Estradiol determine liver lipid deposition in rats fed standard diets unbalanced with excess lipid or protein

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Abstract

The ingestion of excess fat often produces an increased body weight, because of higher adiposity and accumulation of fat in the liver. However, modulation of diet energy partition may affect differently the body metabolic responses and the extent of lipid deposition. Ten-week-old male and female rats were fed with either standard rat chow (SD), standard diet enriched with coconut oil (high-fat diet, HF), standard diet enriched with protein (high-protein diet, HP) or a self-selected "cafeteria" diet (CAF) for one month. Both HF and CAF diets provided the same lipid-derived percentage of energy (40%) HP diet protein-energy derived was twice (40%) than those of the SD diet. After the treatment, CAF groups showed significant weight increases. Hepatic lipid content also showed sex-related differences; triacylglycerol accumulation was significant in HF and CAF fed males. Cholesterol content was higher only in the CAF male group. Plasma estradiol in HF and HP males was higher than in CAF. Circulating cholesterol was inversely correlated with estradiol levels, which were proportional to lactate levels. These changes agreed with the differences found in the expression of key hepatic enzymes of lipid and energy metabolism. The protective effect of estrogens preventing excess liver lipid deposition, is also effective in males with 'normal' diets unbalanced by lipid or protein, but is not sufficient to protect males from the massive changes produced by a markedly obesogenic cafeteria-type diet. Estradiol protective effects are exerted at the root of energy metabolism, on the partition of substrates distributed from or entering the liver.

Introduction

It has been widely established that most of the rodent experimental models using high energy diets have shown that increases in fat intake correlate with body fat accrual.[1] Consequently, diets high in lipids have been used to induce obesity [2], despite a considerable variety of effects (and their quantitative expression) caused by the disparity in the composition of these diets. A critical question is the wide difference established between the animals subjected to the obesogenic manipulated diet and their controls, often fed standards rat chow. The age of animals used,[3] the time course of the intervention,[4] the sex [5] and finally, the palatability of the diet [6] provoke a further degree of heterogeneity in growth, fat deposition, and in the ratio of weight gain to lipid deposition.[7] Although this relationship has also been described for humans,[8] since dietary fat improves diet's palatability, adequate proportions of carbohydrate and other nutrients, are needed to further enhance fat deposition in humans. In addition, varied and savoury flavours increase the hedonic response.[9]

The data obtained using high-fat diets almost invariably result in increased body and liver weights, including a variety of alterations in homeostatic markers, especially overall increased fat deposition, but

also, markedly, in liver, altering glucose and lipid metabolism.[10-11] Furthermore, the incidence of dietary fatty acids in estrogen synthesis,[12] and the role of estrogens signalling metabolic pathways related with lipid metabolism,[13] or inducing non-genomic effects,[14] point to a possible different use of the diets' energy, depending on their fatty acid composition.

Diets with high-protein content have been promoted, especially in humans, to maintain or increase muscle mass,[14] although they have been also recommended for the treatment of obesity and related diseases.[15-16] However, their actual metabolic effects on energy partition and on general regulation of metabolism are largely unknown,[17-18] in parallel to their own metabolic pathways and the factors that regulate their utilization under conditions of normal nutrition. The studies done on malnutrition and starvation have shown us [19] that there are elaborate and resilient mechanisms set in place to preserve body N, for the salvage and turnover of amino N under conditions of starvation.[20] However, high-protein diets, when supplemented with sucrose [21] are known to negatively press on liver metabolism, inducing both steatosis and alterations of main substrate handling metabolic pathways. The high incidence of NAFLD (non-alcoholic fatty liver disease) in humans with metabolic syndrome [22] attests to its first-line position in the fight against excess fat and lipid energy in our diets. The known higher resilience of young women, compared with men, against excess adiposity and liver non-alcoholic steatosis again hints to estrogen as a critical protection factor against the onslaught of excess dietary fat.[23-24] Furthermore, there is a clear sex difference in energy management, since females are more prone to show low energy efficiency than males.[25] As estrogens increase the sensitivity to insulin [26] and androgens also contribute to glycemia homeostasis,[27] the role of both hormones in energy partition must be significant.

Despite liver central position (and function) in handling the substrates derived from the diet, the mechanisms of energy partition have been poorly studied both under normality and excessive feeding (as compared, obviously, with starvation, famine and malnutrition). We expect that lipid and protein content of diet should affect differentially liver metabolism, altering the ability for metabolite synthesis and liver lipid deposition, and the role of estrogens and/or androgens must by seminal and will provoke differential effects between males and females. We devised this experiment using controls which ate in a large proportion the same diet components than the groups with an added burden of protein or lipid, compared with a classical and proven obesogenic diet, cafeteria, which we expected would behave as a 'wild card' to help explain the way energy partition was modulated by 'naturally released' estrogen in both female and male rats.

Materials & Methods

Animals and experimental setup

All animal handling procedures and the experimental setup were carried out in accordance with the

animal handling guidelines of the European, Spanish and Catalan Authorities. The Committee on Animal Experimentation of the University of Barcelona authorized the specific procedures used (# DAAM 6911).

Ten-week-old female (initial weight 233±8 g) and male (initial weight 364±12 g) Wistar rats (Janvier, Le-Genest-Saint-Isle, France) were used (N=52). The animals were randomly divided in four groups (n= 6-8 each) for each sex, and were fed *ad libitum* for 30 days: rat chow (standard diet: SD group), a standard diet mixed with coconut oil (high-fat (HF) diet, the HF group), a simplified cafeteria diet (the CAF group) or a standard diet mixed with proteins (high-protein (HP) diet, the HP group). All animals had free access to water, and they were housed (in same-sex pairs) in a controlled environment (lights on from 08:00 to 20:00, temperature 21.5-22.5ºC, and 50-60% humidity). Body weight and cage food consumption were recorded daily. The calculation of ingested food in rats fed cafeteria diet was done as previously described by weighing the differences in food offered and debris left [28] and correcting for drying.

Diets

Table 1 shows the composition of the diets used. The standard diet (SD) (Teklad 2014, Teklad diets, Madison WI, USA) contained 19% of digestible energy derived from protein, 13% from lipids, and 67% from carbohydrates (including 10% from oligosaccharides). This diet essentially contained plant-derived foods. Diets were prepared as previously described.[29] Thus, HF diet was prepared by the addition of coconut oil to coarsely ground standard chow and contained 14% of digestible energy derived from protein, 37% from lipids, and 49% from carbohydrates (no oligosaccharides). The simplified cafeteria diet was formed by the standard chow pellets, plain cookies spread with liver pâté, bacon, water and milk, which was supplemented with sucrose and a mineral and vitamin. All components were kept fresh (i.e. renewed daily). From the analysis of diet components and the ingested items, we calculated that cafeteria diet contained 40% of energy derived from lipids, 12% from protein, and 49% from carbohydrates (24% from oligosaccharides). The HP diet was obtained by the addition of equal proportions of casein and gelatine to the ground standard chow; in this case, the energy derived from proteins was 40%, and 12% that derived from fat; the energy derived from carbohydrates was 48% (only natural polysaccharides).
The SD, HF and HP diets were given in the form of dry extruded pellets. Aversion tests to this diet gave negative results, not different from control diet as indicated previously.[29]

Experimental procedure

After 30 days of treatment, at the beginning of light cycle, the rats were anesthetized with isoflurane and blood was withdrawn with dry-heparinized syringes, through the exposed aorta until exsanguination. Plasma was obtained by centrifugation and kept at -20ºC until processed. Liver was dissected and immediately frozen in liquid nitrogen, then weighed and stored.

Analytical procedures

Total nitrogen, lipid and energy content of diet components were analysed as previously described.[6] Plasma parameters were measured using standard commercial kits: urea was measured with kit #11537, total cholesterol with kit #11505 and triacylglycerols with kit # 11528 (all from Biosystems, Barcelona, Spain). Lactate was measured with kit #1001330 (Spinreact, Sant Esteve d'en Bas, Spain) and non-esterified fatty acids with kit NEFA-HR (Wako, Neuss, Germany); 3-hydroxybutyrate and acetoacetate were estimated with a ketone bodies kit (Biosentec, Toulouse, France). Glycerol was estimated with kit #F6428 (Sigma-Aldrich, Darmstadt, Germany). Testosterone and estradiol were determined by Elisa kits EIA1559 and EIA2693 (DRG International, Marburg, Germany). Glucose in plasma was measured with a glucose oxidase kit #11504 (Biosystems, Barcelona, Spain) supplemented with mutarotase (490 nkat/mL of reagent) (Calzyme, San Luis Obispo, CA, USA). Mutarotase was added to speed up epimerization equilibrium of α- and β-D-glucose and thus facilitate the complete oxidation of D-glucose by glucose oxidase.[30-31]

Liver determinations

Samples of frozen liver (30-50mg) were powdered under liquid nitrogen. A mix of chloroform-methanol solution (1mL; 2:1 v/v) was added to the liver powder, and was shaken, and left at room temperature for 1 h, with occasional shaking to complete lipid extraction. Water (200µL) was added to the tubes, vortexed and centrifuged at 3000g during 5 min. The upper phase was discarded, and the organic phase was then dried with nitrogen at room temperature and the lipid pellet was dissolved in 2-methyl-2-propanol (60µL) and Triton X-114-methanol (40 µL; 2:1 v/v) mix. [32] Liver triacylglycerol and cholesterol content were measured with glycerol and cholesterol kits, respectively (Biosystems, Barcelona, Spain).

Gene expression analyses

Total tissue RNA was extracted from frozen samples (*ca*. 50 mg) using the Tripure reagent (Roche Applied Science, Indianapolis IN USA), and was quantified in a ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington DE USA). RNA samples were reverse transcribed using the MMLV reverse transcriptase (Promega, Madison, WI USA) system and oligo-dT primers. Real-time PCR (RT-PCR) amplification was carried out using 10 µL amplification mixtures containing Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA USA), 10 ng of reverse-transcribed RNA and 300 nM primers. Reactions were run on an ABI PRISM 7900 HT detection system (Applied Biosystems) using a fluorescent threshold manually set to 0.15 for all runs. A semi-quantitative approach for the estimation of the concentration of specific gene mRNAs per unit of tissue weight was used.[33] *Cyclophyllin A (Ppia)* was used

as the charge control gene. The data were expressed as the number of transcript copies per gram of protein in order to obtain comparable data between the groups, given the uniformity of the samples in that aspect. The genes analysed, and a list of primers used are presented in Table 2. These genes are related with lipid metabolism (*Pgp, Fas, Hmgcs2* and *CPT1α*), energy metabolism (*Cox 4i1* and *Uqcrc 1*) and lipid metabolism transcription factors (*Srebf2* and *Pparα*).

Calculations

Statistical comparisons were performed using two-way ANOVA (sex and diet) and *post hoc* Bonferroni tests, using the Prism 5.0 software (GraphPad Software Inc, La Jolla, CA, USA). Differences obtained with ANOVA or the Bonferroni test were considered statistically significant when the p value was < 0.05. Comparison between groups using Bonferroni's *post-hoc* test: differences between groups are represented by different superscript letters (capital letters for males, and low case for females). Correlations between different parameters were determined by linear regression analysis using the same program, applying a 95% confidence interval and the value of the Pearson correlation coefficient.

Results

Table 1 describes the nutrient composition of the diets. The values for CAF diet were obtained from the actual consumption data and were like those previously described[29] and used to design the lipid content of the HF diet. Both, crude and digestible energy were higher in the HF diet, since its energy density was higher than those of SD, HP and CAF diets. The cafeteria diet had the lowest crude energy value because of its low content of fibre and its abundant food water (as milk) intake, although its digestible energy was akin to the standard diet. Fat content in CAF and HF diets was similar, and three-fold higher than those for SD and HP diets. HP diet showed the highest proportion of protein, with a lipid content in the range of that of the standard diet. HF diet was the richest in C12:0+C14:0 fatty acids, followed by the CAF diet.

Table 3 show that all groups increased their weight in similar proportions, except for the CAF group, showing higher increases for both males and females. Liver weight only showed sex differences. HF diets increased lactate and estradiol levels, and decreased cholesterol levels either in males or females. Female HF diets showed also lower glucose and higher testosterone levels, whereas males showed increases in plasma triacylglycerols. CAF diet induced increases in triacylglycerols in males. The HP diets induced, in both sexes, a marked increase in circulating urea, accompanied by higher lactate and estradiol and lower cholesterol. The effect of diet was significant for all parameters except for acetoacetate and

testosterone. We found differences between both sexes for glycerol, triacylglycerols, hydroxybutyrate, and obviously for estradiol and testosterone.

Liver fat and cholesterol accumulation is presented in Figure 1. Male rats fed CAF diet showed a significant 3-fold increase of triacylglycerol accumulation in their livers compared with standard diet fed animals. Cholesterol accumulation was also higher in male CAF group, but the data for HF and HP were not different from those of the SD group. The females showed lower liver cholesterol and triacylglycerols than the males and did not show differences between groups.

Significant correlations were found, in males, between liver triacylglycerol content and lipid intake ($p=0.0080$) and between cholesterol content and lipid intake ($p=0.0050$). Females did not show any significant correlation for these parameters. Testosterone levels did not correlate neither with liver triacylglycerol ($p=0.5055$) or cholesterol ($p=0.0941$) content in both sexes.

Figure 2 shows a significant inverse correlation between plasma estradiol and plasma cholesterol levels. On the other hand, testosterone levels were not correlated with cholesterol levels ($p=0.6020$).The changes in the expression of different liver enzymes or transcription factors, related with lipid or energy metabolism are shown in Figure 3. The decreases in *Fas* expression in HF, CAF and HP groups in males, contrasts with the lack of changes in *CPT1α*. *PgP* expression followed a similar pattern than *Fas*, including decreases in HF and HP groups in females. *Hmgs2* showed differences between HF and CAF in relation to HP males. Transcription factors showed different patterns, as *Srbf2f* showed a clear tendency to decrease in HF and HP expression in males, whereas *Pparα* showed differences between HF females with respect to CAF and HP groups. A clear difference caused by sex was observed for *Pgp, Cox4i1* and *Uqcrc1* expressions, females showing higher values than males.

Discussion

As expected, the cafeteria diet induced higher weight increases than the other diets; this was caused, mainly, by excess adipose tissue accrual.[34] It is interesting that the HF-treated rats did not increase their weight over that of the SD diet controls despite their high proportion of fat intake, confirming that a high lipid intake *per se* is not associated with a disproportionate increase in reserves.[35] The surplus of dietary energy in the CAF males, was also accumulated in liver as lipids, doubling the normal lipid content. This result already shows that CAF males were unable to export (as VLDL) the excess triacylglycerols synthesized in the liver, despite their increased plasma levels; thus, a part of these lipids end up stored in the liver. This pattern contrasts with that of CAF females, which, despite also high fat ingestion, did not show this accumulation.

The differences in lipid composition between HF and CAF diets (higher proportion of medium-chain fatty acids in the HF diet) may have affected the patterns of distribution of some metabolites, such as estradiol and lactate. Thus, the high levels of estradiol found in the HF group may be –at least in part— a consequence of the presence of high lauric acid [36] which can increase the activity of aromatase.[12]

However, the supplementation of diet with coconut oil reduces lipogenesis (decreases in *Fas* and *Srbf2f* expressions) and tends to increase the oxidation of fatty acids, mediated in part by *Pparα* [37] and *Cpt1.*

Notwithstanding, the marked differences observed in the handling of fat by female rats, suggest that the known influence of medium-chain fatty acids could be in part countered by the low overall unsaturation of the fat in HF diet compared with the small amount of lipid in SD and HP and the large proportion of CAF, since saturated fats have been found to increase fat deposition.[38] Is important to note the radical difference in fat handling pattern shown by the females, since their liver triacylglycerol accumulation did not change irrespective of the level or type of lipid used, and more directly point at estrogen as the main causative effect of this peculiar capacity for lipid handling. This was confirmed also by the analysis of plasma metabolites and agrees with the higher ability of females to handle excess energy [25] by being less 'efficient' but thus safeguarding their energy homoeostasis. The higher liver expressions of *Cox4i* and *Uqrc1c* in all female groups, coupled with lower expression of lipogenic factors, such as *PPARα* attest to a lower energy efficiency than their male counterparts. In fact, this spend thrift characteristic is a net advantage to face situations in which the excess energy can compromise survival, as is the case with metabolic syndrome.[10] This fact was accomplished despite the absence of differences with males for ketogenesis, as indicated by the expression of *Hmgs2.*

The expression of the α-estrogen receptor variants is higher in females than in males.[25] These receptors are needed to limit liver fat deposition; conveyed through membrane receptor signalling.[39] Triacylglycerols and cholesterol are deposited into lipid droplets, metabolically active organelles, where a low level of Cidec/Fsp27β expression has been described in females fed high-energy diets, a fact that induce a lower presence of CIDEC/FSP27 protein,[40] pointing towards a hormonal regulation (estrogens) of their turnover. However, this fact does not seem to be sex-related because HF and HP males also show high levels of estradiol. The effect of estradiol lowering liver lipid content has been also attributed to sequestration of *Srbef1* into membrane.[39] Our results also indicate a substantial decrease in the expression of *Srbef2,* suggesting a decrease in cholesterol synthesis in the male HF group, which may help explain the inverse correlation between cholesterol and estradiol levels. Our results agree with the observed decrease in liver cholesterol synthesis caused by high-fat diets or by cholesterol-supplemented diets.[41]

In our experimental model, testosterone seems not to play a direct role in the regulation of dietary lipid deposition in the liver, since we did not found correlations with lipid metabolism or deposition parameters, including circulating cholesterol, which reinforces our assumption.

A protective role of estrogens against obesogenic diets has also been described in mice,[42] where a dimorphic activity of glucocorticoid metabolizing enzyme may be the base of this different sexual trend. On the other hand, the high plasma levels of lactate showed by HF and HP groups, in a metabolic normoglycemic condition may be a consequence of 3C metabolite sparing in front of 2C metabolite use [43]

because of elevated estradiol levels. Thus, estradiol levels change liver metabolism, modifying energy partition and limiting cholesterol deposition and increasing lactate synthesis.

The postulated effect of estrogen, which we based largely on correlations, is not an exclusive peculiarity of female rats, since males also show a modulation of their estradiol levels in relation to diet and lipid (energy) handling. Long time ago, we already observed anti-obesity effects of estrone derivatives in the face of high-energy diets, irrespective of sex,[44] devoid of patent estrogenic effects despite prolonged treatments.[45] It is well known that estrogens favour the utilization of excess body fat [24] in women and men. In fact, the regulation of sex steroids in plasma via SHBG seems solely limited, in humans, to testosterone,[46] in a context fully disconnected from estrogens. The presence of active 17-hydroxysteroid dehydrogenases in liver,[47] suggests a direct proximity between the regulatory agent and the regulated paths. The marked absence of additional studies on the direct implication of estradiol on the hub of energy partition and its regulation makes further assumptions more difficult, except that estradiol is known to directly affect the oxidative function of mitochondria,[48] thus being able to speed-up the oxidation of acetyl-CoA, a critical point for the removal (despite the overall inefficiency of the process) of unneeded acetyl-CoA and indirectly preventing its incorporation to the lipogenic pathway).

Conclussions

The role of oestrogens protecting on lipid deposition (especially in the liver) operate not only for the females, but also works on HF and HP males, regardless of the great differences between both diets. This uniformity of action suggest that estradiol plays a more general role than usually assumed in the context of dietary energy partition, largely irrespective of sex and influencing also in the handling of cholesterol and the conversion of glucose to lactate (or other 3C fragments) or its use to provide 2C fragments for energy, ketogenesis of lipogenesis.

Conflicts of interest

There are no conflicts to declare.

LEGENDS TO FIGURES

Figure 1. Triacylglycerol and cholesterol liver content. Data are the mean ± SEM of six to eight animals per group. White bars: standard diet (SD); blue bars: high-fat diet (HF); red bars: cafeteria diet (CAF) and brown bars: high-protein diet (HP). Statistical differences between groups: two-way ANOVA (D, diet; S, sex; I, their interaction). Bonferroni post-hoc test: different letters represent statistically significant differences between groups of the same sex.

Figure 2. Correlation of plasma estradiol and plasma cholesterol values. Value of statistical significance of correlations are incorporated as p values.

Figure 3. Liver expression of different liver enzymes or transcription factors: Fatty Acid Synthetase (*Fas*), Carnitine O-palmitoyl transferase 1 (*Cpt1a*), Hydroxymethylglutaryl-CoA synthase 2 (*Hmgs2*), Phosphoglycolate phosphatase (*PgP*), Sterol regulatory element-binding protein 2 (*Srbf2f*), Peroxisome Proliferator Activated Receptor α (*Pparα*), Cytochrome c oxidase I (*Cox4i1*) and Ubiquinol-Cytochrome C Reductase Core Protein 1 (*Uqcrc1*). Data are the mean ± SEM of six to eight animals per group. White bars: standard diet (SD); blue bars: high-fat diet (HF); red bars: cafeteria diet (CAF) and brown bars: high-protein diet (HP). Statistical differences between groups: two-way ANOVA (D, diet; S, sex; I, their interaction). Bonferroni post-hoc test: different letters represent statistically significant differences between groups of the same sex.

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Table 2. Primer sequences used for gene expression analysis.

gene	protein	direction	Sequences	Bp
Pgp	Phosphoglycolate phosphatase	5' >	CCTGGACACAGACATCCTCCT	100
		> 3'	TTCCTGATTGCTCTTCACATCC	
Fas	Fatty Acid Synthetase	5' >	CCCGTTGGAGGTGTCTTCA	117
		> 3'	AAGGTTCAGGGTGCCATTGT	
Cox 4i1	Cytochrome c oxidase subunit 4 isoform 1	5' >		
		> 3'		
Hmgcs2	Hydroxymethylglutaryl-CoA synthase 2, mitochondrial	5' >	CAACCTCTTCCCAGGCACTT	108
		> 3'	CCGGGGAATGGTTGTATGGA	
CPT1a	Carnitine O-palmitoyltransferase 1, liver isoform	5' >	CCGCTCATGGTCAACAGCA	105
		> 3'	CAGCAGTATGGCGTGGATGG	
Srebf2	Sterol regulatory element-binding protein 2	5' >	ACCGTTTAGCAGCCACAGCA	121
		> 3'	CCACAACCCTGACCAACACC	
Pparα	Peroxisome Proliferator Activated Receptor Alpha	5’ >	*GCACAATCCCCTCCTGCAAC*	124
		> 3’	*TTCAATGCCCTCGAACTGGA*	
Uqcrc1	Ubiquinol-Cytochrome C Reductase Core Protein 1	5’ >	TCGCAGCCTCCTGACTTATG	78
		> 3’	ATCTGGGCATCCACCTCCT	
PPIA	Peptidylprolyl isomerase A (cyclophillin A) [housekeeping gene]	5' >	CTGAGCACTGGGGAGAAAGGA	87
		> 3'	GAAGTCACCACCCTGGCACA	

Table 1. Diet composition

	SD	HF	CAF*	HP
Crude energy content (kJ/g)	16.5	18.8	12.4±0.15	17.4
Digestible energy content (kJ/g)	12.1	14.6	12.0±0.14	12.4
% energy intake				
Protein	19.3	14.5	11.7±0.42	40.4
Fat	12.5	36.8	39.5±0.85	11.6
Carbohydrate	67.1	48.6	48.5±0.52	47.7
Sugars (as % of Carbohydrates)	<1	<1	24.1±0.46	<1
Mean fatty acid content (%)				
Saturated	25	74	54	25
C12:0+C14:0	2	53	22	2
Monounsaturated	14	19.5	34	14
Polyunsaturated	61	5	12	61

*Data obtained from the food consumption data of the male animals studied (mean values). Values from female intake did not differ statistically.

Table 3. Weight increase, liver weight, metabolite and hormone plasma values of rats after dietary treatment**.**

	MALES				FEMALES				
	SD diet	HF diet	CAF diet	HP diet	SD diet	HF diet	CAF diet	HP diet	ANOVA
Weight increase (g)	79.1±8.2^{A}	82.2±6.3^{A}	126±3.2^{B}	68.6±1.5^{A}	39.5±4.3^{a}	27.5±1.6^{a}	73.6±6.9^{b}	31.2±1.8^{a}	D S
Liver weight (g)	15.9±1.57^{A}	13.1±0.33^{AB}	16.7±0.85^{AC}	15.1±0.15^{A}	8.56±0.47^{a}	8.14±0.19^{a}	8.16±0.24^{a}	7.66±0.37^{a}	S
Glucose (mM)	9.85±0.31^{A}	9.65±0.51^{A}	10.5±0.7^{A}	11.2±0.6^{A}	10.9±0.7^{a}	8.43±0.31^{b}	10.4±0.6^{ab}	10.1±0.3^{ab}	D
Lactate (mM)	2.07±0.08^{A}	4.71±0.49^{B}	2.66±0.30^{A}	4.40±0.31^{B}	2.27±0.31^{a}	4.01±0.28^{b}	2.70±0.33^{ac}	3.70±0.29^{bc}	D
Glycerol (mM)	0.16±0.02^{A}	0.34±0.06^{A}	0.18±0.03^{A}	0.35±0.06^{A}	0.15±0.03^{a}	0.21±0.01^{a}	0.22±0.02^{a}	0.21±0.01^{a}	D S
Cholesterol (mM)	2.66±0.21^{A}	1.65±0.08^{B}	2.24±0.11^{A}	1.61±0.07^{B}	2.64±0.11^{a}	1.58±0.21^{b}	2.48±0.31^{a}	1.36±0.04^{b}	D
Non-esterified fatty acids (mM)	0.32±0.05^{A}	0.38±0.04^{A}	0.55±0.08^{A}	0.45±0.06^{A}	0.32±0.05^{a}	0.35±0.03^{a}	0.44±0.07^{a}	0.44±0.06^{a}	D
Triacylglycerols (mM)	1.34±0.04^{A}	1.94±0.11^{BC}	1.96±0.18^{B}	1.35±0.19^{AC}	1.01±0.09^{a}	1.04±0.21^{a}	0.98±0.08^{a}	0.77±0.14^{a}	D S
Cholesterol/TAG	1.97±0.13^{A}	0.90±0.10^{B}	1.59±0.15^{AC}	1.13±0.13^{BC}	2.71±0.33^{a}	1.90±0.21^{a}	2.82±0.29^{a}	1.78±0.19^{a}	D S
NEFA/TAG	0.29±0.05^{A}	0.33±0.06^{A}	0.24±0.03^{AC}	0.33±0.04^{A}	0.37±0.07^{a}	0.49±0.08^{a}	0.42±0.05^{a}	0.57±0.11^{a}	S
Urea	2.98±0.31^{AB}	3.55±0.11^{A}	1.98±0.15^{B}	6.62±0.35^{C}	2.26±0.18^{ab}	3.43±0.31^{a}	1.88±0.31^{b}	6.76±0.43^{c}	D
3OH butyrate (µM)	31.2±5.01^{A}	48.9±4.51^{A}	31.1±5.75^{A}	47.2±5.50^{A}	47.1±7.11^{a}	62.4±11.1^{a}	31.4±6.78^{a}	60.6±11.1^{a}	D S
Acetoacetate (µM)	190±43.5^{A}	159±32.4^{A}	128±15.9^{A}	143±12.2^{A}	145±58.5^{a}	183±31.4^{a}	202±49.4^{a}	110±25.2^{a}	
Estradiol (ng/L)	29.1±5.24^{A}	100±8.41^{B}	35.9±3.82^{A}	69.8±6.05^{C}	57.2±4.58^{a}	106±6.96^{b}	59.7±6.07^{a}	111±10.5^{b}	D S
Testosterone (µg/L)	3.18±0.28^{A}	3.51±0.44^{A}	3.69±0.49^{A}	2.96±0.19^{A}	1.16±0.07^{a}	1.72±0.08^{b}	1.26±0.11^{a}	1.47±0.08^{ab}	S
Testosterone/estradiol	111±17.1^{A}	36.9±6.94^{B}	102±8.08^{A}	41.5±5.55^{B}	20.8±2.02^{a}	16.1±1.27^{ab}	21.1±1.38^{a}	13.2±2.11^{b}	D S

Data are expressed as mean ± SEM. Statistical analysis: two-way ANOVA significant p-values for diet (D), sex (S) or Interaction (I): $p < 0:05$. Statistical significance (Bonferroni's post-hoc test) between diets is represented by different superscript letters.

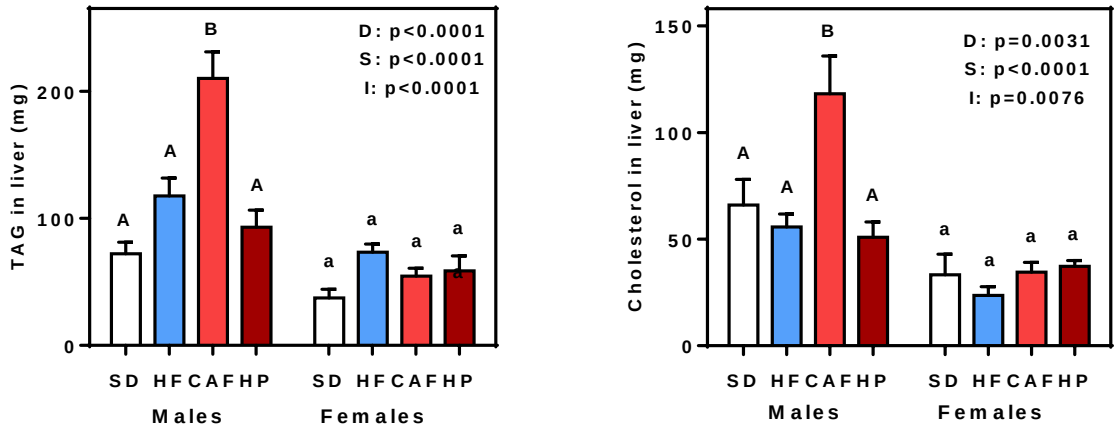


Figure 1. Triacylglycerol and cholesterol liver content. Data are the mean ± SEM of six to eight animals per group. White bars: standard diet (SD); blue bars: high-fat diet (HF); red bars: cafeteria diet (CAF) and brown bars: high-protein diet (HP). Statistical differences between groups: two-way ANOVA (D, diet; S, sex; I, their interaction). Bonferroni post-hoc test: different letters represent statistically significant differences between groups of the same sex.

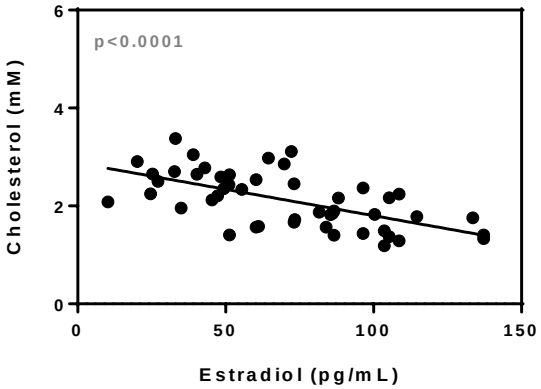
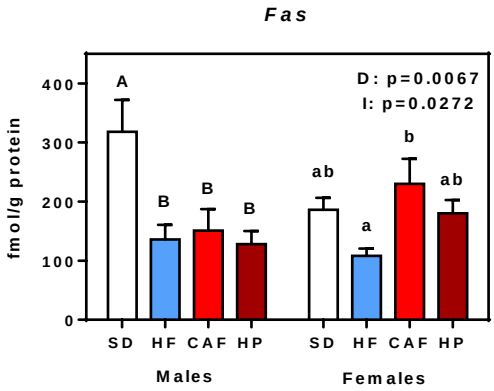


Figure 2. Correlation of plasma estradiol and plasma cholesterol values. Value of statistical significance of correlations are incorporated as p values.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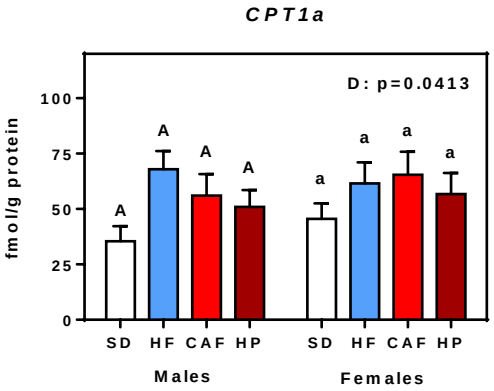
ab
SD HF CAF HP
Males
SD HF CAF HP
Females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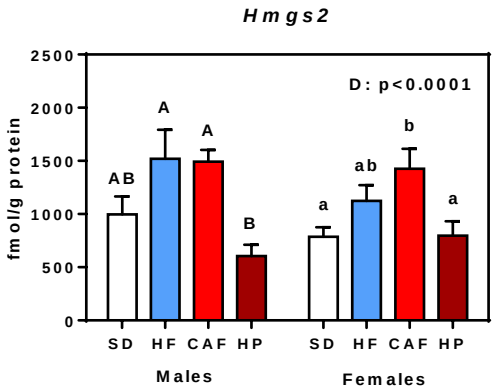
Females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SD HF CAF HP
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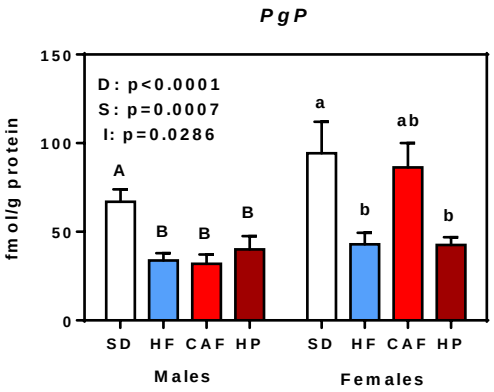
Females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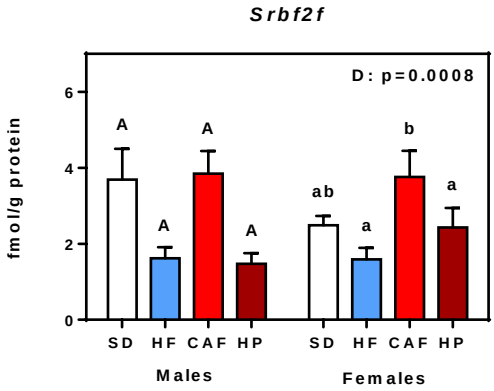
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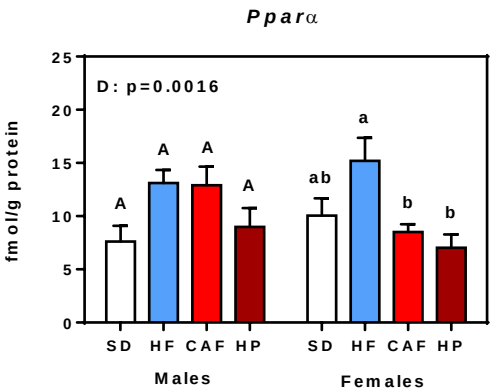
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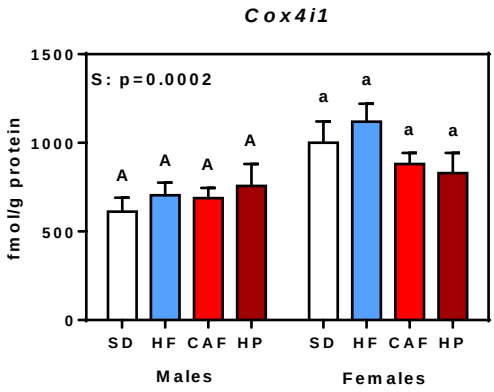
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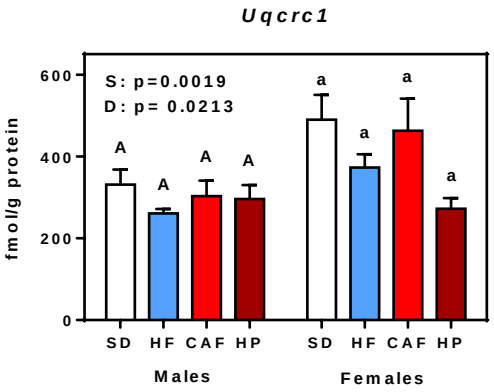
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a
a
a
SD HF CAF HP
Males
SD HF CAF HP
Females

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Figure 2. Correlation of plasma estradiol and plasma cholesterol values. Value of statistical significance of correlations are incorporated as p values.

Figure 3. Liver expression of different liver enzymes or transcription factors: Fatty Acid Synthetase (*Fas*), Carnitine O-palmitoyltransferase 1 (*Cpt1α*), Hydroxymethylglutaryl-CoA synthase 2 (*Hmgs2*), Phosphoglycolate phosphatase (*PgP*), Sterol regulatory element-binding protein 2 (*Srbf2f*), Peroxisome Proliferator Activated Receptor α (*Pparα*), Cytochrome c oxidase I (*Cox4i1*) and Ubiquinol-Cytochrome C Reductase Core Protein 1 (*Uqcrc1*). Data are the mean ± SEM of six to eight animals per group. White bars: standard diet (SD); blue bars: high-fat diet (HF); red bars: cafeteria diet (CAF) and brown bars: high-protein diet (HP). Statistical differences between groups: two-way ANOVA (D, diet; S, sex; I, their interaction). Bonferroni post-hoc test: different letters represent statistically significant differences between groups of the same sex.

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