body weight and (in order of increasing statistical significance) LNA, ARA and DPAn-6.

Discussion

The present study showed that newborns of rats given a cafeteria diet from 22 days prior to pregnancy and throughout pregnancy have lower body weights compared to those from mothers on a standard control diet; this effect disappeared when pups were studied at the time of weaning even though the dams remained on the cafeteria diet during lactation. We also tested the effects of a dietary supplement with fish oil to the dams given the cafeteria diet, but maintaining the same energy intake. We found that supplementation did not affect the decreased newborns' body

Table 8 Concentration of plasma triacylglycerols (TAG), non-esterified fatty acids (FFA), cholesterol, 3-hydroxybutyrate and specific fatty acids of 2-day-old pups of dams that were given either standard diet (STD), cafeteria diet during pre-gestation, gestation and lactation (CD), cafeteria diet during pre-gestation, cafeteria diet supplemented with fish oil during the first 12 days of pregnancy and cafeteria diet during the remaining pregnancy and lactation (CD-FO12), or cafeteria diet during pre-gestation and cafeteria diet supplemented with fish oil during pregnancy and lactation (CD-FO)

	STD	CD	CD-FO12	CD-FO
TAG (μmol/L)	1.77 ± 0.29	1.81 ± 0.37	1.54 ± 0.34	1.71 ± 0.26
FFA (μmol/L)	731 ± 27	815 ± 85	758 ± 85	710 ± 80
Glycerol (μmol/L)	490 ± 44	559 ± 93	510 ± 116	562 ± 73
3-Hydroxybutyrate (μmol/L)	1883 ± 313	2940 ± 345	3229 ± 425	2633 ± 425
Fatty acids (μmol/L)				
MA, 14:0	79.3 ± 18.1^{A}	79.0 ± 13.6^{A}	57.1 ± 15.9^{A}	140 ± 11^{B}
PAM, 16:0	2093 ± 103	2441 ± 255	2320 ± 235	2603 ± 138
STA, 18:0	797 ± 50	1071 ± 143	960 ± 66	892 ± 70
PAO, 16:1n-7	183 ± 16^{A}	169 ± 15^{A}	175 ± 27^{A}	288 ± 34^{B}
OLA, 18:1n-9	1123 ± 118^{A}	2273 ± 287^{B}	1840 ± 255^{AB}	1965 ± 114^{AB}
LNA, 18:2n-6	1343 ± 113	1111 ± 175	1097 ± 89	1086 ± 250
DGLA, 20:3n-6	91.2 ± 6.9	104 ± 11	105 ± 7	105 ± 6
GLN, 18:3n-6	75.6 ± 7.9	54.3 ± 5.9	48.3 ± 7.3	49.3 ± 5.4
ARA, 20:4n-6	2411 ± 82^{A}	2141 ± 197^{B}	1964 ± 123^{B}	1462 ± 109^{C}
DPAn-6, 22:5n-6	222 ± 12^{A}	141 ± 8^{B}	21.8 ± 2.0^{C}	16.6 ± 2.5^{C}
ALA, 18:3n-3	25.9 ± 3.2	22.1 ± 4.1	18.8 ± 5.1	33.6 ± 3.4
EPA, 20:5n-3	66.4 ± 18.3^{A}	79.5 ± 22.5^{A}	246 ± 36^{B}	654 ± 103^{C}
DPAn-3, 22:5n-3	116 ± 9^{A}	109 ± 14^{A}	216 ± 12^{B}	334 ± 18^{C}
DHA, 22:6n-3	389 ± 34^{A}	345 ± 32^{A}	1011 ± 32^{B}	1547 ± 214^{C}
Product/precursor indices				
Δ^{5} *d* ARA/DGLA	26.9 ± 1.9^{A}	20.8 ± 0.9^{B}	18.9 ± 0.7^{B}	14.0 ± 1.3^{C}
Δ^{6} *d* GLN/LNA	0.0561 ± 0.002	0.0526 ± 0.0039	0.0430 ± 0.0029	0.0711 ± 0.0331
Δ^{9} *d* OLA/STA	1.44 ± 0.22^{A}	2.17 ± 0.14^{B}	1.89 ± 0.18^{AB}	2.23 ± 0.19^{B}
Elongase DGLA/GLN	1.35 ± 0.11^{A}	2.14 ± 0.14^{B}	2.52 ± 0.20^{B}	2.47 ± 0.29^{B}

Values correspond to mean ± SEM (n = 6–9). Statistical comparisons between the groups are shown by upper-case superscript letters (different letters indicate $P < 0.05$)

d Index of desaturase

weight on the CD, but that this effect was maintained until weaning only when the supplementation of the maternal diet was also maintained during that period. The decreased body weight of the offspring could be related to decreased plasma concentrations of ARA in the pups, which correlated to the pups' body weights tested at days 2 and 21 postpartum. In fact, the decreased body weight in pups of the CD-FO group was maintained until 41 days of age (data not shown) despite their just being given the standard diet from the time of weaning (day 21 of age).

Although in humans obese women are most likely to give birth to macrosomic children [5, 39, 40], in rats made obese by cafeteria or other high-fat diets, it has been consistently reported that newborns have unchanged or decreased body weight [14, 15, 41–43]. The reason for this effect has not been so far adequately explained. One explanation might be maternal protein intake. Despite increased total energy intake, cafeteria-fed dams usually show a decrease in their protein intake, which by itself could reduce the offsprings' birth weights [44, 45]. In the current study, however, although the cafeteria diet had a lower protein content than the standard diet, the actual protein intake was above the recommendations for growing and pregnant animals [46].

Our attention was focused on the fatty acid status, and we found for the first time a reduction in the ARA concentration in liver and lumbar adipose tissue in dams given the CD; this change was also found, and was even greater, in the plasma of those dams given the cafeteria diet supplemented with fish oil (CD-FO). The decrease in ARA concentrations in the CD group and its further decrease in the fish oil-supplemented group were also found in plasma of pups, 2 and 21 days postpartum. Arachidonic acid (or ARA) has been shown to be essential for fetal development in humans [47, 48], being specifically involved in fetal growth as shown by the significant linear correlation coefficients previously observed between birth weight and plasma ARA in preterm infants [31, 49].

The reasons for the decline in ARA levels in maternal and pup plasma of the CD group is not clear because there was more ARA in the CD than in the STD diets. Since this fatty acid is quite low in both diets, a possible reason for the decline in ARA concentrations could be a reduction in the availability of LNA, the precursor for its endogenous

Table 9 Concentrations of plasma triacylglycerols (TAG), non-esterified fatty acids (FFA), cholesterol, 3-hydroxybutyrate and specific fatty acids of 21-day-old pups of dams that were given either standard diet (STD), cafeteria diet during pre-gestation, gestation and lactation (CD), cafeteria diet during pre-gestation, cafeteria diet supplemented with fish oil during the first 12 days of pregnancy and cafeteria diet during the remaining pregnancy and lactation (CD-FO12), or cafeteria diet during pre-gestation and cafeteria diet supplemented with fish oil during pregnancy and lactation (CD-FO)

	STD	CD	CD-FO12	CD-FO
TAG (μmol/L)	1.19 ± 0.11^{A}	4.20 ± 0.82^{B}	2.54 ± 0.23^{AB}	2.99 ± 0.87^{AB}
FFA (μmol/L)	256 ± 21^{A}	612 ± 69^{B}	537 ± 45^{B}	520 ± 72^{B}
Glycerol (μmol/L)	245 ± 14^{A}	784 ± 126^{B}	506 ± 75^{AB}	648 ± 127^{AB}
3-Hydroxybutyrate (μmol/L)	304 ± 68^{A}	563 ± 41^{B}	705 ± 53^{B}	611 ± 67^{B}
Fatty acids (μmol/L)				
MA, 14:0	687 ± 137	873 ± 112	670 ± 75	710 ± 196
PAM, 16:0	3888 ± 333^{A}	7399 ± 796^{B}	5708 ± 424^{AB}	5340 ± 793^{AB}
STA, 18:0	1656 ± 106^{A}	2987 ± 244^{B}	2599 ± 199^{B}	1773 ± 221^{A}
PAO, 16:1n-7	160 ± 32^{A}	538 ± 76^{B}	226 ± 78^{A}	588 ± 126^{B}
OLA, 18:1n-9	1514 ± 54^{A}	7583 ± 1246^{B}	4504 ± 478^{A}	3683 ± 768^{A}
LNA, 18:2n-6	3809 ± 330	4469 ± 336	3968 ± 248	3248 ± 421
DGLA, 20:3n-6	113 ± 10^{A}	95.7 ± 11.7^{A}	76.1 ± 12.2^{A}	24.6 ± 4.9^{B}
GLN, 18:3n-6	69.6 ± 6.7^{A}	108 ± 6^{B}	121 ± 9^{B}	73.6 ± 7.2^{A}
ARA, 20:4n-6	2959 ± 296^{A}	2288 ± 87^{B}	1820 ± 136^{C}	772 ± 42^{D}
DPAn-6, 22:5n-6	138 ± 9^{A}	105 ± 8^{B}	35.0 ± 3.2^{C}	18.2 ± 2.2^{D}
ALA, 18:3n-3	61.7 ± 4.7	127 ± 19	85.8 ± 11.5	126 ± 34
EPA, 20:5n-3	34.7 ± 3.6^{A}	70.7 ± 6.8^{A}	70.8 ± 10.6^{A}	1599 ± 191^{B}
DPAn-3, 22:5n-3	75.9 ± 10.8^{A}	81.8 ± 9.6^{A}	77.6 ± 10.4^{A}	344 ± 78^{B}
DHA, 22:6n-3	523 ± 208^{A}	252 ± 10^{A}	375 ± 33^{A}	1731 ± 363^{B}
Product/precursor indices				
Δ^5 *d* ARA/DGLA	43.0 ± 4.1^{A}	21.5 ± 0.9^{B}	15.2 ± 1.5^{C}	10.7 ± 0.6^{C}
Δ^6 *d* GLN/LNA	0.0300 ± 0.00^{A}	0.0214 ± 0.0026^{A}	0.0200 ± 0.0041^{A}	0.0100 ± 0.00^{B}
Δ^9 *d* OLA/STA	0.91 ± 0.04^{A}	2.45 ± 0.24^{B}	1.71 ± 0.08^{B}	1.99 ± 0.20^{B}
Elongase DGLA/GLN	0.68 ± 0.04^{A}	1.33 ± 0.14^{B}	1.89 ± 0.29^{C}	2.71 ± 0.17^{D}

Values correspond to mean ± SEM ($n = 4$–7). Statistical comparisons between the groups are shown by upper-case superscript letters (different letters indicate $P < 0.05$)

d Index of desaturase

biosynthesis. However, the amount of LNA was similar in the CD and STD diets and no differences were found between these two groups in the plasma concentrations of dams at day 21 of lactation or of pups at 2 or 21 days old. The reason therefore could be a decreased capacity for the synthesis of ARA at the level of the Δ^5 and Δ^6 desaturases as is suggested by the consistent decline in the Δ^5 desaturase index ratio (ARA/DGLA) found here in the plasma of CD dams at day 21 of lactation and in 2- and 21-day-old pups of the same dams. One possible explanation for the decreased Δ^5 desaturase activities in these CD animals could be their higher concentrations of oleic acid, since an inverse relationship between oleic acid and ARA at the level of the Δ^5/Δ^6 desaturases has been reported in healthy human subjects [50], although its mechanism remains to be established.

Pups of dams on the fish oil-supplemented cafeteria diet during the first 12 days of pregnancy or throughout pregnancy and lactation had even lower body weights than pups from dams fed either the standard diet or just the cafeteria diet, especially at day 21 of age. A similar effect, together with a delay in neurodevelopment, had previously been seen in pups of dams given a fish oil-supplemented standard diet throughout pregnancy and lactation [30, 51]; the effect was explained by the low concentrations of ARA [30] probably resulting from the well-known effect of fish oil inhibiting the Δ^5 and Δ^6 desaturase activities [52, 53]. Decreased values of ARA concentrations and of the ARA/DGLA ratio in the presence of increased concentrations of EPA and DHA were consistently seen here in the groups that had received fish oil supplements, which supports the hypothesis. It is interesting to note that some of these changes have been seen here in the dams' adipose tissue at the end of the lactation period; these changes were even evident in rats on the cafeteria diet, supplemented with fish oil for only the first 12 days of pregnancy, which corresponds to the anabolic phase of the pregnant rat [54]. This finding together with the increase in plasma DPAn-3 and DHA concentrations in the same dams, as well as in plasma of the 2-day-old pups, clearly agree with findings previously reported in both pigs [55] and rats [56] showing the capacity of adipose tissue to store dietary-derived fatty acids during the first half of gestation, which are latterly (i.e., at

Table 10 Linear correlations of body weight and specific plasma polyunsaturated fatty acid concentrations in pups of dams that were given either standard diet (STD), cafeteria diet during pre-gestation, gestation and lactation (CD), cafeteria diet during pre-gestation, cafeteria diet supplemented with fish oil during the first 12 days of pregnancy and cafeteria diet during the remaining pregnancy and lactation (CD-FO12), or cafeteria diet during pre-gestation and cafeteria diet supplemented with fish oil during pregnancy and lactation (CD-FO)

Fatty acids	*n*	*r*	*P*
Pups 2 days of age			
LNA, 18:2n-6	24	0.543	0.0061
ARA, 20:4n-6	24	0.636	0.0008
DPAn-6, 22:5n6	24	0.334	0.1111
ALA, 18:3n-3	24	0.219	0.3033
EPA, 20:5n-3	23	0.003	0.9875
DHA, 22:6n-3	24	−0.111	0.6044
Pups 21 days of age			
LNA, 18:2n-6	20	0.520	0.0187
ARA, 20:4n-6	20	0.615	0.0039
DPAn-6, 22:5n-6	20	0.685	0.0009
ALA, 18:3n-3	20	0.153	0.5188
EPA, 20:5n-3	20	−0.338	0.1453
DHA, 22:6n-3	20	−0.226	0.3381

late pregnancy and during early lactation) released into plasma becoming available to the suckled neonates.

Some of the changes found in the plasma concentrations of other lipid components deserve further comment. It was seen here that plasma TAG, FFA, and glycerol levels are higher at the end of lactation in rats on the cafeteria diet groups as compared to those of the standard diet. This effect was expected on the basis of increased high-fat dietary intake in the cafeteria-fed dams and is in agreement with previous reports that found an increase in plasma TAG concentrations in rats given cafeteria diets [57] although not all reports agree [58, 59]. These different responses may be the result of differences in the duration of treatment. A hyperlipidemic condition was also seen here in the CD pups at weaning. At the same time, they also showed that the fish oil supplement had decreased the TAG and glycerol concentrations while maintaining increased values for both FFA and 3-hydroxybutyrate as seen in those from the cafeteria diet. The plasma triacylglycerol-lowering effects of fish oil supplements in rats is well known [60] and has been associated with diminished fatty acid biosynthesis and increased fatty acid oxidation and ketogenesis [61], which fits with the maintained high levels of 3-hydroxybutyrate in pups of the fish oil-supplemented cafeteria diet group at the end of lactation seen here.

It has previously been reported that over-nutrition in laboratory rats may affect milk production and composition [62]; therefore, one cannot discard the possibility that altered milk yield or composition in dams given the cafeteria diets, with or without supplementation, might cause the metabolic changes found in pups at the time of weaning. However, when a high-lipid diet, similar to the one used here, was introduced at the time of conception (in the current study it commenced 22 days prior to conception) and consequently the rats were not obese (as occurred here), milk lipid concentration was increased without changes in other components [63]. This finding, together with the fact that milk consumption by our litters did not differ between the groups, is consistent with the hyperlipidemic condition of the pups of CD dams.

At birth, newborns of dams given CD weighed less than those on STD, but at the time of weaning, pups of the CD group had a body weight that was similar to that of the STD group. This indicates that those of the CD group gained weight more rapidly, which also fits with the study of Del Prado et al. [63]. However, the lower body weight, seen also at weaning in pups of the group given the CD supplemented with fish oil, could be related to the lower concentrations of ARA in their plasma. In fact the significant correlations found here between pups' body weights and plasma ARA and LNA in case of 2-day-old pups, or between body weight and any of ARA, LNA and DPAn-6 in 21-day-old pups, points to the major role of ARA in postnatal development in rats, as has previously been proposed in humans [31]. Practically the only n-6 fatty acid present in the diets in appreciable amounts is LNA; it is therefore the main precursor for the endogenous synthesis of ARA. DPAn-6 is also synthesized from LNA, and is also a precursor of ARA via its retro-conversion [64]. The growth-promoting effect of ARA could be related to its function as a precursor of prostaglandins and other eicosanoids or to structural roles in membrane phospholipids. Therefore, the amount of ARA during the perinatal stages should be clearly preserved by the sufficient availability of its EFA precursor, LNA, as well as by the active synthetic n-6 pathway. This pathway can, however, be inhibited by an excess of n-3 LCPUFA in the diet, as was the case when supplements of fish oil were applied to the cafeteria diet.

In conclusion, this study shows that a moderate high-fat and highly palatable diet, such as the cafeteria diet, given to rats for 22 days prior to gestation and during pregnancy and lactation decreases the pups' newborn body weights, which recover during lactation. However, the effect is maintained, at least until weaning, in those pups whose mothers had received a supplement of fish oil, and this phenomenon could be related to the decreased concentration of ARA, which seems to be caused by an inhibition at the Δ^5 and Δ^6 desaturase reactions in the pathway for the biosynthesis of the n-6 LCPUFA.

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Compliance with ethical standard

Conflict of interest None of the authors had any financial or personal conflicts of interest.

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Dietary fish oil supplementation during early pregnancy in rats on a cafeteria-diet prevents fatty liver in adult male offspring

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ABSTRACT

We studied in rats the effects of cafeteria diet (CD) supplemented (or not) with fish oil (FO) during just the first 12 days of pregnancy, or during the whole of pregnancy and lactation in 14-month old offspring. Female rats were given standard diet (STD) or CD and after mating some animals remained on STD or CD; for some CD rats the diet was supplemented with 8.78% FO. After 12 days, half of the CD-FO group returned to CD (CD-FO12) and the others remained on CD-FO. From weaning all offspring were given STD. The adiposity index of male offspring of CD dams increased but was normal in CD-FO males. Plasma triacylglycerols (TAG) and individual fatty acid concentrations were similar among the groups. Liver total lipids, TAG, fatty acid concentrations, Δ9-desaturase indices and the mRNA expression of fatty acid synthase were higher in male offspring of CD than in those of STD; most of these differences disappeared in male offspring of CD-FO12 and CD-FO dams. Female offspring showed smaller changes. Thus, a moderate supplement with FO during just the first half of gestation or during pregnancy and lactation in rats on CD decreases the liver steatosis in male adult offspring.

1. Introduction

It is now well-known that maternal over-nutrition during pre-pregnancy, pregnancy and lactation influences offspring development and may cause permanent changes in adults, including the susceptibility for chronic diseases such as obesity, type-2 diabetes and cardiovascular disease; that is to say that the changes have been programmed (Hull et al., 2011; Boney et al., 2005; Schack-Nielsen et al., 2010; Godfrey and Barker, 2000; Lucas et al., 1999). In this sense, the composition of the diet may play an important role, as is the case for the polyunsaturated fatty acids (PUFA), which have a direct effect regulating the gene expression (Clarke, 2000) and major dietary implications during pregnancy (Herrera et al., 2012). To date there are limited data on the long-term influence of high-calorie processed foods during the perinatal stages. Recently we have studied the influence of a cafeteria diet, which is a highly palatable diet containing high fat and sugars, during pregnancy and lactation on pups' body weight and fatty acid profiles in rats, and also determined how a fish oil supplement rich in n-3 long-chain polyunsaturated fatty acids (LCPUFA) such as eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22-6n-3) modified the response (Sanchez-Blanco et al., 2016). Dietary depletion of DHA is known to cause adverse neurological outcomes in animals (Carlson and Neuringer, 1999) and it has been suggested that increasing the consumption of fish oil could prevent certain diseases (Hodge et al., 1996; Black and Sharpe, 1997). These findings have prompted the issuing of advice to pregnant and lactating women to consume fish oil supplements (Escolano-Margarit et al., 2013; Ribeiro et al., 2012; Palmer et al., 2012).

It has also been shown, however, that supplementing diets with n-3 LCPUFA in adult subjects decreases arachidonic acid (ARA, 20:4n-6) levels (Buckley et al., 2004; Grimsgaard et al., 1997; Burns et al., 2007). It has been proposed that ARA has a growth-promoting effect during the perinatal stage (Koletzko and Braun, 1991; Clandinin and VanAerde, 2003). In fact, we found in rats that both dietary fish oil (Amusquivar et al., 2000) or cafeteria diet with or without fish oil supplement during pregnancy decreases pups body weight that could be related to decreased plasma ARA levels (Sanchez-Blanco et al., 2016; Amusquivar et al., 2000). On the other hand, it has been shown in rats that fish oil alleviated high-fat diet-induced non-alcoholic fatty acid disease (Yuan et al., 2016) and we have previously found that fish oil supplements during pregnancy and lactation in rats have a long-term effect reducing the age-dependent insulin resistance in male but not female offspring (Sardinha et al., 2013).

Stearoyl-CoA desaturase-1 (SCD-1) is a lipogenic Δ9-desaturase (Ntambi et al., 2002) that is the rate-limiting enzyme in the biosynthesis of monounsaturated fatty acids. SCD-1 converts saturated fatty

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acids (16-carbon PAM, 16:0 and 18-carbon STA, 18:0) to monounsaturated fatty acids (palmitoleic, PAO, 16:1n-7, and OLA, 18:1n-9, respectively). The PAO/PAM and OLA/STA ratios have been widely used as desaturation indices for SCD-1 in both human (Sjogren et al., 2008; Warensjo et al., 2009; Vessby et al., 2013; Yee et al., 2014) and rat (Hofacer et al., 2012; Cedernaes et al., 2013) tissues. When female rats were on a high-fat diet prior to and throughout pregnancy and lactation, their 6-month old male pups had liver desaturation indices that were higher than in controls (Seet et al., 2015).

In the current study we followed the same protocol as before, where rats were given the cafeteria diet supplemented or not with fish oil during either the first 12 days of their 21.5-day pregnancy or throughout pregnancy and lactation (Sanchez-Blanco et al., 2016). The main objective on this occasion was to study the long-term effects for the offspring of changing dietary composition during perinatal life.

2. Materials and methods

2.1. Animals

Female Sprague Dawley rats of 40–45 days of age were obtained from the animal quarter of University San Pablo CEU, Madrid, Spain. Rats were given a standard pellet diet (Harlan Global Diet, 2014; Madison, WI) and maintained for 5 days in shared cages (5 per cage) under controlled conditions (22 ± 2 °C, 55 ± 10% relative humidity and 12-hourly cycling of light and dark). The experimental protocol was approved by the Animal Research Committee of the University San Pablo CEU (reference 220113).

Rats were then randomly assigned to one of two dietary treatment groups: the standard diet group (STD) and the cafeteria diet group (CD). After 21 days on their respective diet rats were mated with males that had been given the standard diet and the day that spermatozoa appeared in vaginal smears was considered to be day 0 of pregnancy. At this time pregnant rats were placed in individual cages and the CD group was subdivided into two, one maintained on the same diet and the other on a diet supplemented with fish oil (CD and CD-FO groups). On the 12th day of pregnancy half of the CD-FO group was returned to the cafeteria diet without the fish oil supplement (CD-FO12) and the other half remained on the fish oil supplemented cafeteria diet throughout the whole of pregnancy and lactation (CD-FO). Rats had free access to the assigned diet and tap water. Pups were maintained with their mothers until weaning and all litters were culled to 9 pups within 48 h of delivery by selecting the excess number of pups at random and removing them. At weaning (day 21 post-natal) female and male pups from each experimental group were separated from each other and housed in collective cages (3–4 per cage) and given the standard diet until the end of the experiment.

When the offspring were 14 months old, they were fasted for 12 h then decapitated under CO_2 sedation; trunk blood from the neck wound was collected into ice-chilled receptacles containing Na_2EDTA. Livers and the different fat depots were rapidly dissected and placed into liquid nitrogen for weighing and kept at −80 °C until analysis. Plasma from fresh blood was separated by centrifugation at 1500 × *g* for 30 min at 4 °C and stored at −80 °C until analysis.

2.2. Diets

Each diet was prepared as a homogeneous paste by mechanically blending the components shown in Table 1 and they were stored at −20 °C until use. The amount of fish oil present in the diet of the CD-FO group (8.78g/100g) was based on previous studies by us (Sanchez-Blanco et al., 2016; Amusquivar et al., 2000; Sardinha et al., 2013), being lower than the amount used by others for high-fat diet in rats (Nakashima, 2008; Viggiano et al., 2016; Hernandez-Casaer et al., 2017).

2.3. Analytical methods

Plasma triacylglycerols (TAG), cholesterol (Spinreact Reactives, Spain) and non-esterified fatty acids (NEFA) (Wako Chemicals, Germany) were determined by enzymatic methods using commercial kits. Liver TAG and cholesterol were determined in purified lipid extracts (Folch et al., 1957) as described elsewhere (Carr et al., 1993). For the analysis of the fatty acids profile, nonadecenoic acid (19:1) (Sigma Chemical Co.) was added as the internal standard to fresh aliquots of each diet, of frozen plasma and frozen livers, which were used for lipid extraction and purification (Folch et al., 1957). The final lipid extract was evaporated to dryness under vacuum and the residue resuspended in methanol/toluene and subjected to methanolysis in the presence of acetyl chloride at 80 °C for 2.5 h as previously described (Amusquivar et al., 2011). Fatty acid methyl esters were separated and quantified on a Perkin-Elmer gas chromatograph (Autosystem) with a flame ionization detector and a 20 m Omegawax capillary column (internal diameter 0.25 mm). Nitrogen was used as carrier gas, and the fatty acid methyl esters were compared with purified standards (Sigma Chemical Co.). Quantification of the fatty acids in the sample was performed as a function of the corresponding peak areas compared to that of the internal standard.

To extract protein from the tissues, between 50 and 60 mg of frozen liver were homogenized in a lysis buffer using a Tissue-Lyzer (Qiagen, Hilden, Germany) and after three thermic shocks at 37 °C. The homogenate was centrifuged at 4 °C for 30 min at 12000 rpm. After removing the upper fat layer the supernatant was used for protein analysis using a commercial kit (Pierce BCA Protein Assay kit, Thermo Fisher Scientific, Waltham, MA, USA) and stored at −20 °C until use for Western blot analysis. Proteins were combined with molecular weight markers (Precision Blue, Bio-Rab Laboratories, Hercules, CA, USA) and subjected to electrophoresis in 10% polyacrylamide gel at 90 V. Proteins were transferred to a nitrocellulose membrane (Bio-Rab Laboratories, Hercules, CA, USA) for the expression of fatty acid synthase (FAS). The membranes were incubated with different antibodies and latterly with secondary antibody marked with horseradish peroxidase (HRP). Immunoreactive bands were visualized by chemiluminiscence using a commercial kit (Clarity Western ECL Kit, Bio-Rab Laboratorioes, Hercules, CA, USA) and quantified by densitometry (Quantity One Analysis Software, Bio-Rab Laboratories, Hercules, CA, USA).

2.4. Desaturation index

After determining the amounts of the different fatty acids, the Δ9 desaturation index (SCD-1) was calculated as the ratio of the monounsaturated fatty acid to the corresponding saturated fatty acid: PAO (16:1n-7)/PAM (16:0) or OLA (18:1n-9)/STA (18:0).

2.5. Adiposity index

Adiposity index was determined by the sum of inguinal, mesenteric, perirenal, lumbar and epididymal pad weights divided by body weight × 100%, and expressed as adiposity percentage.

2.6. Statistical analysis

Results are expressed as mean ± standard error. Statistical analysis was carried out using GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, CA, USA). One-way analysis of variance (ANOVA) was used to compare the changes due to the different diets. When treatment effects were significantly different ($P < 0.05$) values were analyzed by the Newman-Keuls post hoc test. For TAG concentrations, a log transformation was carried out before statistical testing. When two groups were compared the Student *t*-test was applied.

Table 1
Composition of the diets.

	Diet		
	Standard (STD)	Cafeteria (CD)	Cafeteria plus Fish Oil (CD-FO)
Components (g/100g)			
Pellets*	100	25.2	25.2
Condensed milk (Nestlé, Barcelona)	–	11.0	11.0
Sucrose (Azucarera, Barcelona)	–	17.2	17.2
Muffins (Panrico SA, Barcelona)	–	6.58	6.58
Croissants (Bimbo, Barcelona)	–	6.58	6.58
Powdered milk (Central Lechera Asturiana, Asturias)	–	24.7	24.7
Lard (El Pozo, Murcia)	–	8.78	–
Fish oil (Pugnos Iberica, Barcelona)	–	–	8.78

* From Harlan, Global Diet 2014, Madison, MI.

Table 2
Fatty acid (FA) composition of the experimental diets.

Fatty acids (μmol/g)	Diet		
	Standard (STD)	Cafeteria (CD)	Cafeteria plus Fish Oil (CD-FO)
MA, 14:0	N.D.^{A}	40.7 ± 1.23^{B}	49.4 ± 1.6^{C}
PAM, 16:0	28.2 ± 1.6^{A}	185 ± 26^{B}	151 ± 3^{B}
STA, 18:0	3.62 ± 0.13^{A}	73.5 ± 2.3^{B}	36.8 ± 1.0^{C}
PAO, 16:1n-7	0.611 ± 0.042^{A}	15.1 ± 0.4^{B}	31.8 ± 0.6^{C}
OLA, 18:1n-9	33.4 ± 1.3^{A}	232 ± 6^{B}	156 ± 2^{C}
LNA, 18:2n-6	83.3 ± 3.1^{A}	77.9 ± 0.7^{A}	63.4 ± 2.2^{B}
DGLA, 20:3n-6	N.D.	N.D.	N.D.
GLA, 18:3n-6	N.D.^{A}	N.D.^{A}	0.69 ± 0.03^{B}
ARA, 20:4n-6	N.D.^{A}	0.835 ± 0.024^{B}	1.58 ± 0.06^{C}
DPAn-6, 22:5n-6	N.D.^{A}	N.D.^{A}	0.543 ± 0.008^{B}
ALA, 18:3n-3	5.67 ± 0.22^{A}	6.35 ± 0.07^{B}	7.49 ± 0.18^{C}
EPA, 20:5n-3	N.D.^{A}	0.712 ± 0.040^{B}	20.2 ± 0.3^{C}
DPAn-3, 22:5n-3	N.D.^{A}	N.D.^{A}	3.32 ± 0.08^{B}
DHA, 22:6n-3	N.D.^{A}	N.D.^{A}	22.5 ± 0.2^{B}
Sum of saturated FA	32.8 ± 1.7^{A}	304 ± 26^{B}	243 ± 6^{B}
Sum of monounsaturated FA	34.5 ± 1.3^{A}	252 ± 6^{B}	244 ± 2^{B}
Sum of n-6 polyunsaturated FA	83.3 ± 3.1^{A}	78.2 ± 2.5^{A}	66.6 ± 3.7^{B}
Sum of n-3 polyunsaturated FA	5.67 ± 0.22^{A}	7.06 ± 0.06^{B}	53.5 ± 0.5^{C}

Values correspond to mean of triplicate samples ± SEM. Statistical comparisons between the diets are shown by upper-case superscript letters (different letters indicate $P<0.05$). N.D. = not detected.

3. Results

The fatty acid composition of the diets is shown in Table 2 which shows that the CD diets have a higher content of fatty acids than the STD diet, the greatest difference being in the total amounts of saturated and monounsaturated fatty acids. The CD- FO diet has higher amounts of myristic (MA, 14:0), palmitoleic (PAO, 16:1n-7), gamma-linolenic (GLA, 18:3n-6), arachidonic (ARA, 20:4n-6), docosapentaenoic n-6 (DPAn-6, 22:5n-6), alpha-linolenic (ALA, 18:3n-3), docosapentaenoic n-3 (DPAn-3, 22:5n-3) acids, EPA, and DHA as well as total n-3 PUFA, but less linoleic acid (LNA, 18:2n-6) and total n-6 PUFA than the others. Body and tissue weights at different time points during pregnancy and lactation and of pups at birth and at weaning (21 days of age) have previously been reported (Sánchez-Blanco et al., 2016). When pups were studied at 14 months of age there were no statistical differences between the groups in terms of the weights of their body, liver and adipose tissues (data not shown). The adiposity index in males (Fig. 1A) was higher in pups whose mothers were given the cafeteria diet (CD) than in those with mothers fed the standard diet (STD) ($P < 0.05$ by *t*-test). Pups from dams fed CD supplemented with fish oil during the first 12 days of pregnancy (CD-FO12) also had higher adiposity indices than STD pups ($P < 0.05$ by *t*-test) and that difference disappeared in pups from CD dams receiving the fish oil supplement for the whole pregnancy and lactation (CD-FO). In females similar tendencies were seen (Fig. 1B), although the difference was significant only between CD and CD-FO groups.

When studying the plasma concentrations of TAG, NEFA, glucose, cholesterol or any of the individual fatty acids, including the sum of saturated, monounsaturated, n-6 fatty acids or n-3 fatty acids, no differences between the groups were found, with the only exception of a higher concentration of gamma-linolenic acid (GLA, 18:3n-6) in those male pups of the CD, CD-FO12 or CD-FO groups vs. those of the STD group and a lower concentration of alpha-linolenic acid (ALA, 18:3n-3) also in male CD, CD-FO12 and CD-FO groups vs. STD with no differences in females (supplement Table 1). However, major differences between groups were found in liver. As shown in Fig. 2A, the total concentration of lipids in livers from males was higher in those pups from dams fed the CD during pregnancy and lactation than those of the STD group ($p < 0.001$). Pups of cafeteria diet dams that received dietary fish oil supplement for just the first 12 days of pregnancy (CD-FO12) had lower liver lipid concentrations than those of the CD group ($p < 0.001$) and no difference was found between CD-FO12 and those of the STD group. This decrease in the liver total lipid concentration was even more pronounced in those male pups from CD dams that were given the fish oil supplement during pregnancy and lactation (CD-FO) ($p < 0.001$). These differences between the groups in males were similar to those of liver TAG concentrations (Fig. 2B), with values in the CD group being much higher than in the STD group ($p < 0.001$). Again, these values decrease in the CD-FO12 group ($p < 0.01$) and even more so in the CD-FO group ($p < 0.001$) where the values did not differ from those in the STD group. As shown in Fig. 2C and D no significant statistical differences between the groups were found in the female pups.

The concentrations of individual fatty acids in liver were determined (supplement Table 2). In male pups, stearic acid (STA, 18:0), dihomo-gamma-linolenic acid (DGLA, 20:3n-6), docosapentaenoic acid n-6 (DPAn-6, 22:5n-6), EPA and DHA liver concentrations showed no differences between the 4 groups, whereas the concentration of ARA was lower in the CD-FO than in the rest of the groups ($p < 0.01$). However, the concentration of the remaining fatty acids was significantly higher in liver of CD than in the STD group, an effect which decreased or even disappeared in those of the CD-FO12 and CD-FO groups. In female pups these differences between the groups were once again much smaller for all the studied fatty acids in liver. In Fig. 3 the sums of the concentrations of all saturated, monounsaturated, n-3 PUFA and n-6 PUFA are summarized; it can be seen that in males (Fig. 3A) the pattern for all the groups of fatty acids again resembles that found for the liver TAG (see above): i.e., significant increases in CD vs. STD, a decline in CD-FO12 and completely normalized in pups from the CD-FO

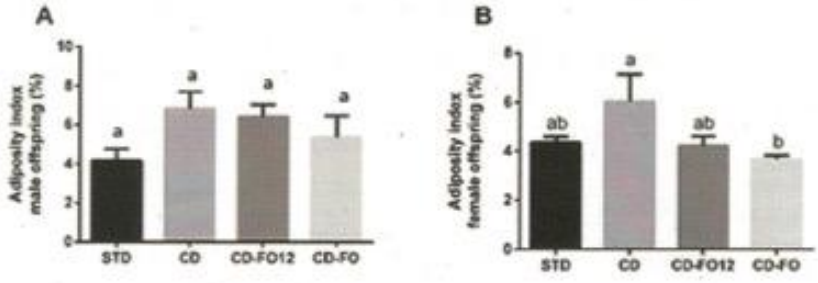

Fig. 1. Adiposity index of 14-month old male (A) and female (B) offspring of dams that were given either standard diet (STD) or cafeteria diet during pre-gestation, gestation and lactation (CD), or the cafeteria diet during pre-gestation followed by cafeteria diet supplemented with fish oil during only the first 12 days of pregnancy and cafeteria diet thereafter until weaning (CD-FO12), or the cafeteria diet during pre-gestation followed by cafeteria diet supplemented with fish oil throughout pregnancy and lactation (CD-FO). All pups were given standard diet from weaning. Statistical comparisons by ANOVA between the groups are shown by the lower case letters (different letters indicate $P < 0.05$). Number of 14-month old pups: 5/group.

group. In the case of females (Fig. 3B), the sum of all the n-6 PUFA was lower in those of the CD-FO group than in CD, but no significant differences appeared between the groups for the remaining fatty acid groups.

The SCD-1 indices were derived from the liver fatty acid concentration profiles and, as shown in Fig. 4A and B (corresponding to the PAO/PAM and OLA/STA ratios in males respectively), they were significantly higher in CD pups than in STD both for SCD-1 ($p < 0.01$). These effects were lower or had completely disappeared in both CD-FO12 and CD-FO pups. No differences between the groups were found in females (Fig. 4C and D). In order to relate these findings to lipogenesis, the expression of fatty acid synthase (FAS) was determined in the livers of males and as shown in Fig. 5, its amount changed between the groups in parallel to the SCD-1 values, being higher in CD pups than in STD ($p < 0.05$) and the effect was completely abolished in both CD-FO12 and CD-FO pups.

4. Discussion

We had previously found that, when 21-day-old pups of dams that were given a cafeteria diet during pre-gestation, gestation and lactation, they showed hypertriacylglycerolemia that completely disappeared when the cafeteria diet was supplemented with fish oil during just the first 12 days of pregnancy or throughout pregnancy and lactation (Sanchez-Blanco et al., 2016). Using the same protocol here, we have shown in 14-month old offspring that there were no differences between the studied groups either in plasma TAG concentrations or in their plasma fatty acid profiles. However, it was found that 14-month old male pups from dams given a cafeteria diet during pregnancy and lactation have increased both hepatic TAG and concentrations of all the groups of fatty acids in the liver. When the cafeteria diet dams also received a dietary supplement with fish oil during just the first half of pregnancy or throughout pregnancy and lactation these variables were completely normalized back to the values found in STD group, the effect being gender-dependent since it did not appear in females. The 14 month-old male pups of dams given the cafeteria diet during pregnancy and lactation also showed higher liver SCD-1 indices and higher amounts of FAS protein, which indicated an increased lipogenic activity that was normalized in those of dams that received the fish oil supplement during just the first 12 days of pregnancy or throughout the whole pregnancy and lactation. Since both SCD-1 and FAS have been positively associated to lipogenesis (Ntambi et al., 2002; Seet et al.,

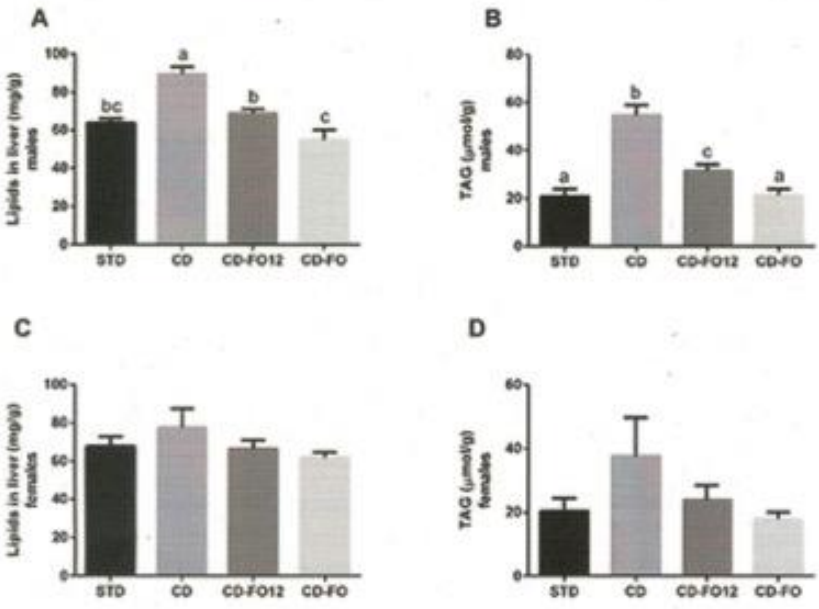

Fig. 2. Total lipids and TAG in liver of 14-month old male (A and B) and female (C and D) offspring of dams that were given either standard diet (STD) or cafeteria diet during pre-gestation, gestation and lactation (CD), or the cafeteria diet during pre-gestation followed by cafeteria diet supplemented with fish oil during only the first 12 days of pregnancy and cafeteria diet thereafter until weaning (CD-FO12), or the cafeteria diet during pre-gestation followed by cafeteria diet supplemented with fish oil throughout pregnancy and lactation (CD-FO). All pups were given standard diet from weaning. Statistical comparisons by ANOVA between the groups are shown by the lower case letters (different letters indicate $P < 0.05$. No letters in the graph indicate no statistical significance). Number of 14-month old pups: 5/group.

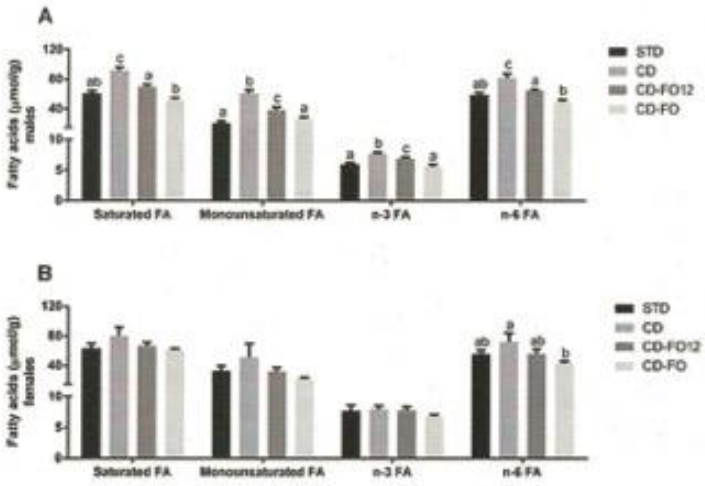

Fig. 3. Groups of fatty acid (FA) concentrations in liver of 14-month old male (A) and female (B) offspring of dams that were fed either standard diet (STD) or cafeteria diet during pre-gestation, gestation and lactation (CD), or the cafeteria diet during pre-gestation followed by cafeteria diet supplemented with fish oil during the only the first 12 days of pregnancy and cafeteria diet thereafter until weaning (CD-FO12), or the cafeteria diet during pre-gestation followed by cafeteria diet supplemented with fish oil throughout pregnancy and lactation (CD-FO). All pups were given standard diet from weaning. Statistical comparisons by ANOVA between the groups are shown by the lower case letters (different letters indicate $P < 0.05$. No letters in the graph indicate no statistical significance). Number of 14-month old pups: 5/group.

2015; Srable and Ntambi, 2010) current findings indicate that those FO supplements in dams on cafeteria diet during just the first 12 days of pregnancy or throughout pregnancy and lactation prevented the increased lipogenic effect in 14-month-old male pups. These findings extend those previously recorded by others in just 6-month old offspring of rats fed high fat diet during pregnancy and lactation showing increased liver SCD-1 indices (Seet et al., 2015) and in those of rats showing that 4 months of fish oil protects against the fatty liver developed by high-fat diet (Yuan et al., 2016). Although fish oil supplementation in rats during pregnancy has been shown to reduce the risks of adult disease in offspring (Joshi et al., 2003), this is the first time that the effect has been shown to be produced with a dietary fish oil supplement for just the first half of pregnancy.

The mechanism by which maternal fish oil supplements during just the first half of pregnancy in rats fed the cafeteria diet during pregnancy and lactation reverses the increased liver concentration of TAG and of most fatty acids as well as lipogenesis in male offspring of 14 months of age is unknown. However there is already enough experimental support to show that fatty acids, in particular polyunsaturated fatty acids, can modify the epigenome (Burdge and Lillycrop, 2014), and we have previously shown that the type of fatty acid consumed by the rat mother during early pregnancy elicits epigenetic mechanisms through miRNAs modulation in male offspring (Casas-Agustench et al., 2015). The present findings also show that the response to fish oil during the perinatal stage reverses those long-term effects of cafeteria diet on fatty acid metabolism was sex-dependent, since it was clearly found in males but not in females. This finding agrees with previous findings in the sense that there are sex differences in response to high-fat diet feeding in rats (Amengual-Cladera et al., 2012) and that fish oil intake during early pregnancy in rats reduces the age-dependent insulin resistance also in a sex-dependent manner (Sardinha et al., 2013).

As a result of studies in both humans and rats it is well known that diets with high fat contents, especially saturated fatty acids, promote the development of non-alcoholic fatty liver disease and consequent

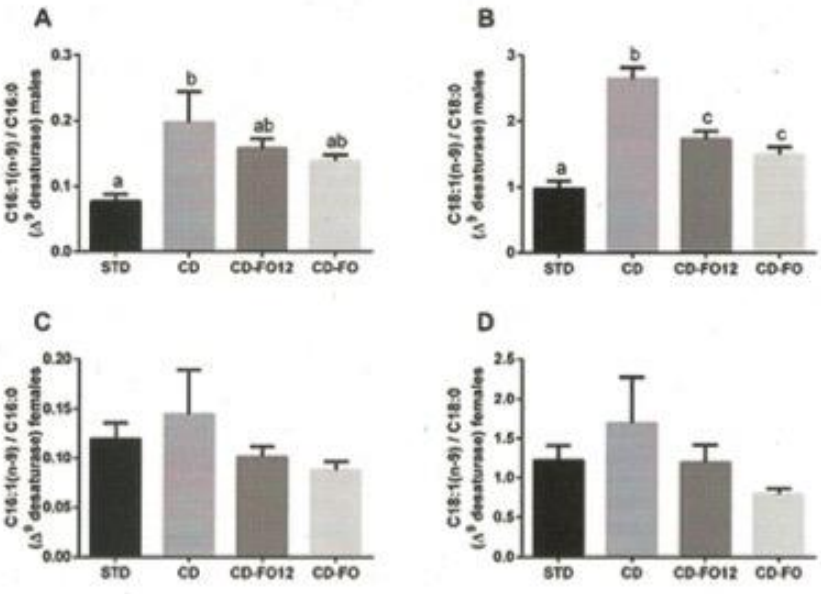

Fig. 4. Liver stearoyl-CoA desaturase-1 (SCD-1) indices of 14-month old male (A and B) and female (C and D) offspring of dams that were fed either standard diet (STD) or cafeteria diet during pre-gestation, gestation and lactation (CD), or the cafeteria diet during pre-gestation followed by cafeteria diet supplemented with fish oil during only the first 12 days of pregnancy and cafeteria diet thereafter until weaning (CD-FO12), or the cafeteria diet during pre-gestation followed by cafeteria diet supplemented with fish oil throughout pregnancy and lactation (CD-FO). All pups were given standard diet from weaning. Statistical comparisons by ANOVA between the groups are shown by the lower case letters (different letters indicate $P < 0.05$. No letters in the graph indicate no statistical significance). Number of 14-month old pups: 5/group.

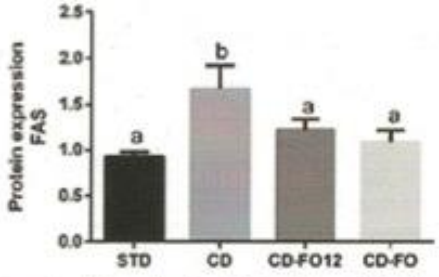

Fig. 5. Expression of fatty acid synthase in liver of 14-month old male offspring of dams that were fed either standard diet (STD) or cafeteria diet during pre-gestation, gestation and lactation (CD), or the cafeteria diet during pre-gestation followed by cafeteria diet supplemented with fish oil during only the first 12 days of pregnancy and cafeteria diet thereafter until weaning (CD-FO12), or the cafeteria diet during pre-gestation followed by cafeteria diet supplemented with fish oil throughout pregnancy and lactation (CD-FO). All pups were given standard diet from weaning. Statistical comparisons by ANOVA between the groups are shown by the lower case letters (different letters indicate $P < 0.05$). Number of 14-month old pups: 5/group.

increased hepatic TAG concentration (Yuan et al., 2016; Musso et al., 2003; Gentile and Pagliassotti, 2008). This effect has been mainly attributed to a promotion of lipogenesis *de novo* associated with a high-fat diet-induced overexpression of the sterol regulatory element binding protein-1 (SREBP1) encoded by the gene sterol regulatory element binding factor 1 (Srebf1), which is inhibited by fish oil consumption (Yuan et al., 2016; Ou et al., 2001; Levy et al., 2004). The current findings in 14-month old offspring are also consistent with reported findings in adult rat offspring of dams given a high-fat diet during pregnancy and lactation and then given a control diet from weaning; they developed a metabolic syndrome-like phenotype (Srinivasan et al., 2006; Desai et al., 2014) as well as increased liver SCD-1 (Seet et al., 2015), which has been positively correlated with lipogenesis and adiposity (Ntambi et al., 2002; Yoo et al., 2014; Jeyakumar et al., 2009; Kotronen et al., 2011).

In conclusion, although current findings cannot be extrapolated to the human condition by obvious reasons, we demonstrate for the first time that a moderate supplement of dietary fish oil during just the first 12 days of pregnancy is able to decrease the development of liver steatosis in male adult offspring of rats given a cafeteria diet during pregnancy and lactation; the effect was even greater when the fish oil supplement was maintained throughout gestation and lactation and can be related to a reduction in lipogenic activity.

Conflicts of interest

None of the authors had any financial or personal conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2018.12.006.

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