Intake of grape procyanidins during gestation and lactation impairs reverse cholesterol transport and increases atherogenic risk indexes in adult offspring.

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Abstract

Cardiovascular disease (CVD) is one of the most prevalent noncommunicable diseases in humans. Different studies have identified dietary procyanidins as bioactive compounds with beneficial properties against CVD by improving lipid homeostasis, among other mechanisms. The aim of this work was to assess whether grape seed procyanidin consumption at a physiological dose during the perinatal period could influence the CVD risk of the offspring. Wistar rat dams were treated with a grape seed procyanidin extract (GSPE, 25 mg kg-1 of body weight/day) or vehicle during gestation and lactation. The adult male offspring of GSPE-treated dams presented decreased HDL-cholesterol levels, increased total cholesterol to HDL-cholesterol ratios and an exacerbated fasting triglyceride to HDL-cholesterol ratios (atherogenic index of plasma) compared to the control group. Impaired reverse cholesterol transport (RCT) was evidenced by the accumulation of cholesterol in skeletal muscle and by decreased fecal excretion of cholesterol and bile acids, which was consistent with the observed mRNA downregulation of the rate-limiting enzyme in the hepatic bile acid synthesis pathway *Cyp7A1*. Conversely, GSPE programming also resulted in upregulated gene expression of different key components of the RCT process, such as hepatic *Npc1, Abcg1, Abca1, Lxra, Srebp2, Lcat, Scarb1* and *Pltp*, and the repression of micro RNA miR-33a expression, a key negative controller of hepatic RCT at the gene expression level. Our results show that maternal intake of grape procyanidins during the perinatal period impacts different components of the reverse cholesterol transport process, resulting in increased CVD risk in the adult offspring.

Keywords: Grape seed procyanidins; metabolic programming; cardiovascular disease; reverse cholesterol transport

1. Introduction

According to the World Health Organization, 37% of deaths associated with noncommunicable diseases are due to cardiovascular disease (CVD) [1]. Among the main risk factors, including blood pressure, endothelial function or platelet aggregation, abnormalities in lipoprotein metabolism stand out [2,3]. Thus, low concentrations of high density lipoprotein cholesterol (HDL-C) are an independent risk factor for CVD [6]. HDLs are the primary effectors of reverse cholesterol transport (RCT), which consists of the efflux of cholesterol from peripheral tissues and subsequent transport to the intestine for direct excretion by means of the transintestinal cholesterol efflux [9] or to the liver for conversion to bile acids and subsequent excretion through the bile canaliculi into the intestinal lumen [10]. Thus, the concentration of cholesterol in HDL particles has been considered by some authors as a surrogate analysis of RCT efficiency [6]. Together with LDL-C and HDL-C, previous research has shown that serum TG levels are statistically independent predictors of atherosclerosis and incident CVD events, likely due to the role of TG-rich lipoproteins in the generation of atherosclerosis [12,13]. These observations has prompted the definition of different cardiovascular risk indexes, such as the total cholesterol to HDL-C ratio, which is widely accepted [2], and the atherogenic index of plasma (AIP), which combines serum TG and HDL-C values. Initially, this index was proposed as an indirect measure of LDL particle size and was defined as a good indicator of the atherogenic lipoprotein phenotype in several human cohorts [14]. Recently, different studies have shown that the AIP might be a good predictor of subclinical atherosclerosis as well as a biomarker of high blood pressure [15], diabetes and vascular events [16].

The origin of CVD is multifactorial, conditioned by genetic and environmental factors. Among the different factors that might cause predisposition to CVD, the impact of the

environment during fetal development and early life has gained relevance. Initially, Barcker and collaborators established an association between maternal undernutrition, low birth weight and rates of death from ischemic heart disease in the adult offspring [18,19]. More recently, different epidemiological studies in humans and controlled interventions in pre-clinical models have shown that programming in early periods of life is relevant for the development of metabolic alterations in adulthood, leading to the Developmental Origins of Health and Disease Hypothesis [20]. Thus, both maternal under- and overnutrition during the perinatal period have been associated with an increased risk of obesity, diabetes or CVD in the adult offspring [21,22]. These observations are currently explained by the effect of maternal nutritional status on fetal development, shaping the metabolism of the offspring even into adulthood.

Due to the tight link between maternal nutritional status and the perinatal origin of some cardiometabolic alterations, assessing the metabolic programming effects of food bioactive compounds has gained much attention. One of such family of compounds is the polyphenols, which present a wide range of beneficial effects for health [23–26]. Together with these properties, different studies have demonstrated that polyphenols can exert metabolic programming effects when consumed during the perinatal period. Thus, the offspring of dams treated with a grape skin extract rich in polyphenols were protected against obesity [27,28]. In another study, consumption of Azuki bean polyphenols during the lactation period counteracted different deleterious effects of maternal undernutrition [29]. Finally, the treatment of obese dams with quercetin protected the dams against hypercholesterolemia and the offspring against insulin resistance and endoplasmic reticulum stress [30]. On the other hand, some studies have suggested negative effects of maternal intake of polyphenols on fetal health and bird weight [31,32]. Thus, the outcome of metabolic programming by polyphenols remains a subject of controversy.

Among polyphenols, procyanidins, a family of flavonoids, have different beneficial effects on health, such as anti-inflammatory [33] and hypolipidemic actions [34,35], and being effective against diet-induced dyslipidemia [36] or CVD [37,38]. In both rats and mice, these polyphenols are effective at ameliorating cardiovascular risk indexes and exerting beneficial effects in the metabolism of cholesterol [34,35,39]. Moreover, we have shown that procyanidins administered to pregnant rats can reach the fetus [40] and exert metabolic programming effects, increasing adiposity and improving the inflammatory response in the 30-day-old offspring of diet–induced obese rats [41]. In the adult offspring, maternal grape seed procyanidin (GSPE) consumption resulted in a shift in the whole-body energetic substrate utilization toward lipid catabolism and, in parallel, induced the phosphorylation of AMPK and the expression of genes related to fatty acid uptake and β-oxidation in the muscle [42]. Importantly, the dose of polyphenols used in those and the present study is equivalent to the average daily consumption of procyanidins in the Spanish population [43]. The aim of this work was to further explore the same cohort of animals described previously [42] and to assess whether GSPE programming affects biomarkers of CVD. To achieve that aim, dams were treated with a dose of GSPE equivalent to normal human consumption during gestation and lactation. The offspring were monitored during the first 170 days of life, until adulthood, revealing that the metabolic imprinting of procyanidins resulted in different alterations of the reverse cholesterol transport process.

2. Materials and methods

2.1 Procyanidin extract

The grape seed procyanidin extract (GSPE) was kindly provided by Les Dérives Résiniques et Terpéniques (Dax, France) and was previously described [44]. Briefly, the total phenolic content of the extract was approximately 517 mg of gallic acid equivalents/g of fresh GSPE. Furthermore, this extract contained gallic acid (1.8%), epicatechin (5.5%), epicatechin gallate (5.5%), and catechin (9.1%), as well as dimeric (18.4%) and trimeric (2.8%) procyanidins.

2.2 Animals

The Animal Ethics Committee of the University Rovira i Virgili (Tarragona, Spain) approved all of the procedures. All animals (Harlan Laboratories, Barcelona, Spain) were housed at 22 °C with a light/dark cycle of 12 hours (lights on at 09:00 am) and were given free access to food and water.

The experimental design and procedures have been described previously [42]. Briefly, pregnant rats were housed individually and were divided into two groups (n=10) depending on the treatment received during pregnancy and lactation. Every day, at 09:00 p.m., one group was orally supplemented with low-fat condensed milk (D-vehicle group), and the other group received an oral supplement at 25 mg kg^{-1} of body weight .(D-GSPE group). The dose of GSPE was calculated according to the Reagan-Shaw formula [45] and is equivalent to the daily human consumption of GSPE of 330 mg, which is approximately the estimated average daily human intake of procyanidin in Spain (240-450 mg) [46]. During the entire experimental period, both groups of dams were fed ad libitum with a standard diet (2.99 kcal/g; A04, Panlab, Barcelona, Spain). On day 21 of

lactation, the dams received no treatment and were euthanized under anaesthesia (pentobarbital sodium, 60 mg /kg^{-1} of body weight) during the first 2 h of of the light cycle. Blood was collected by cardiac puncture, and plasma was obtained by centrifugation and stored at -20°C until analysis. Tissues were rapidly removed after death, weighed, frozen in liquid nitrogen and stored at -80°C until analysis.

On postnatal day 30, one male pup from each litter was single-caged and fed with the same diets as their mothers until the age of 170 days. This method resulted in two experimental groups (n=10): the vehicle group, corresponding to the offspring of D-vehicle dams, and the GSPE group, corresponding to the offspring of the D-GSPE dams. At the age of 140 days, blood samples were collected by saphenous vein puncture. At the age of 170 days, the animals were euthanized, and the blood was collected by cardiac puncture. In addition, the liver and skeletal muscle from the leg was collected and stored until analysis.

2.3 Serum lipids

Enzymatic colorimetric kits were used for the determination of triglycerides (QCA, Barcelona, Spain), total phospholipids (QCA, Barcelona, Spain) and total cholesterol (QCA, Barcelona, Spain). Two different colorimetric kits were used for the determination of the parameters HDL-cholesterol and VLDL/LDL-cholesterol (Bioassay systems, CA, USA and Spinreact, Girona, Spain). The plasma atherogenic index was computed as the logarithm of the ratio of fasting triglycerides to fasting HDL-cholesterol.

2.4 Extraction and quantification of tissue lipids

Lipids were extracted from the liver (80 mg), muscle (100 mg) and dried feces (100 mg) using methods described previously [47]. Lipids were determined by gravimetry, and triglycerides, total cholesterol and total phospholipids were quantified by colorimetric

kits (Spinreact, Girona, Spain). The retention of cholesterol was computed as the difference between ingested and excreted cholesterol with respect to the cholesterol ingested over 24 hours.

2.5 Fecal bile acid analysis.

Total bile acids were extracted according to the method described by Hagio *et al.* [48], with some modifications [49]. Fecal samples collected during the 24 hours previous to sacrifice were lyophilized and ground. 100 mg of grounded feces were subjected to extraction using absolute ethanol. The samples were dried under a nitrogen steam and diluted with methanol. Total bile acids were quantified by a colorimetric kit based on 3-α hydroxysteroid dehydrogenase activity (Spinreact, Barcelona, Spain). Cholic, muricholic, ursodeoxycholic, hyodeoxycolic, chenodeoxycholic, deoxycholic and lithocholic bile acids were quantified by LC-MS/MS. The same extraction protocol as described above was used, but cholic acid-2,2,3,4,4-d5 (Sigma-Aldrich) in methanol was included as the internal standard for this analysis. Bile acids were quantified using a 1290 Infinity LC coupled to a 6490 QqQ/MS (Agilent Technologies, Palo Alto, U.S.A.). The chromatographic column was an Extend C18 column (50 mm length x 2.1 mm i.d., 1.8 µm particle size) from Agilent Technologies. The mobile phases were H_2O and 20 mM ammonium acetate (phase A) or acetonitrile (phase B). The flow rate was 0.4 ml/min, and the sample volume was 2 µl. The elution gradient was 0-1 min, 25% B; 1-8 min, 25-50% B; 8-9 min, 50-100% B; 9-11 min, 100% B; 11-13 min, 100-25% B; and 14 min 25% B. QqQ was operated in the negative mode. The acquisition was in the MRM (multiple reaction monitoring) mode. Individual bile acids were quantified by a seven-point regression curve using standards obtained from Sigma-Aldrich (St. Louis, MO, USA) and Steraloids (Newport, Rhode Island, USA).

2.6 RNA extraction

Liver total RNA was extracted using Tripure Reagent (Roche Diagnostic Barcelona, Spain) and purified using Qiagen RNeasy Mini Kit spin columns (Izasa, Barcelona, Spain) according to the manufacturer's instructions. The RNA yield was quantified in a Nanodrop ND 1000 spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA), and the integrity of the RNA was confirmed using agarose gel electrophoresis.

2.7 Gene expression The cDNA was synthesized using MuLV reverse transcriptase (Applied Biosystems, Madrid, Spain) and was subjected to Q-PCR using the LightCycler 480 II System with SYBR Green I Master Mix (Roche Diagnostic Barcelona, Spain). The primers for the different genes are described in Supplementary Table 1 and were obtained from Biomers.net (Ulm, Germany). The relative expression of each mRNA was calculated as a percentage of the vehicle group using the $2^{-\Delta\Delta Ct}$ method [50], with *hprt* and *tfrc* as reference genes. Each PCR was performed at least in duplicate. The miR-33a and miR-122 analyses have been described previously [51].

2.8 Statistical analysis

The data are expressed as the mean ± SEM. The differences between the groups were analyzed using Student's t-test. Grubbs' test was used to detect outliers. The statistical analyses were performed using the software R 3.1.2 [52]. P-values < 0.05 were considered statistically significant.

3. Results

3.1 Serum and liver lipids in the dams

As previously described [42], no differences were observed in plasma triglycerides, total cholesterol and phospholipids between dams treated with the vehicle and dams treated with GSPE. Additional plasma and biometric parameters have been described previously [42]. The analysis of the liver samples revealed no effect of the GSPE treatment on triglycerides, cholesterol, phospholipids or total lipid content (Table 1).

3.2 Serum lipids in the offspring

No differences in body weight and food intake were detected between the groups during the experimental period (data not shown). At day 142 after weaning (table 2), fasting HDL-cholesterol levels were 27% lower in the GSPE group than in the control group (p=0.008). The triglycerides concentration was 36% higher in GSPE animals than in their counterparts, although this difference did not reach statistical significance (p=0.057). Both fasting triglycerides and HDL-cholesterol levels are used to compute the atherogenic index of plasma (AIP). Thus, as a consequence of the changes described above, the AIP, which was negative for the control animals, significantly increased for the GSPE group, reaching positive values (p=0.009). Due to the lower HDL-C levels, the GSPE animals had a higher total cholesterol to HDL-cholesterol ratio (p=0.036), another index of cardiometabolic disease risk. No differences were found between groups in fasting total cholesterol, VLDL/LDL-cholesterol and phospholipids. At day 170 after weaning, in *ad libitum* conditions, HDL-cholesterol levels were still 26% lower (p=0.009) in the GSPE group, but no differences were observed in the levels of serum triglycerides (p=0.895). Moreover, GSPE animals presented statistically significant lower levels of

total cholesterol (p=0.044) but no differences in the concentration of VLDL/LDL-cholesterol. Again, total phospholipids were not different between groups.

3.3 Cholesterol and bile acid excretion in the offspring.

In order to determine whether the lower levels of HDL-cholesterol in the GSPE group translated into decreased cholesterol excretion, the lipid and cholesterol balance was analyzed for both groups (table 3). No differences were found between groups in the daily intake and excretion of total lipids. Nevertheless, GSPE animals presented a significant lower excretion of cholesterol (p=0.04) compared to controls, although cholesterol intake did not differ between groups (p=0.33). Therefore, cholesterol balance resulted in a significant increase in cholesterol retention in GSPE animals (p=0.02).

The excretion of bile acids was also quantified (table 4). The feces of GSPE animals contained less total bile acids than the feces of their counterparts (47%; p=0.016). In agreement with these findings, the analysis of different species of fecal bile acids revealed that GSPE animals excreted less deoxycholic acid (p=0.032) and lithocholic acid (p=0.006). Moreover, a clear tendency towards reduced excretion of hyodeoxycholic (p=0.051) and chenodeoxycholic acid (p=0.081) was found in the GSPE group compared to the control. As a consequence, the sum of the secondary bile acids (i.e., deoxycholic, lithocholic and hyodeoxycholic) was reduced by 35% in GSPE animals compared to the control group (p=0.028). In turn, the sum of the primary bile acids (i.e., muricholic, cholic and chenodeoxycholic acids) was 24% lower in GSPE animals, although the difference with the control group was not statistically significant (p=0.179). Importantly, the ratio of chenodeoxycholic acid to cholic acid was significantly lower in the feces of the GSPE than in the feces from the control rats (p=0.022).

The link between HDL-C and fecal total bile acids and cholesterol was further explored (figure 1). When fecal total bile acids were compared to serum HDL-C (figure 1A), an excellent positive correlation was found, with a Pearson coefficient of 0.8, a confidence interval of 0.553 to 0.917 and a p-value of 2.33×10^{-5}, confirming that the levels of fecal bile acids and HDL-C levels are associated. The analysis of HDL-C versus fecal cholesterol (figure 1B) did not reveal a statistically significant correlation ($p=0.165$).

3.4 Metabolic programming effects of GSPE in the liver.

The liver presides over the metabolism of serum lipoproteins and couples the RCT to its elimination by means of both the bile acid synthesis pathway and the direct excretion of cholesterol into bile. Therefore, we analyzed the expression of different genes in order to assess whether the metabolic programming of GSPE could be seen at the transcriptional level in the liver.

The metabolic programming of GSPE affected different genes related to HDL metabolism (figure 2 A). Thus, the cholesterol transporters *Abca1* and *Abcg1* were significantly upregulated in the GSPE group compared to the control group by 50% ($p=0.002$) and 60% ($p=5 \times 10^{-5}$), respectively. The expression of *Lcat* and *Pltp* were significantly higher in GSPE animals, reaching a 37% ($p=0.004$) and a 22% ($p=0.047$) increase, respectively, compared to the control group. The HDL receptor *Scarb1* was significantly upregulated in GSPE animals ($p= 0.009$), together with the intracellular cholesterol transporter *Npc1*, with a 68% higher ($p= 2 \times 10^{-7}$) expression in the GSPE group compared to the control group. We also found higher expression levels of the cholesterol metabolism regulators *Lxra* (72% higher, $p=2 \times 10^{-5}$) and *Srebp2* (36% higher, $p=0.003$) in GSPE rats compared to their control counterparts. No effects were found in other genes related to cholesterol and HDL metabolism, such as *Apoa1*, *Hmgcr* or *Lipc*.

We also analyzed the expression of ten genes related to bile acid synthesis and excretion (figure 2B). Significant changes were found in *Cyp7a1* and *Lrh1*, which were repressed (45% and 28%, respectively) in the GSPE group versus the control group.

Genes related to fatty acid and triglyceride synthesis and VLDL assembly and secretion were also analyzed (figure 2C). Only one out of seven genes, *Srebp1*, presented a significant increase in its expression level (27%) when GSPE was compared to the control group (p=0.03). These results cannot explain the increased levels of serum TG found in GSPE animals in the fasted state.

GSPE has been shown to modulate the levels of the miRNAs miR-33a and miR-122, regulators of RCT and fatty acid metabolism, respectively [51,53]. The analysis of these regulators in the liver revealed a significant downregulation of miR-33a (p=0.041) and no changes in the levels of miR-122 (p=0.14) (figure 2D).

3.5 Lipid composition of liver and muscles

In order to assess whether the lower HDL-cholesterol levels and decreased excretion of cholesterol and bile acids found in the GSPE group could be associated with altered levels of cholesterol in peripheral tissues, we analyzed the lipid composition of liver and muscle samples. The results (table 5) revealed that liver lipids were not affected by the metabolic programming of GSPE. Nevertheless, we found that muscles of GSPE animals presented 19% more cholesterol than their counterparts, although the difference did not reach statistical significance and it remained as a clear tendency (p=0.051). These results suggest that metabolic programming induced by GSPE results in increased cholesterol retention in muscles.

4. Discussion

The beneficial effects of grape procyanidins have been widely described, identifying these polyphenols as promising bioactive compounds for the prevention of CVD. Beyond their antioxidant and anti-inflammatory properties, procyanidins might exert some of their beneficial effects by modulating the metabolism of lipids and lipoproteins [38]. Previous studies have shown that the perinatal consumption of different polyphenols exerts metabolic programming effects in the offspring, some of them related to cardiovascular protection [27–30,54–56]. Therefore, this work explored the metabolic imprinting effects of a grape seed procyanidin extract on serum lipids and atherogenic risk indexes.

Despite the beneficial health effects ascribed to dietary polyphenols, our results show that daily consumption of GSPE at a physiological dose during pregnancy and lactation results in deleterious alterations of serum lipids in the adult offspring. The difference with unexposed animals is primarily in decreased levels of fasting and non-fasting serum HDL-C and an increased fasting TC/HDL-C ratio, which have been defined as good predictors of CVD [2]. Moreover, fasting TG were increased in rats exposed to GSPE during the perinatal period, though the differences did not reach statistical significance. Increased TG and lowered HDL-C resulted in increased AIP in GSPE animals. Therefore, the different serum parameters suggest that the offspring of GSPE-treated rats present a more CVD-prone phenotype than the offspring of control rats at adulthood.

The decrease in HDL-C suggests reduced reverse transport and excretion of cholesterol. This hypothesis is confirmed by the different analyses of the fecal samples. The lower

cholesterol and bile acid concentrations found in the feces of GSPE animals are consistent with a decreased influx of cholesterol into the liver and subsequent reduction of bile acid synthesis. This hypothesis is reinforced by the excellent correlation between fecal bile acids and HDL-C levels. The observed repression of hepatic *Cyp7a1* in the GSPE group is in good agreement with these findings, since it is the rate-limiting enzyme in the bile acid synthesis pathway [10]. Moreover, we also found a clear repression of *Lrh1*, which controls the basal expression of CYP7A1 [10]. Together, these observations suggest that GSPE programming results in a lowered basal rate of bile acid synthesis due to diminished HDL-mediated delivery of cholesterol to the liver.

The decreased fecal bile acid pool in the GSPE group prompted us to quantify individual bile acids. Surprisingly, we found that the most affected species were secondary bile acids, which result from gut microbiota activity [10]. It has recently been shown that changes in either the protein or fiber content of the mother's diet can modify the composition of milk and the microbiota of the offspring, even in adulthood [57]. Moreover, previous research has documented that changes in the gastrointestinal tract of dams can influence the microbiome colonization or development in the offspring [58,59], and it is known that polyphenols can modulate the composition of the gut microbiota [60]. Thus, it is tempting to speculate that maternal GSPE intake can influence the microbiota of the offspring by an unknown mechanism. Although more research is needed to determine the mechanisms leading to these results, it is known that the lack of secondary bile acids in germ-free rats results in the increased absorption of cholesterol [61]. Moreover, we also show that GSPE animals had a lower CDCA/CA ratio, and previous studies have reported that a lower ratio increases the intestinal absorption of cholesterol [62,63]. The changes in the fecal bile acid pool composition suggests that GSPE animals present a higher tendency towards intestinal cholesterol absorption than their

counterparts. Although we have not observed increased serum non-HDL-cholesterol in fasting or in *ad libitum* conditions, the relevance of the alterations described here could be further explored by exposing these animals to a cholesterol-rich diet.

Since cholesterol intake was similar in both groups, the lower cholesterol excretion observed in GSPE rats compared to the control group could be attributed to lower cholesterogenesis. The analysis of gene expression in the liver revealed increased mRNA levels of SREBP2 and decreased levels of miR33, key controllers of cholesterol synthesis, and no changes in the expression of the gene encoding for the cholesterol synthetic enzyme HMGCoAR. Although repression of cholesterol synthesis in tissues other than the liver cannot be ruled out, our results suggest that in the liver, cholesterol synthesis is not repressed at the gene expression level. A second possibility to explain the lowered cholesterol excretion in the GSPE group is an increased accumulation of cholesterol in tissues. Indeed, although we found no effects in hepatic cholesterol levels, the muscle of GSPE animals was enriched in cholesterol compared to the control group. These findings might be indicative of an impaired efflux of cholesterol from muscle to HDL particles. Together with impaired cholesterol efflux from tissues, alterations of RCT in GSPE animals could be due to changes in the hepatic metabolism of HDL. Gene expression analyses in the liver showed important changes in the expression of key genes related to RCT. Thus, *Abcg1* and *Abca1*, involved in lipidization and maturation of nascent HDL particles [64]; *Pltp*, which encodes for the enzyme involved in phospholipidization of blood HDL [11]; *Lcat*, which encodes the enzyme that catalyzes the esterification of free cholesterol in plasma lipoproteins and plays a critical role in HDL metabolism and maturation [65]; *Npc1*, encoding for an intracellular transporter of cholesterol [66]; and *Scarb1*, the main hepatic receptor of HDL [11], were all upregulated in GSPE animals compared to the control group, which might be interpreted as an enhancement of key

components of the RCT. Consistently, hepatic levels of miR-33a, a key controller of cholesterol efflux and HDL metabolism [67], were downregulated. Emerging evidence has revealed that some components in the maternal diet can directly or indirectly affect miRNA expression in the offspring [68]. microRNAs (miRNAs) are small RNAs that negatively regulate protein-coding genes at the post-transcriptional level. Thus, miRNAs are a mechanism of epigenetic regulation. In fact, miRNAs have also emerged as a new molecular mechanism by which GSPE could modulate lipid metabolism [69]. Specifically, these GSPE modulation is thought to be by the repression and direct binding of liver miR-33a and miR-122, which in turn increase the expression of Abca1 and decrease Fas mRNA as their target genes, respectively [70,71]. In this study, miR-33a was repressed and consistently their target genes, like *Abca1* and *Abcg1*, increased. Although *Srebp2* was also increased, the modulation of miR-33a is thought to be independent of the expression of its host gene [70]. Also in this study miR-122 was not modulated by maternal GSPE intake and, consequently, neither its target gene *Fasn* was altered. In view of these results, it is tempting to speculate that GSPE consumption during the perinatal period can affect the machinery that controls microRNAs of foetuses and, subsequently, the genetic program of different components of the RCT. However, these results contrast with all the physiological evidence (i.e., serum, muscle and feces analyses), that depict a scenario in which the RCT is impaired. Although we cannot explain this contrasting evidence, it could be hypothesized that in GSPE rats, the RCT is impaired due to the inefficient efflux of cholesterol from peripheral tissues rather than due to impaired hepatic metabolism of HDL.

In conclusion, we report here that the consumption of grape seed procyanidins at a physiological dose during gestation and lactation programs the offspring towards a CVD-prone phenotype, characterized by increased AIP and the TC to HDL-C ratio. The

lowered serum HDL-C and decreased cholesterol and bile acid excretion concomitant with *Cyp7a1* repression and the observed accumulation of cholesterol in muscles suggests an impairment of the RCT due to altered cholesterol efflux from peripheral tissues. Paradoxically, grape seed procyanidins, that were previously shown to ameliorate atherogenic risk indexes and to upregulate CYP7A1 in healthy rats [35], exert the opposite effects in the context of the metabolic programming phenomenon. Results from our group and others have shown both positive and negative effects of metabolic programming by polyphenols [27–29,31,32,41,42]. These contrasting results point to the need for more research in order to underscore which factors (i.e., dose, family or source of polyphenols, and consumption during gestation, lactation or both periods) contribute to the final outcome of perinatal exposition to polyphenols. Our results show that the metabolic programming effects of bioactive compounds found in foods might be relevant to the origin, and therefore to the prevention or amelioration, of CVD. Further studies focused on the metabolic programming effects of procyanidins under altered homeostatic situations (i.e., obesity, high cholesterol diet) could corroborate the impact of our findings.

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Table 1. Plasma and liver lipids of dams treated with the vehicle or GSPE during gestation and lactation.

	D-vehicle	D-GSPE	*p-value*
Serum			
Triglycerides (mmol/L)	0.41±0.03	0.45±0.04	*0.45*
Total Cholesterol (mmol/L)	1.99±0.15	2.21±0.16	*0.33*
Phospholipids (mmol/L)	1.87±0.14	2.11±0.17	*0.30*
Liver			
Triglycerides (mg/g)	3.5±0.3	4.2±0.4	*0.19*
Total Cholesterol (mg/g)	1.6±0.1	1.8±0.1	*0.13*
Phospholipids (mg/g)	11.4±0.5	13.3±0.3	*0.13*
Total lipids (mg/g)	28.4±0.6	28.8±0.7	*0.69*

Female rats were fed with chow and received a daily oral dose of GSPE (D-GSPE, 25 mg kg^{-1} of body weight) or the vehicle (D-vehicle) during the gestation and lactation periods (43 days). Data are mean ± SEM (n = 9-10). The p-value resulting of a Student's t test is reported.

Table 2. Blood lipids and atherogenic indexes in male offspring of rats that were treated with GSPE or vehicle during the gestation and lactation period.

	Vehicle	GSPE	*p-value*
Fasted			
Total Cholesterol (mmol/L)	2.58±0.07	2.25±0.20	*0.549*
HDL-cholesterol (mmol/L)	1.92±0.16	1.32±0.10	*0.005*
LDL/VLDL- cholesterol (mmol/L)	0.82±0.12	0.58±0.12	*0.333*
Triglycerides (mmol/L)	1.44±0.15	1.96±0.21	*0.057*
Phospholipids (mmol/L)	2.15±0.11	2.10±0.12	*0.779*
Atherogenic index of plasma (AIP)	-0.08±0.04	0.16±0.07	*0.009*
Total cholesterol / HDL-cholesterol	1.40±0.14	1.83±0.13	*0.036*
Ad libitum			
Total Cholesterol (mmol/L)	2.34±0.12	1.92±0.16	*0.044*
HDL-cholesterol (mmol/L)	1.68±0.08	1.25±0.12	*0.009*
LDL/VLDL- cholesterol (mmol/L)	0.59±0.06	0.50±0.07	*0.392*
Triglycerides (mmol/L)	1.59±0.24	1.63±0.19	*0.895*
Phospholipids (mmol/L)	2.03±0.13	1.92±0.09	*0.495*

Male offspring of rats treated with a daily oral dose of GSPE (25 mg $kg^{-1}$of body weight) or the vehicle during the gestation and lactation periods (43 days). Weaning was conducted at 21 days of life. At day 30, one male of each litter (n=10 per group) was single caged and maintained under the same diet of their mothers until sacrifice (day 170). Blood samples were obtained on day 142 in fasted conditions and on day 170 in *ad libitum*

conditions. The data are expressed as the mean ± SEM (n = 10). The p-value resulting of a Student's *t* test is reported.

Table 3. Lipids and cholesterol intake and excretion balance in male offspring of rats that were treated with GSPE or vehicle during the gestation and lactation period.

	Control	GSPE	*p-value*
Lipids intake (mg/day)	421±18	356±16	*0.32*
Lipids excretion (mg/day)	139±26	108±12	*0.36*
Lipids retention (%)	75±2	74±2	*0.42*
Total Cholesterol intake (mg/day)	13.7±0.6	13.0±0.5	*0.32*
Total Cholesterol excretion (mg/day)	4.0±0.5	2.9±0.2	*0.04*
Total Cholesterol retention (%)	68±3	78±2	*0.02*

Dams were treated with a daily oral dose of GSPE (25 mg kg^{-1} of body weight) or the vehicle during the gestation and lactation periods (43 days). Weaning was conducted at 21 days of life. At day 30, one male of each litter (n=10 per group) was single caged and maintained under the same diet of their mothers until sacrifice (day 170). Faeces of a 48 hours period were collected before sacrifice, lipids were extracted and quantified and cholesterol was analysed. The data are expressed as the mean ± SEM (n = 10). The p-value resulting of a Student's *t* test is reported.

Table 4. Faecal bile acids in male offspring of rats that were treated with GSPE or vehicle during the gestation and lactation period.

	Control	GSPE	*p-value*
Total bile acids (µmol/day)	15±3	8±1	*0.016*
Muricholic acid (µmol/day)	0.37±0.08	0.41±0.12	*0.779*
Cholic acid (µmol/day)	0.026±0.005	0.023±0.004	*0.785*
Hyodeoxycholic acid (µmol/day)	4.88±0.80	2.80±0.40	*0.051*
Chenodeoxycholic acid (µmol/day)	4.87±0.59	3.53±0.35	*0.081*
Deoxycholic acid (µmol/day)	1.41±0.18	0.91±0.09	*0.032*
Lithocholic acid (µmol/day)	1.10±0.11	0.62±0.05	*0.006*
Secondary bile acids (µmol/day)	6.77±0.78	4.38±0.48	*0.028*
Primary bile acids (µmol/day)	4.90±0.62	3.73±0.36	*0.179*
Chenodoxycholic acid : cholic acid	258±26	169±20	*0.022*

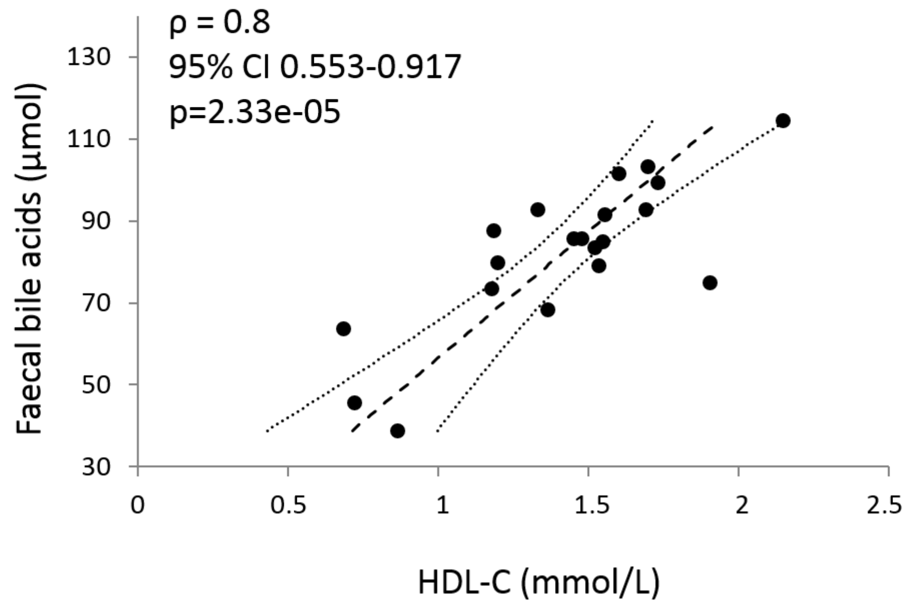
Dams were treated with a daily oral dose of GSPE (25 mg kg^{-1} of body weight) or the vehicle during the gestation and lactation periods (43 days). Weaning was conducted at 21 days of life. At day 30, one male of each litter (n=10 per group) was single caged and maintained under the same diet of their mothers until sacrifice (day 170). Faeces of a 48 hours period were collected before sacrifice. Bile acids were extracted and quantified. Secondary bile acids were computed as the sum of hyodeoxychoilic, deoxycholic and lithocholic acids and primary bile acids as the sum of muricholic, cholic and chenodeoxycholic. The data are expressed as the mean ± SEM (n = 10). The p-value resulting of a Student's t test is reported.

Table 5. Liver and muscle lipids in male offspring of rats that were treated with GSPE or vehicle during the gestation and lactation period.

	Control	GSPE	*p-value*
Liver			
Total lipids (mg/g)	29.2±0.9	31.7±1.6	*0.18*
Total Cholesterol (mg/g)	5.0±0.4	6.0±0.5	*0.15*
Triglycerides (mg/g)	2.3±0.1	2.4±0.1	*0.25*
Phospholipids (mg/g)	12.5±0.6	12.3±0.6	*0.79*
Muscle			
Total lipids (mg/g)	13.9±0.4	17.0±1.5	*0.54*
Total Cholesterol (mg/g)	0.80±0.04	0.95±0.05	*0.05*
Triglycerides (mg/g)	2.7±0.4	3.9±0.7	*0.92*
Phospholipids (mg/g)	5.6±0.2	6.4±0.3	*0.56*

Dams were treated with a daily oral dose of GSPE (25 mg kg^{-1} of body weight) or the vehicle during the gestation and lactation periods (43 days). Weaning was conducted at 21 days of life. At day 30, one male of each litter (n=10 per group) was single caged and maintained under the same diet of their mothers until sacrifice (day 170). Liver and muscle were collected and snap frozen in liquid nitrogen until analysis. Lipids were extracted and analysed by colorimetric kits. The data are expressed as the mean ± SEM (n = 10). The p-value resulting of a Student's t test is reported.

A



B

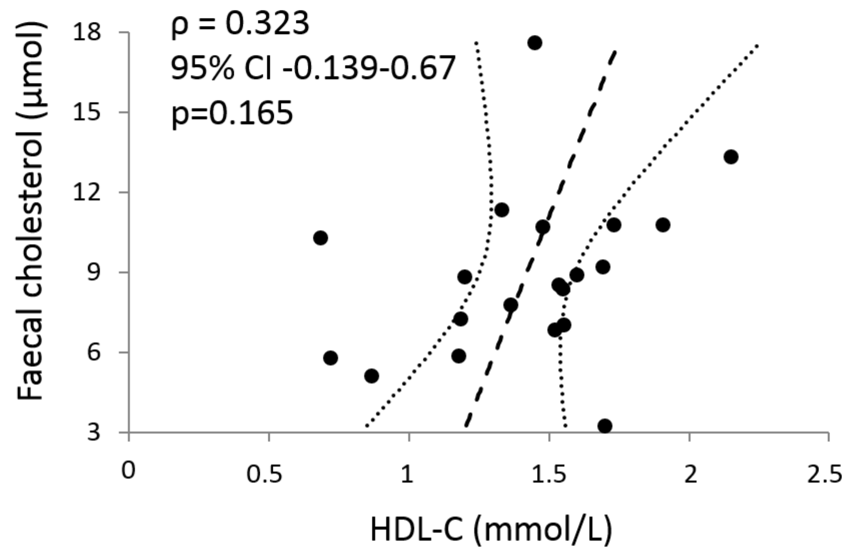


Figure 1. Correlation between serum HDL-cholesterol (HDL-C) and fecal bile acids (A) or fecal cholesterol (B) in the offspring of dams treated with GSPE or the vehicle during the gestation and lactation periods. Dashed lines represent the least squares regression fit and dotted lines show the confidence intervals. The Pearson correlation coefficient (ρ) and its confidence intervals are presented together with the p-value of the correlation analysis.

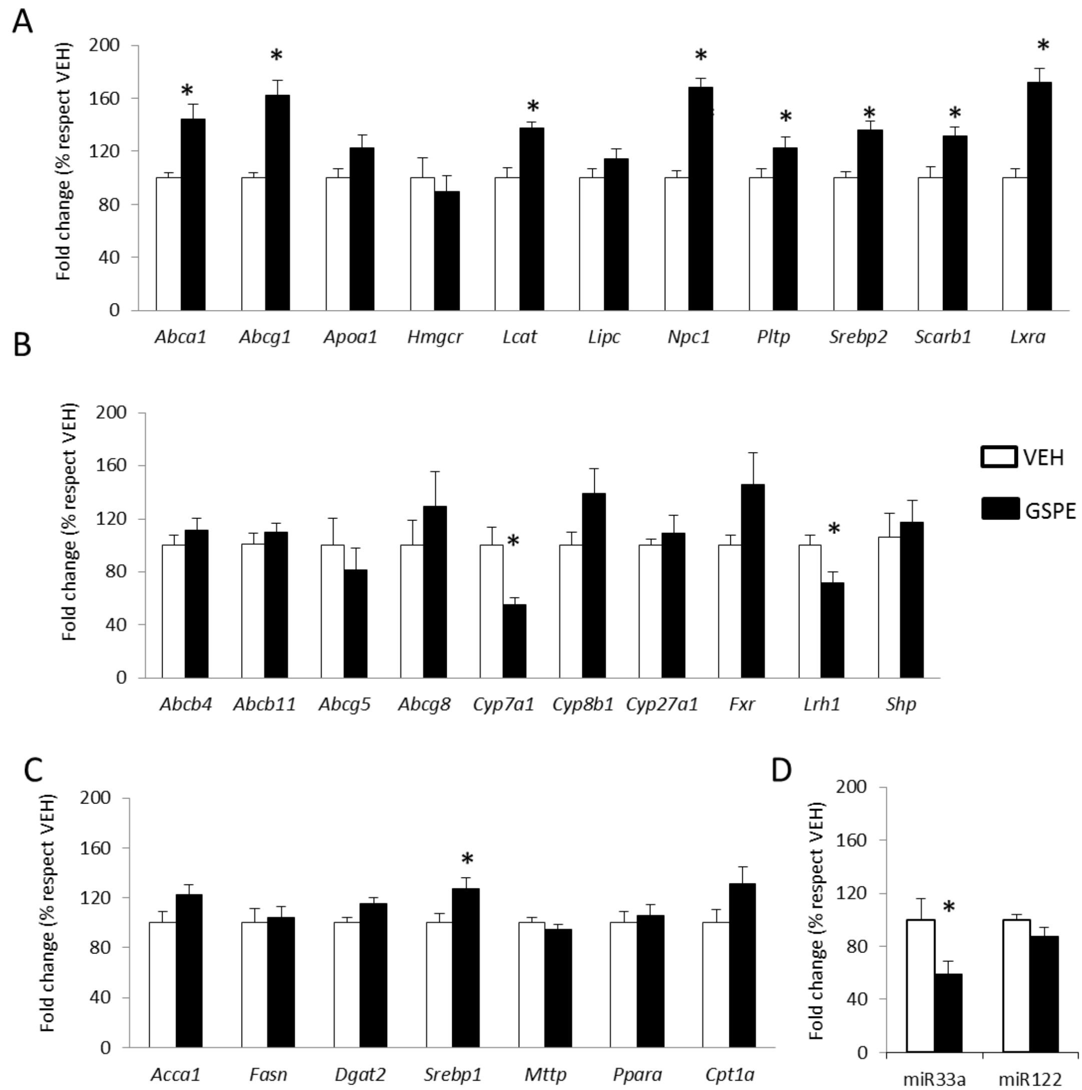


Figure 2.

The expression levels of genes involved in cholesterol metabolism and reverse cholesterol transport (A), bile acid synthesis and excretion (B) and lipogenesis and fatty acid oxidation (C) in livers from the offspring of dams that were treated with GSPE or the vehicle (VEH). The data are the mean ± SEM (n = 10). * Metabolic programming effect of GSPE ($p<0.05$, Student's t-test). *Abca1*, ATP-binding cassette transporter A1; *Abcg1*, ATP-binding cassette transporter G1; *Apoa1*, apolipoprotein A1; *Hmgcr*, 3-hydroxy-3-methylglutaryl-CoA reductase; *Lcat*, lecithin-cholesterol acyltransferase; *Lipc*, hepatic lipase; *Npc1*, Niemann-Pick disease, type C1; *Pltp,* phospholipid transfer protein; *Srebp2*,

sterol regulatory element binding transcription factor 2; *Scarb1*, scavenger receptor class B, member 1; *Lxra*, nuclear receptor subfamily 1, group H, member 2 or liver x receptor alpha; *Abcb4*, ATP-binding cassette, sub-family B (MDR/TAP), member 4*; Abcb11*, ATP-binding cassette transporter B11; *Abcg5*, ATP-binding cassette, sub-family G, member 5; *Abcg8*, ATP-binding cassette, sub-family G, member 8; *Cyp7a1*, cytochrome P450, family 7, subfamily A, polypeptide 1; *Cyp8b1*, cytochrome P450, family 8, subfamily B, polypeptide 1*; Cyp27a1*, cytochrome P450, family 27, subfamily A, polypeptide 1; *Fxr*, Nuclear Receptor Subfamily 1, Group H or Farnesoid X receptor; *Lrh1*, Nuclear Receptor Subfamily 5, Group A, Member 2 or Liver receptor homolog-1; *Shp*, Nuclear Receptor Subfamily 0, Group B, Member 2 or Small heterodimer partner; *Acca1*, acetyl-CoA carboxylase alpha; *Fasn*, fatty acid synthase; *Dgat2*, diacylglycerol O-acyltransferase 2; *Mttp*, microsomal triglyceride transfer protein; *Ppara*, peroxisome proliferator-activated receptor alpha; *Cpt1a*, carnitine palmitoyltransferase 1A.

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