RESEARCH ARTICLE

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Inherited Metabolic Memory of High-Fat Diet Impairs Testicular Fatty Acid Content and Sperm Parameters

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Scope: Exposure to a high-fat diet (HFD) from early-life is associated with a testicular metabolic signature link to abnormal sperm parameters up to two generations after exposure in mice. Hereby, this study describes a testicular lipid signature associate with "inherited metabolic memory" of exposure to HFD, persisting up to two generations in mice.

Methods and Results: Diet-challenged mice ($n = 36$) are randomly fed after weaning with standard chow (CTRL); HFD for 200 days or transient HFD (HFD_t) (60 days of HFD + 140 days of standard chow). Subsequent generations (36 mice per generation) are fed with chow diet. Mice are euthanized 200 days post-weaning. Glucose homeostasis, serum hormones, testicular bioenergetics, and antioxidant enzyme activity are evaluated. Testicular lipid-related metabolites and fatty acids are characterized by ^{1}H-NMR and GC-MS. Sons of HFD display impaired choline metabolism, mitochondrial activity, and antioxidant defenses, while grandsons show a shift in testicular $\omega 3/\omega 6$ ratio towards a pro-inflammatory environment. Grandsons of HFDt raise 3-hydroxybutyrate levels with possible implications to testicular insulin resistance. Sperm counts decrease in grandsons of HFD-exposed mice, regardless of the duration of exposure.

Conclusion: HFD-induced "inherited metabolic memory" alters testicular fatty acid metabolism with consequences to sperm parameters up to two generations.

1. Introduction

The epidemical proportions of acquired metabolic disorders, namely overweight/obesity and type 2 diabetes (T2D), raise several challenges to health care systems worldwide. Although efforts to curb the incidence of these diseases have been taken globally, the current projections by the World Health Organization and other non-governmental organizations predict an increase in their prevalence in the decades to come.[1,2] This trend promotes the early-onset of obesity and T2D,[1,2] raising concerns regarding the possible consequences of these disorders to the sexual health of men of reproductive age.[3,4] In addition, there is strong evidence that the consumption of HFDs by the father can condition the progeny health for several generations (inter- and transgenerational effects).[5,6] These effects have even been reported in humans,[7] suggesting that the signature of ancestral exposure persists over the course of a lifetime, and requires more than one window of epigenetic erasure to be reset. Intergenerational effects of a low-protein diet and transgenerational effects of obesogens in lipid metabolism have been described in mouse liver.[8,9]

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Another study demonstrated that paternal HFD was correlated to increased gene expression levels of adiponectin and leptin in visceral white adipocytes of the progeny, in mice.[10] However, the knowledge of the transgenerational effects of HFD in testicular lipid metabolism and function is limited.

"Metabolic memory" has been coined in the context of metabolic syndrome, to describe the phenomenon where new cells, generated from insulin-resistant cells, inherited the same phenotypical characteristics. We have previously advocated the adaptation of this term to the context of transgenerational inheritance of characteristics acquired due to environmental variables and lifestyle choices, such as the adoption of HFDs with concurrent development of metabolic disorders.[11] We demonstrated that eating an HFD, even transiently, creates an "inherited metabolic memory" in the metabolome of mice testes for up to two generations (father-son-grandson).[11] In this study, we further explore the effects caused by an ancestral exposure to an HFD in the testicular content of fatty acids (FAs) and lipid-related metabolites. We apply a bottom-up exploratory data analysis (EDA) strategy[12] based on multivariate and discriminant analysis to highlight testicular metabolic changes associated with detrimental testicular function and sperm parameters caused by the adoption of HFD by the male ancestor.

2. Experimental Section

2.1. Animal Model

This study was performed in three generations of *Mus musculus* C57BL6/J mice as described.[11] In brief, the first generation (Generation F_0), was originated from normoponderal progenitors (both male and female), fed with a standard chow (#F4031, BioServ, USA—carbohydrate: 61.6%, protein: 20.5%, fat: 7.2% [16.3% kcal]) and water *ad libidum*. After weaning (21–23 days), F_0 mice ($n = 36$) were randomly divided in three groups: control (CTRL) ($n = 12$), HFD ($n = 12$), and HFD_t ($n = 12$). Mice from the CTRL group were fed with standard chow. Mice from the HFD group received a fat-enriched diet (#F3282, BioServ, USA—carbohydrate: 35.7%, protein: 20.5%, fat: 36.0% [59.0% kcal]). The mice from the HFD_t group were fed with a fat-enriched diet for 60 days (#F3282, BioServ, NJ, USA), then switched to standard chow (#F4031, BioServ, NJ, USA). Despite different fatty acid content, the proportion of fatty acids families to the total fatty acid content was the same between diets. The age of dietary switch had chosen to restrict the influence of this lifestyle intervention to adult, sexually mature mice.[13] By this age mice had already completed two to three spermatogenic cycles,[14] corresponding to a young man (≈20 years old).[15] F_0 mice were mated starting at 120 days of age with normoponderal, chow-fed, same-age randomly selected lean females to generate the F_1 generation. Mating lasted for 8 days and consisted of placing a male and a female in the same cage for 6 h each day, without water or food supply. After weaning, F1 mice were assigned to the same experimental group as their fathers: CTRL_F1—offspring of CTRL ($n = 12$); HFD_F1—offspring of HFD ($n = 12$); HFD_t_F1—offspring of HFD_t ($n = 12$). In this generation (F_1), all mice were fed with standard chow. Food and water were supplied without restrictions. The mating of F_1 mice was performed under the same conditions as their progenitors (Generation F_0). Mice from the resulting generation F_2 were assigned to experimental groups in the same way as their fathers (12 animals per group). All mice of generation F_2 were fed with standard chow after weaning. Food and water were supplied without restrictions. Mice from all generations were killed by cervical dislocation 200 days after weaning, and tissues were collected for further analysis. Total body weight, water, and food intake were monitored weekly from weaning to sacrifice. The animal model was compliant with the ARRIVE guidelines and was licensed by the Portuguese Veterinarian and Food Department (0421/000/000/2016).

2.2. Endocrine and Metabolic Function

Glucose tolerance and insulin resistance were assessed by the intraperitoneal glucose tolerance test (ipGTT) and intraperitoneal insulin resistance test (ipITT) protocols,[16] 196 and 198 days post-weaning. Fasting glycemia was measured before euthanasia (200 days post-weaning), after overnight fast (8 h), using a glucometer (One Touch Ultra Lifescan-Johnson, Milpitas, CA, USA) by collecting a drop of blood from the tail vein. Blood was then collected by cardiac puncture and centrifuged at 1500 × *g*, 4 °C, for 10 min to collect the serum. Insulin was quantified in serum using a Rat/Mouse Insulin ELISA assay (EZRMI-13K, Millipore). Similarly, FSH, LH, 17β-estradiol (E_2), and testosterone were quantified in the purified serum using ELISA kits (respectively, ENZ-KIT108-0001, ENZ-KIT107-0001, ADI-900-174, and ADI-900-176, Enzo Life Sciences, USA).

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2.3. Evaluation of Epididymal Sperm Parameters

Epididymides were isolated and placed in pre-warmed (37 °C) Hank's Balanced Salt Solution (HBSS) pH 7.4, minced with a scalpel blade and the suspension was incubated for 5 min (37 °C). Sperm parameters were evaluated as previously described.[13,17] Sperm motility was calculated as the average proportion of motile sperm in 10 random microscope fields, observing a drop of sperm suspension on a warmed slide (37 °C) using an optical microscope (×100 magnification). Epididymal sperm concentration was determined using a Neubauer counting chamber and an optical microscope (×400 magnification), diluting the sperm suspension 1:50 in HBSS. Sperm viability and morphology were assessed in differently stained epididymal sperm smears, counting 333 spermatozoa in random fields using an optical microscope (×400 magnification). Sperm viability smears were stained with eosin-nigrosin,[18] as membrane-compromised spermatozoa stain pink. Sperm morphology smears were stained with Diff-Quick (Baxter Dale Diagnostics AG, Dubinger, Switzerland). Sperm morphology categories were mutually exclusive, i.e., spermatozoa displaying more than one defect were assigned according to the most serious defect category (decapitated > pinhead > flattened head > bent neck > coiled tail).[18]

2.4. NMR Spectroscopy

A combined extraction of polar and nonpolar metabolites from testicular tissue was performed as described.[13,19] Testes were not perfused with saline before extraction, as contamination by serum lipids and metabolites is negligible,[20,21] but they were decapsulated before homogenization. The aqueous phase containing polar water-soluble metabolites was lyophilized and analyzed by ^{1}H-NMR spectroscopy as described.[22] The organic phase containing less polar lipids was dried by a nitrogen stream and analyzed by GC-MS. Lipid-related metabolites were identified according to the Metabolomics Standards Initiative (MSI) guidelines.[23] Recorded spectra were compared to reference spectra of the Human Metabolome Database (HMDB).[24] Identification levels are indicated in Table S1, Supporting Information. ^{1}H spectra were processed using previously described methods.[22] Peak areas were obtained using AMIX software (Bruker BioSpin GmbH, Rheinstetten, Germany) and normalized to the total spectral area.

2.5. GC-MS Analysis

FA methyl esters of total lipids were obtained by base-catalyzed transmethylation (2 M KOH in methanol) in the presence of nonadecanoic FA (C19:0), used as the internal standard. The obtained hexane FA methyl esters solution was analyzed by gas chromatography using a Shimadzu GC–MS QP2010 UltraGas Chromatograph Mass Spectrometer (Shimadzu, Kyoto, Japan), equipped with a capillary column BPX70 (0.25 mm internal diameter, 0.25 μm film thickness, 30 m long, SGE, Austin, TX, USA). The injector temperature was 250 °C, and 1 μL of each sample was injected with a split ratio of 1:80. Helium was used as the carrier gas, and the linear velocity was 35 cm s^{-1}. The initial column temperature was 155 °C, followed by a heating rate of 1 °C min^{-1} up to 170 °C, 4 °C min^{-1} up to 220 °C and 40 °C min^{-1} until reaching 250 °C, maintained for 5 min. Linear velocity was 35 cm s^{-1}, interface temperature: 250 °C, ion source temperature: 225 °C, mass range: 45—500, and event time: 0.3 s. All the experimental measurements were repeated three times and the average values were reported. FAs were identified by retention time and fragmentation profile and quantified by the internal standard procedure. Results were expressed as a percentage of total FAs.

2.6. Lipid Peroxidation and Activity of Antioxidant Enzymes

A single testis from each animal was homogenized in 2 mL of ice-cold extraction buffer (100 mM sucrose, 10 mM Tris–HCl, pH 7.4) supplemented with a protease inhibitors cocktail (#B14001, Bimake, Munich, Germany) (1:10, p/v) using a glass-Teflon Potter Elvejhem (Kimble, Millville, NJ, USA) as described previously.[25] The resulting homogenate was split into two fractions: one was used for adenine nucleotides (ATP, ADP, and AMP) extraction and quantification by high-performance liquid chromatography (HPLC), as described[26]; the other was fractionated by differential centrifugation to obtain a mitochondria-free cytosolic fraction and a mitochondria-enriched fraction, as described.[25] The final protein content of each fraction was determined by the bicinchoninic acid method.[27] The mitochondria-enriched fraction was used to assess the activity of the mitochondrial enzymes (complexes I, II, and IV, and citrate synthase), while the mitochondria-free cytosolic fraction was used to evaluate the activity of the enzymes of the antioxidant defense system [CAT, SOD, glutathione peroxidase (GPx), GSR] and to assess the levels of lipid peroxidation.

CAT activity was polarographically determined following oxygen production resulting from H_2O_2 decomposition using a Clark-type oxygen electrode (Hansatech, Norfolk, UK),[28] and expressed in nmol O_2 min^{-1} mg^{-1} protein.

SOD, GPx, and GSR activities were evaluated in 96-well plates, at 37 C, as described.[25,29] SOD activity was evaluated as the reduction of nitro blue tetrazolium (NBT) and expressed as U min^{-1} mg^{-1} protein, where U is the enzyme activity that inhibited the NBT reduction to blue formazan by 50%. GSR activity was determined by the difference between the rate of NADPH oxidation in the presence and absence of GSSG and expressed in nmol min^{-1} mg^{-1} of protein. GPx activity was evaluated by the rate of NADPH oxidation associated with H_2O_2 reduction, expressed in nmoles min^{-1} mg^{-1} of protein.

Testicular lipid peroxidation levels were evaluated by the production of thiobarbituric acid reactive species (TBARS) assay. Shortly, 50 μL testicular cytosolic fraction were mixed with 600 μL of Reaction Solution [thiobarbituric acid 0.38% (m/V), trichloroacetic acid 37% and 2,6-ditertbutyl-4-methylphenol 0.02% (m/V)[30]] and incubated at 95 °C for 30 min. Malondialdehyde (MDA) formation was measured by colorimetric methods (ϵ = 156 mM^{-1} cm^{-1}), and results were expressed in nmol MDA mg^{-1} protein.

2.7. Activity of Mitochondrial Enzyme Complexes

Activities of the mitochondrial complexes I, II, and IV, and citrate synthase (CS) were assessed, at 37 °C, in a 96-well plate

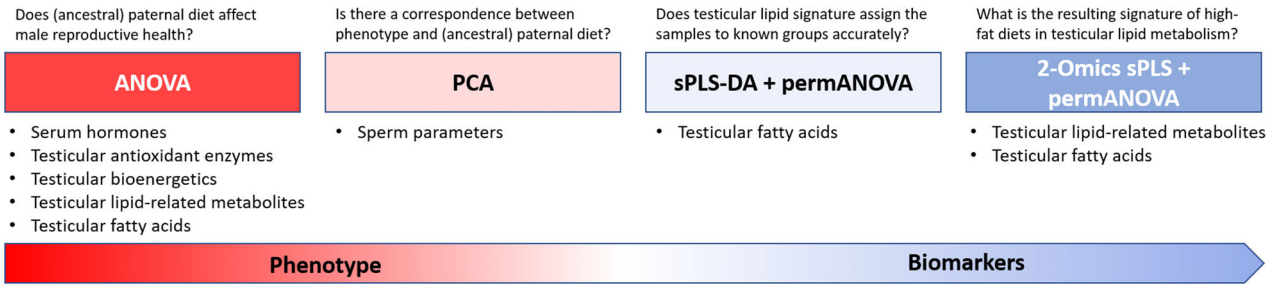

Figure 1. Bottom-up exploratory data analysis workflow. We started by investigating whether serum hormones and testicular antioxidant enzymes, bioenergetics, lipid-related metabolite content, and fatty acid content were different across groups, in each generation. This hypothesis was tested using Univariate ANOVA as previously.[13,25,31] Then we evaluated whether samples clustered in groups corresponding to their diet (or ancestral diet) in relation to their sperm parameters. We have tested this hypothesis using PCA complemented by PERMANOVA as previously.[25,31] Next, we have performed a Discriminant Analysis of the testicular FAs according to diet (or ancestral diet), using sPLS-DA, to evaluate if experimental groups could also be clustered according to their testicular content in FAs. Besides, sPLS-DA is more robust as a confirmatory multivariate method than PCA, as it downweighs non-discriminant variables. Finally, we assessed if the samples could be clustered according to diet (or ancestral diet), considering the interaction between testicular FA and lipid-related metabolite content. To test this hypothesis, we have used 2omics-sPLS complemented by PERMANOVA. Similarly, to PCA, 2omics-sPLS is not a discriminant method, but it combines two related datasets and, like sPLS-DA, it downweighs variables with low influence in the model. PERMANOVA was then used to calculate the overlap between groups, in each generation, granting confirmatory property to sPLS. Moreover, we have used the correlations established between the variables in the 2omics-sPLS model to estimate networks of testicular lipid metabolism.

by adapting previously described protocols.[25,26] CS activity was measured spectrophotometrically and calculated by the reduction rate DTNB ($\epsilon = 13.6$ mM^{-1} cm^{-1}), determined in the linear range of the plot, and expressed as nmol min^{-1} mg^{-1} protein.

Complex I activity was assessed following NADH oxidation, and calculated by the difference between the NADH oxidation rate of rotenone-inhibited mitochondria-enriched fraction and non-inhibited mitochondria-enriched fraction. This value was expressed in nmol NADH min^{-1} mg^{-1}.

Complex II activity was assessed monitoring 2,6-dichlorophenolindo-phenol (DCPIP) reduction at 600 nm ($\epsilon = 20.7$ mM^{-1} cm^{-1}). Complex II activity was expressed as the DCPIP reduction rate (corrected for spontaneous reduction) in nmol DCPIP min^{-1} mg^{-1} protein.

Complex IV activity was calculated by the cytochrome c (CytC, $\epsilon = 29.5$ mM^{-1} cm^{-1}) oxidation rate, corrected by the spontaneous oxidation rate obtained in the KCN-inhibited wells, and expressed as nmol CytC min^{-1} mg^{-1} protein.

2.8. Determination of Testicular Adenosine Nucleotides Levels by HPLC

Testicular adenosine nucleotides levels were assessed in a Waters 600 HPLC system (Waters, Milford, MA, USA) equipped with 2487 dual-λ Absorbance Detector. ATP, ADP, and AMP were separated on a reverse-phase chromatography column (Lichrospher RP-18 HPLC Column, 5 µm particle size, L x I.D. 25 cm x 4.6 mm), using a gradient mobile phase that consisted of Phase A (100 mM KH_2PO_4 buffer with 1.2% methanol v/v, pH = 7.0) and Phase B (100 mM KH_2PO_4 buffer with 10% methanol v/v, pH = 7.0). The elution program was the following: 100% of A from zero to 20 min followed by a linear gradient up to 100% of B until 25 min, and from 25 to 28 min down to 0% B (initial conditions). The flow was 1 mL min^{-1}, and the column temperature was maintained at 25 °C during the run. Chromatograms were recorded at 254 nm and analyzed using the Waters Millennium32 (Waters, Milford, MA, USA). Peaks were identified by their retention times, comparing them with samples of standard compounds. ATP, ADP, and AMP levels were quantified using standard curves obtained with a serial of known concentrations of each of the adenine nucleotides run on the same day and conditions of the samples. The results were expressed as mol g^{-1} wet tissue.

2.9. Statistical and Data Analysis

Our bottom-up EDA design included univariate and multivariate analysis (**Figure 1**). Phenotypic features were first compared between groups, within each Generation in the study, based on parametric univariate methods of analysis. The assumptions for parametric methods were tested by Kolmogorov-Smirnoff (KS) test with Lillefor's correction (normality) and by the Levene test (homoscedasticity). Data were considered suitable for parametric statistics if at least one of the assumptions was met. Extreme values were considered as outliers using the normality criterion, i.e., values were excluded if their exclusion permitted to achieve a normal distribution. Pairwise comparisons were then performed using ANOVA corrected by Tukey's Honest Significant Differences (HSD). Additionally, transgenerational comparisons between F_1 and F_2 generations were performed using two-way ANOVA corrected by the Šidak method. Significance was considered when $p < 0.05$. A multivariate analysis was performed for sperm parameters, using Principal Component Analysis (PCA) based on the correlation matrix. Data were tested for normality using the KS test ($p > 0.05$) and for data reduction adequacy using Kaiser-Meyer-Olkin Measure of Sample Adequacy (KMO) ($0.5 < KMO < 0.8$). Two principal components (PC) were extracted by PCA, rotated by the varimax method with Kaiser normalization. Samples were then plotted in the two-dimensional Euclidean space defined by the two components. All these methods were performed in IBM SPSS Statistics v26 (Armonk, NY, USA).

Testicular biomarkers associated with the phenotype were identified using multivariate analysis based on sparse Projection

of Latent Structures (sPLS) and sPLS Discriminant Analysis (sPLS-DA). These methods were implemented using the R package mixOmics.[32] The "sparse" version of these methods, implemented by the mixOmics package introduces variable selection via implementation of the LASSO (ℓ_1) regularization.[33] Testicular FA content was tested by sPLS-DA, a supervised statistical method, to classify samples according to their experimental group. Testicular FAs and lipid-related metabolites were then integrated using (2-Omics) sPLS, an unsupervised method, to evaluate the combined effect of the two omics. For both methods, 2 components were extracted, and variables with component loading weight ≥|0.2| were used to estimate the number of variables to be affected by the ℓ_1 parameter of the "sparse" method.[34] Samples were plotted in the space spanned by the extracted components. Group separation was evaluated using Permutational Multivariate ANOVA (PERMANOVA), using the adonis2 function provided in the vegan R package.[35] PERMANOVA was performed based on the Euclidean distance with 999 permutations. sPLS-DA and sPLS were considered discriminant when the PERMANOVA test had $p < 0.05$. All these methods were performed in R 4.0.3 (R Foundation for Statistical Computing, Vienna, Austria).

3. Results

3.1. Exposure of the Male Progenitor to HFD Induces Changes in the Reproductive Axis for up to Two Generations

No changes were found in serum hormones in Generation F_0,[25] but differences were found in the descendants (**Figure 2**). In Generation F_1, sons of HFD_t fed mice show lower E_2 levels (201 ± 99 pg mL^{-1}) than CTRL (333 ± 73 pg mL^{-1}) and HFD fed mice sons (341 ± 97 pg mL^{-1}). Notably, in generation F_2 serum FSH levels were increased in grandsons of HFD_t fed mice (84.2 ± 55.7 ng mL^{-1}) comparing to the levels detected in CTRL and HFD fed grandsons (16.2 ± 5.6 and 35.8 ± 24.3 ng mL^{-1}, respectively) while serum LH in grandsons of HFD_t fed mice presented decreased levels (330 ± 28 ng mL^{-1}) compared to the grandsons of HFD fed mice (401 ± 38 ng mL^{-1}). E_2 levels were increased in the grandsons of HFD (309 ± 132 pg mL^{-1}), comparing to other groups (CTRL: 151 ± 53 pg mL^{-1}; HFD_t: 252 ± 202 pg mL^{-1}, respectively). No changes in fasting insulin or glycemia were found in generations F_1 and F_2. Regarding glucose homeostasis, we found that the sons of mice fed with HFD had higher serum glucose levels at 90 and 120 min on the ipITT.[11] No changes were found in Homeostatic Model Assessment for Insulin Resistance 2 (HOMA2) metrics in sons and grandsons of the HFD-challenged mice.

3.2. Paternal Adoption of HFDs Promotes Negative Outcomes on the Testicular Antioxidant Defenses and Mitochondrial Function of the Progeny

We have previously reported a decrease in the activity of catalase (CAT) and glutathione-disulfide reductase (GSR) in the testis of mice fed a life-long HFD.[25] Here, we observed a decrease in the activity of CAT in testes of their offspring (generation F_1, HFD: 952 ± 192 nmol O_2 min^{-1} mg^{-1} protein), comparing to other groups (**Table 1**). Superoxide dismutase (SOD) activity was increased in sons of HFDt-fed mice (21.1 ± 3.2 U mg^{-1} protein) compared to the sons of HFD fed mice. Interestingly, testicular mitochondrial Complex I and IV activities are reduced in the sons of HFD fed mice (generation F_1: 46.54 ± 19.21 nmol NADH min^{-1} mg^{-1} protein, and 29.32 ± 9.03 nmol Cyt c min^{-1} mg^{-1} protein, respectively) comparing to the sons of the other groups (Table S2, Supporting Information). We have further characterized testicular bioenergetics in all generations, notably testicular content of ATP, ADP, and AMP using HPLC, but no differences were found (Table S3, Supporting Information).

Table 1. Enzymatic activity of antioxidant enzymes in testes of mice fed standard chow (CTRL), life-long high-fat diet (HFD), and those subjected to diet correction after 60 days (HFDt), and their progeny (Generation F1 and F2).

		CTRL	HFD	HFD_t
Generation F0	*TBARS*[a]	32.5 ± 3.4	35.3 ± 12.1	33.2 ± 3.8
	GPx activity[b]	1549 ± 117	1394 ± 225	1548 ± 174
	SOD activity[c]	10.8 ± 2.1	9.5 ± 0.8	9.7 ± 0.7
	Cat activity[d]	748 ± 141	**552 ± 92***	670. ± 107
	GSR activity[e]	133.1 ± 11.3	**114.0 ± 11.5***	120.4 ± 9.1
Generation F1	*TBARS*[a]	34.8 ± 8.9	33.0 ± 7.9	41.0 ± 12.1
	GPx activity[b]	733 ± 119	739 ± 184	861 ± 96
	SOD activity[c]	16.0 ± 4.2	14.24 ± 3.4	**21.0 ± 3.2#**
	Cat activity[d]	1356 ± 255	**952 ± 192***	1220 ± 205
	GSR activity[e]	142.5 ± 7.5	149.2 ± 19.4	142.9 ± 6.0
Generation F2	*TBARS*[a]	30.6 ± 1.5	30.6 ± 4.0	**30.2 ± 5.0§**
	GPx activity[b]	737 ± 129	608 ± 119	**636 ± 98§§**
	SOD activity[c]	13.3 ± 2.2	13.4 ± 1.9	**12.4 ± 2.4§§§§**
	Cat activity[d]	1696 ± 238	**1879 ± 461§§§§**	1594 ± 348
	GSR activity[e]	147.2 ± 20.2	124.0 ± 33.5	116.5 ± 27.5

Results are expressed as mean ± standard deviation. Experimental groups ($n = 6$) were compared by one-way ANOVA with Tukey's HSD. Equivalent groups between generations F1 and F2 were compared using two-way ANOVA corrected by Šidak method. Significance was considered when $p < 0.05$. * versus CTRL; # versus HFD; § versus Generation F1. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Abbreviations: Cat, catalase; GPx, glutathione peroxidase; GSR, glutathione S-reductase; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive species assay. [a] Units: nmol MDA mg^{-1} protein [b] Units: nmol NADH min^{-1} mg^{-1} protein [c] Units: U mg^{-1} protein [d] Units: nmol O_2 min^{-1} mg^{-1} protein [e] Units: nmol NADPH min^{-1} mg^{-1} protein.

3.3. HFDs Cause Intergenerational and Transgenerational Signatures in Testicular Fatty Acids and Lipid-Related Metabolites

We have demonstrated that HFDs, even transient, cause a characteristic FA signature in mice testes.[25] We investigated how these signatures are imprinted in the progeny and found inter- and transgenerational effects. Regarding lipid-related metabolites, testicular content of choline in the sons of HFD fed mice (0.36 ± 0.12 log_2 FC) was the only intergenerational effect found in our study (**Figure 3**A). 3-hydroxybutyrate (3HO-But) was over-represented in testes of the grandsons (generation F_2) of HFD (0.41 ± 0.21 log_2 FC) and HFD_t fed mice (0.44 ± 0.26 log_2 FC) compared to the levels found in the testis of grandsons of CTRL mice (Figure 3B). Ethanolamine ($EtNH_2$) was increased

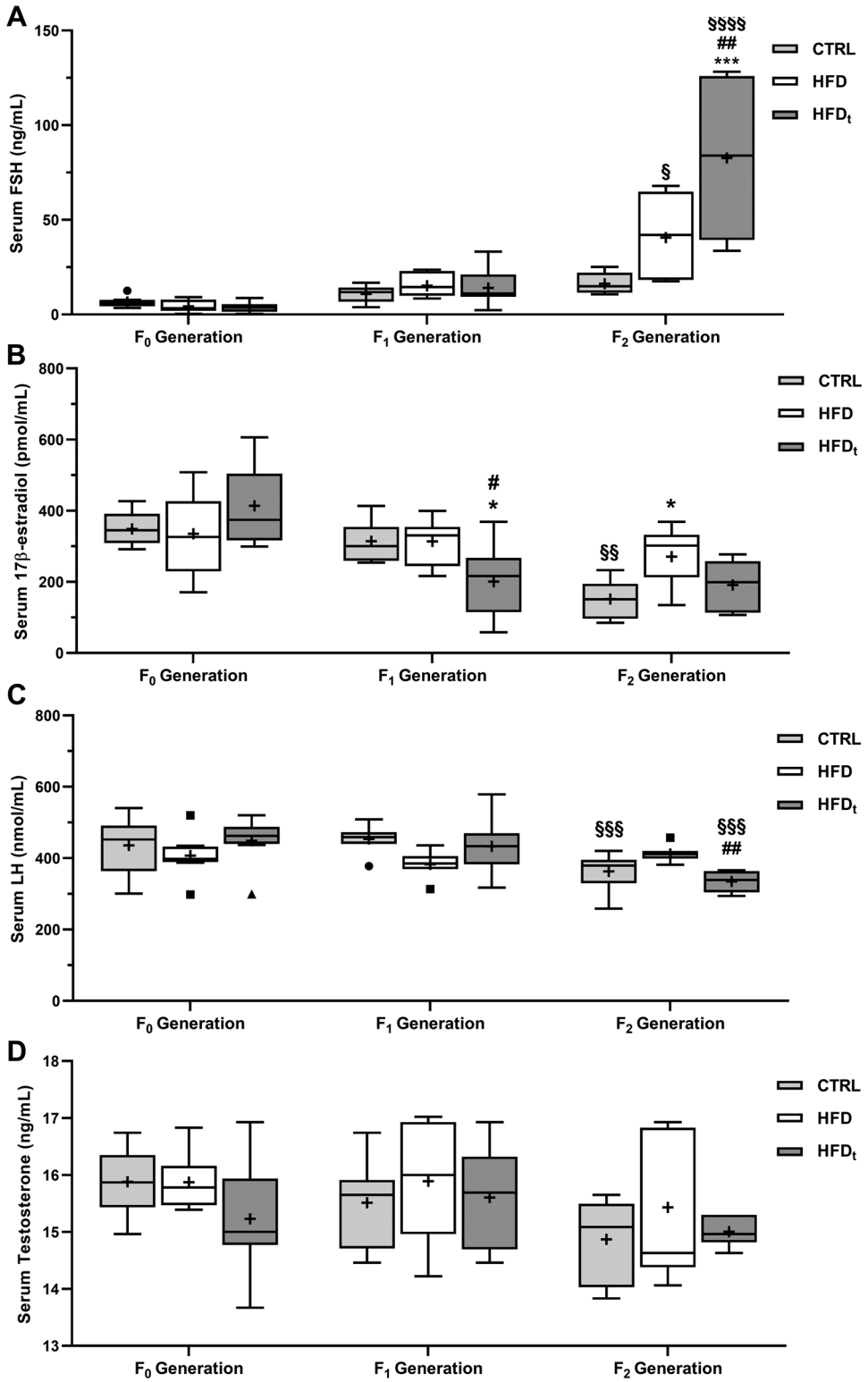

Figure 2. Serum hormones quantified in mice fed standard chow (CTRL) or life-long high-fat diet (HFD) or subjected to diet correction after 60 days (HFD_t), and in their progeny (Generation F_1 and F_2). Results are expressed as Tukey's whisker boxes (median, 25th to 75th percentiles ± 1.5 IQR). Extreme values are represented individually (● CTRL—standard chow; ■ HFD—high-fat diet; ▲ HFD_t—transient high-fat diet). Pairwise comparisons were tested using ANOVA corrected by Tukey's HSD ($n = 8$). Equivalent groups in generations F_1 and F_2 were compared using two-way ANOVA corrected by the Šidak method. Significance was considered when $p < 0.05$. $^+$ group average; * versus CTRL; $^\#$ versus HFD. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

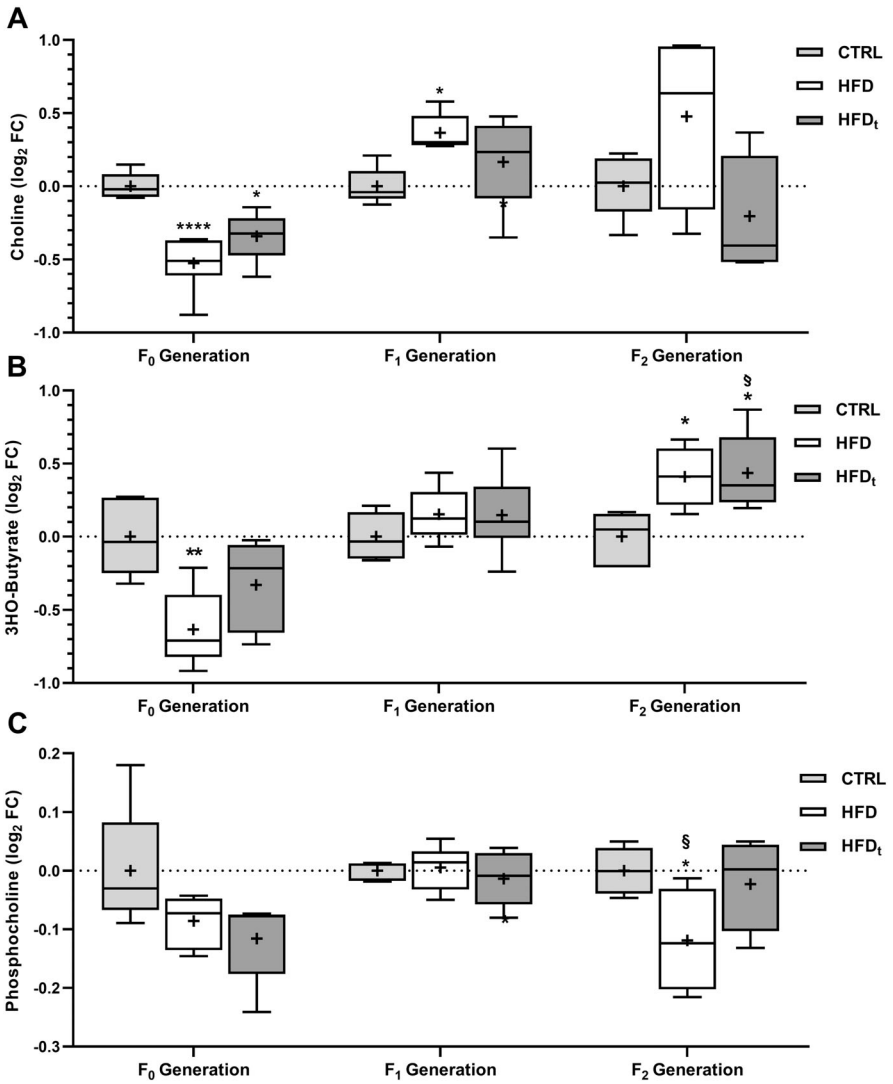

Figure 3. Testicular content in lipid-related metabolites quantified in mice fed standard chow (CTRL) or life-long high-fat diet (HFD) or subjected to diet correction after 60 days (HFD_t), and in their progeny (Generation F_1 and F_2). Results are expressed as Tukey's whisker boxes (median, 25th to 75th percentiles ± 1.5 IQR). Pairwise comparisons were tested using ANOVA corrected by Tukey's HSD (*n* per group disclosed in Table S4, Supporting Information). Equivalent groups in generations F_1 and F_2 were compared using two-way ANOVA corrected by the Šidak method. Significance was considered when $p < 0.05$. $^{+}$ group average; * versus CTRL; $^{\#}$ versus HFD. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Abbreviations: FC, fold change.

in the testes of the grandsons of HFD fed mice (0.76 ± 0.59 $\log_2$ FC), comparing to CTRL (Figure 3C). Similarly, grandsons of HFD fed mice had a higher testicular acetate concentration (1.33 ± 0.16 $\log_2$ FC) than grandsons of CTRL mice (Table S4, Supporting Information). Opposingly, grandsons of HFD fed mice had lower content of testicular phosphocholine (P-choline, −0.08 ± 0.06 $\log_2$ FC) and phosphoethanolamine (P-$EtNH_2$, –0.12 ± 0.09 $\log_2$ FC) than grandsons of CTRL mice (Table S4, Supporting Information). Results are detailed in supplemental Table S4, Supporting Information. No intergenerational effects of ancestral HFD were found in testicular FA content (**Figure 4**). Yet, a transgenerational FA signature was observed in the grandsons (generation F_2) of HFD mice. Summarily, grandsons of HFD fed mice had lower testicular levels of polyunsaturated fatty acids (PUFAs) (−0.24 ± 0.16 $\log_2$ FC), ω3 PUFA (−0.50 ± 0.32 $\log_2$ FC), and ω6 PUFAs (−0.19 ± 0.14 $\log_2$ FC). The grandsons of HFD-fed mice further showed reduced values for several lipid ratios: ω3/ω6 (−0.31 ± 0.18 $\log_2$ FC), C22:6n3/C20:4n6 (−0.47 ± 0.31 $\log_2$ FC), and C22:5n6/C20:4n6 (−0.42 ± 0.25 $\log_2$ FC), comparing to the grandsons of other groups. No changes in testicular FA composition were found between grandsons of HFD_t mice and CTRL. Detailed values are provided in supplemental Table S5, Supporting Information.

3.4. Ancestor's Diet Influences Sperm Parameters of the Grand-Offspring

Sperm parameters were used to classify the samples, by plotting them in the two-dimensional space defined by two PCs extracted

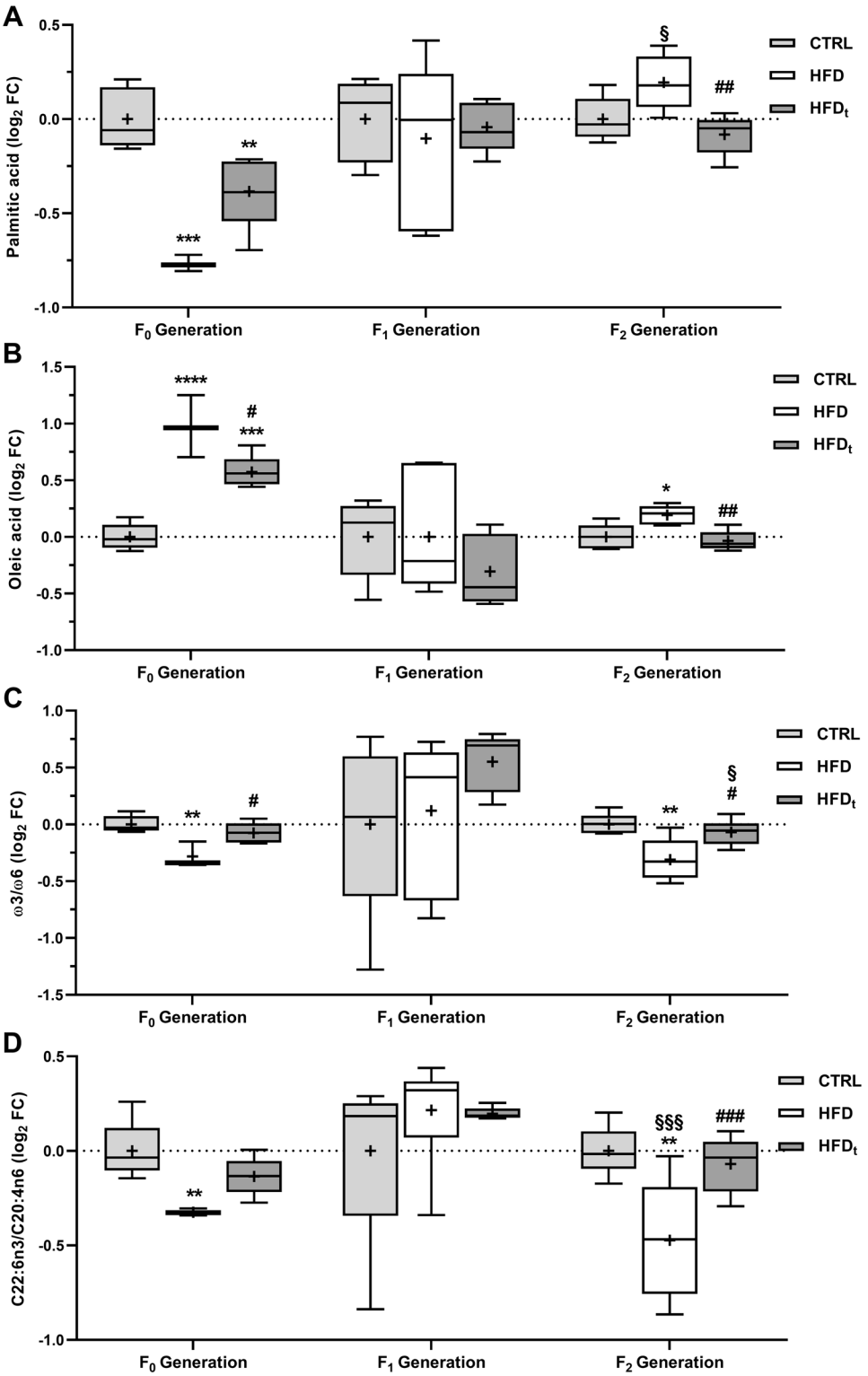

Figure 4. Testicular FA content quantified in mice fed standard chow (CTRL) or life-long high-fat diet (HFD) or subjected to diet correction after 60 days (HFD_t), and in their progeny (Generation F_1 and F_2). Results are expressed as Tukey's whisker boxes (median, 25th to 75th percentiles ± 1.5 IQR). Pairwise comparisons were tested using ANOVA corrected by Tukey's HSD (*n* per group disclosed in Table S5, Supporting Information). Equivalent groups in generations F_1 and F_2 were compared using two-way ANOVA corrected by the Šidak method. Significance was considered when $p < 0.05$. $^{+}$ group average; * versus CTRL; $^{\#}$ versus HFD. *$p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Abbreviations: FC, fold change.

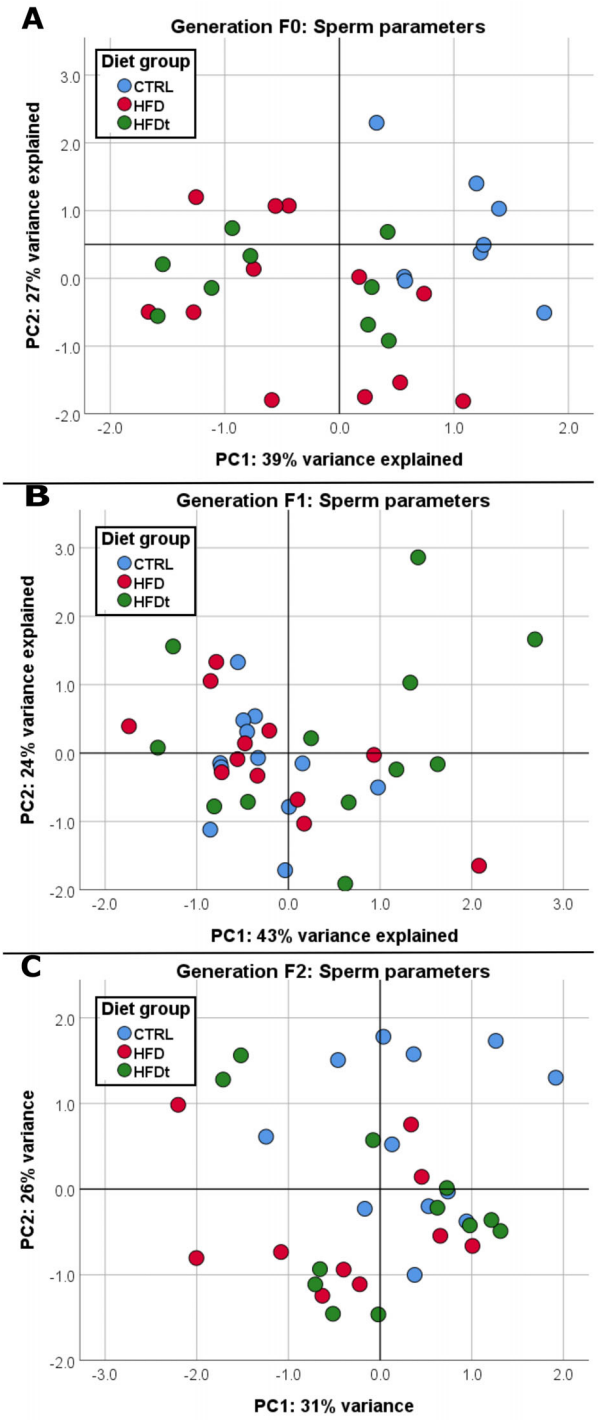

Figure 5. Sample distribution in the space defined by the two principal components extracted by PCA, based on sperm parameters. A) Generation F_0; B) Generation F_1; C) Generation F_2.

by PCA (**Figure 5**). To avoid multicollinearity, only four variables were considered for dimension reduction: sperm counts (millions per mL), viability (%), motility (%), and normal morphology (%). This method was used to demonstrate that samples of the same group displayed a similar phenotype when considering sperm parameters. Generation F_0 has achieved the best separation, with CTRL samples clustering almost to no overlap to other groups. Yet, samples from HFD and HFD_t fed mice were overlapped. In Generation F_1 (sons), samples were unable to be sorted according to sperm parameters. However, in Generation F_2 (grandsons), the samples of the grandsons of CTRL are clustered on the right side of the PC 1, while the samples of the grandsons of HFD fed mice are clustered mostly to the left side of this axis. Samples obtained from the grandsons of HFD_t fed mice overlap both groups, occupying the centermost position in the plot (Figure 5).

3.5. Ancestral Paternal HFD Correlates Sperm Quality to Testicular Fatty Acid Signature

Independent testicular FA variables (i.e., variables not dependent on other variables, like ratios) were used to classify samples taking the experimental groups as predictors, using sPLS-DA. First, PLS-DA was performed to extract two components and estimate the number of relevant variables for the "sparse" method; n variables with component loading $\geq|0.2|$ were selected per component (Table S6, Supporting Information). Samples were plotted in the two-dimensional space defined by the components. Projected group areas were calculated based on the distance to the group's centroid. In generation F_0, nine and five variables met the selection criterion for components 1 and 2, respectively. Using sPLS-DA, the separation between groups is evident; and not a single sample is found in the area of another group (**Figure 6**A). PERMANOVA confirms that the differences between groups are significant ($p < 0.05$). However, in generation F_1 (sons), sPLS-DA does not accurately assign subjects to the experimental group of their fathers, based on the testicular FA signature (Figure 6B). In this generation, 13 and 4 variables per component were selected for sPLS-DA, but the groups could not be separated ($p = 0.147$). Interestingly, the method is discriminant for samples of generation F_2 (grandsons) (Figure 6C). Ten variables were selected in component 1, and seven in component 2. Although some samples are plotted over areas of another group and the overall discriminative power of the sPLS-DA is significant ($p < 0.001$).

3.6. Testicular Fatty Acids and Lipid-Related Metabolites Are Predictors of Anomalies in Sperm Parameters Caused by HFD Even if Transiently

To identify lipid metabolism signatures of ancestral HFD in the paternal lineage, we integrated testicular FAs and lipid-related metabolite content using two-omics sPLS. Contrary to sPLS-DA, sPLS does not classify samples towards known predictors; it highlights commonalities between data sets. Nonetheless, it is possible to group subjects according to a set of variables in a two-dimensional space and tests the discriminative power of the resulting projection using PERMANOVA. Like sPLS-DA, the non-sparse PLS method was performed before sPLS to estimate the number of meaningful variables in each component, for each dataset (Table S7, Supporting Information). The experimental design does not support the discrimination between fatty

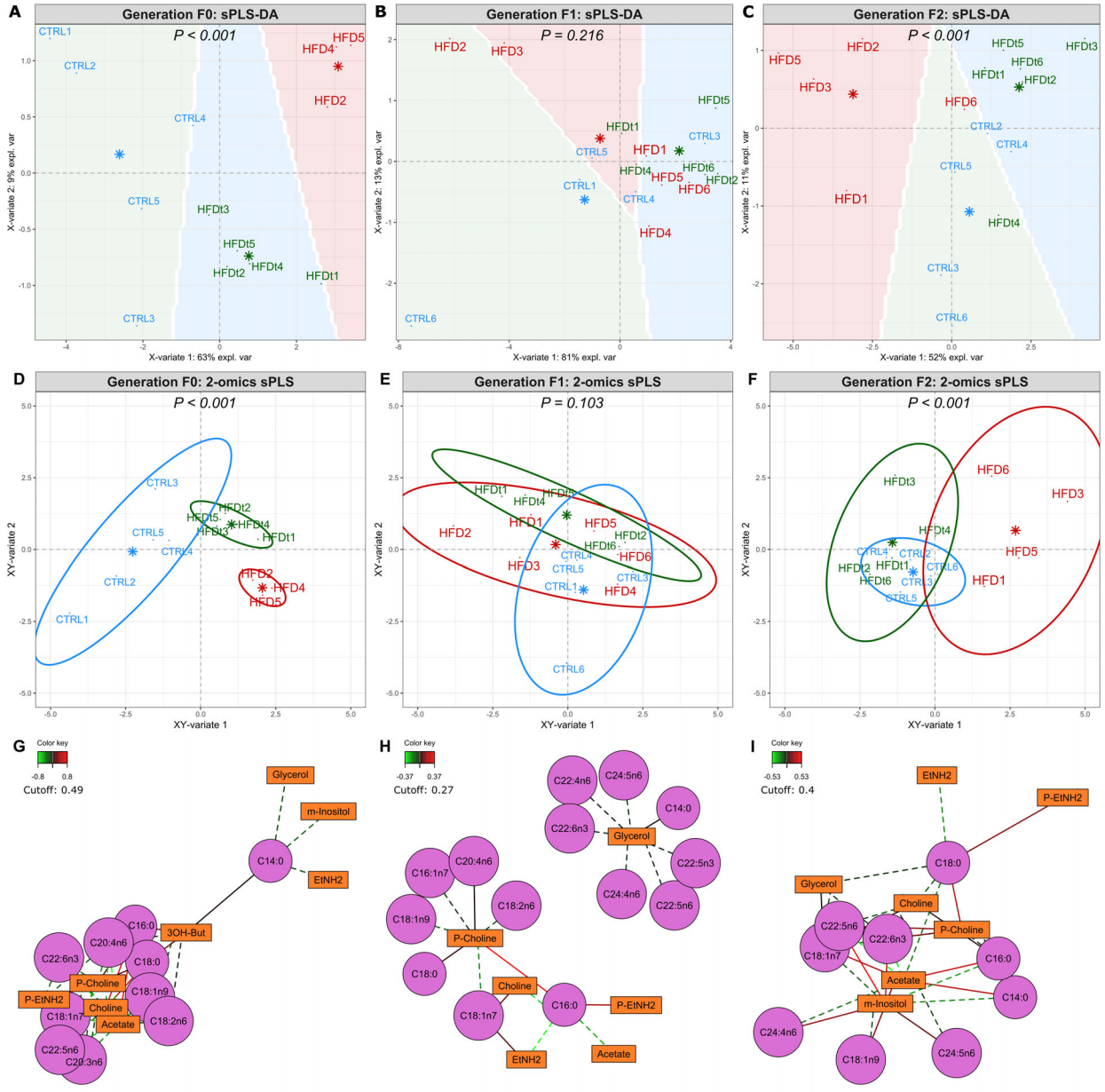

Figure 6. Metabolite and lipid profile of mice testis in different generations. A, B, and C) Sample distribution in the space defined by the two principal components extracted by sPLS-DA of testicular FAs. The background color was calculated according to the Euclidean distance to the group's centroid. The separation between groups was tested based on the Euclidean distances to centroid using PERMANOVA. Significance was considered when $p < 0.05$. D, E, and F) Sample distribution in the space defined by the combined result of the two principal components of the FA and lipid-related metabolites datasets, using 2-omics sPLS. The separation between groups was tested based on the Euclidean distances to centroid using PERMANOVA. Significance was considered when $p < 0.05$. G, H, and I) Testicular metabolic network of the variables considered in the 2-omics sPLS. These networks are generated from the Pearson's correlation coefficients between the variable scores obtained by 2-omics sPLS (FAs versus lipid-related metabolites). Therefore, this mathematical model indirectly illustrates molecular interactions based on their covariance. The correlation cut-off was adjusted to improve data visualization.

acids that come from triacylglycerols and from membrane polar lipids (e.g., phospholipids). However, the interaction network generated from the sPLS result permits an indirect assessment of molecular associations, contemplating the variables with the strongest covariation. In generation F_0 the separation of diet groups is evident ($p < 0.001$) (Figure 6D). A molecular network was established with 3HO-But, choline, P-choline, acetate, and P-$EtNH_2$ taking a central role between SFAs, and MUFAs and PUFAs (Figure 6G). In generation F_1 (sons) no changes were found between groups ($p = 0.103$) (Figure 6E). The metabolic network (Figure 6H) of relevant FAs and lipid-related metabolites is divided into two nuclei, eliciting triacylglycerol and phospholipid

Table 2. Intergenerational and transgenerational effects of HFD in the paternal lineage (comparing to CTRL).

Intergenerational effects	Transgenerational effects
↓ serum E_2 (HFDt)	↑ serum FSH (HFDt)
↓ testicular Catalase activity (HFD)	↑ serum E_2 (HFD)
↓ testicular Complex I activity (HFD)	↑ testicular 3-hydroxybutyrate (HFD/HFDt)
↓ testicular Complex IV activity (HFD)	↑ testicular acetate (HFD)
↓ testicular choline (HFD)	↑ testicular ethanolamine (HFD)
	↓ testicular phosphocholine (HFD)
	↓ testicular phosphoethanolamine (HFD)
	↑ testicular C18:1n9 (HFD)
	↓ testicular C22:5n6 (HFD)
	↓ testicular C22:6n3 (HFD)
	↓ testicular C24:4n6 (HFD)
	↓ testicular C24:5n6 (HFD)
	↓ testicular PUFAs (HFD)
	↓ testicular ω3 PUFAs (HFD)
	↓ testicular ω6 PUFAs (HFD)
	↓ testicular ω3/ω6 (HFD)
	↓ C22:6n3/C20:4n6 (HFD)
	↓ C22:5n6/C20:4n6 (HFD)

The affected lineage is reported in brackets.

tail composition. Choline and P-choline establish relevant interactions with C18:0, C18:1n7, and C20:4n6, while glycerol is positively correlated to C14:0. Groups were well-defined in generation F_2 ($p < 0.001$) (Figure 6F). The resulting metabolic network (Figure 6I) evidences that choline and P-choline (metabolites related to phospholipids) can be associated with different FA classes. Besides, *myo*-inositol is positively correlated with long ω6 PUFAs. Different cutoffs were applied to generate Figure 6G-I for visualization purposes.

4. Discussion

An association between the prevalence of metabolic disorders (obesity and T2D) and a decline in sperm parameters and male fertility potential has been suggested.[36–38] Although the biochemical cues linking these phenomena are still debatable, evidence has brought a new layer of complexity into this equation: transgenerational inheritance.[39,40] Recent studies by our research group have shown that the early exposure to HFD induces metabolomic and lipidomic changes in testes of mice, even after reverting to a balanced diet, with deleterious effects on sperm parameters.[13,25] We have further shown that an HFD, even transiently, causes deleterious effects to glucose homeostasis, sperm parameters, and is responsible for a distinctive testicular metabolic fingerprint.[11] Those deleterious effects persist not only in sons (intergenerational effects) but also in grandsons (transgenerational effects).[11] Hereby we further describe this "inherited metabolic memory," reporting the inter- and transgenerational effects of ancestral paternal HFD on the testicular FA metabolism of the progeny (**Table 2**). Moreover, we characterize the molecular fingerprints of this metabolic memory, relating the testicular content in FAs and lipid-related metabolites employing a bottom-up EDA design (Figure 1).

We identified key biomarkers of testicular lipid metabolism associated with the phenotypic features in testis and sperm in descendants of males fed with HFD. We observed that testicular choline and P-choline are significantly correlated with C18 FAs. This suggests that C18 FAs, regardless of their saturation, are a common element of the phospholipid tails of testicular cells. Interestingly, the direction of this correlation changes across generations, which elicits a dependence upon FA availability (Figure 6). There is a significant positive correlation between choline and linoleic acid (C18:2n6), likely due to dietary intake, as both choline and linoleic acid are found in significant amounts in the diet (standard and high-fat), and there is no other source for linoleic acid as it is an essential FA in mammals. Despite that, the sons of HFD mice have increased levels of choline in testes, contrary to their progenitors who had a diet richer in choline (the HFD contains more choline than the standard diet, per unit of mass). Abnormally high concentration of testicular choline has been associated with abnormal sperm motility in mice.[41] Nevertheless, we have not observed such an effect.[11] Yet, this excess in testicular choline may act as an anti-inflammatory mechanism in the sons of HFD mice, considering its positive correlation with MUFAs. These mice showed lower testicular enzymatic activity of CAT and decreased activity of mitochondrial complexes I and IV, but no differences in the levels of lipid peroxidation products were found.

The phospholipid precursor *myo*-inositol establishes a relevant correlation with SFAs and MUFAs, especially evidenced in Generation F_0 and Generation F_2. Testicular *myo*-inositol is negatively correlated to C14:0, an SFA (Figure 6G) but positively correlated to the pro-inflammatory ω6 PUFAs (Figure 6I). Inositol-containing phospholipids (PI) commonly contain arachidonic acid, particularly in the C2 position of glycerol.[42] Also, the ω6 elongation pathway from arachidonic acid is highly active in testicular cells, where ω6 FAs play signaling and structural roles.[43] Hence, our data elicits that testicular *myo*-inositol is correlated with the content of ω6 PUFAs in the hydrophobic tails of testicular phospholipids and the activation of the ω6 signaling. Arachidonic acid (C20:4n6, AA), ω6 docosapentaenoic acid (C22:5n6, ω6 DPA), and docosahexaenoic acid (C22:6n3, DHA) are essential structural elements of SC phospholipids, granting the SC membrane the flexibility needed to form invaginations, thus creating the microenvironment for germ cells through spermatogenesis.[43,44] Interestingly, grandsons of HFD fed mice have lower testicular content of ω6 DPA, DHA, C24:4n6 and C24:5n6 than grandsons of other groups. Considering the prevalence and typology of sperm defects previously reported,[11] it is likely that the reduction of testicular availability of these PUFAs has a pivotal role in the structural integrity of the cell membrane of spermatozoa. Indeed, it has been shown that the disruption of the FA elongation causes severe defects in testicular function and sperm parameters, whereas PUFA supplementation can rescue.[43,45]

The grandsons of HFD fed mice have proportionally lower testicular content of PUFAs, and specially ω3 PUFAs, comparing to grandsons CTRL, which promotes a pro-inflammatory environment. Interestingly, we have reported a similar phenomenon in their grandfathers (HFD, generation F_0) although in that group testicular PUFA content was ~50% higher than CTRL.[25] The decrease in PUFAs proportion does not improve sperm morphological quality; sperm head defects are more prevalent in this group.[25] Although no changes were found in markers of

oxidative stress, the prevalence of abnormal sperm morphology may be related to this pro-inflammatory environment. Indeed, the changes in testicular content of acetate, P-choline, $EtNH_2$, and P-$EtNH_2$ found in the grandsons of HFD-fed mice are correlated to depletion of C18 lipids, and to the inhibition of $\omega 3$ elongation pathways. Considering the metabolic correlation network, SFAs and MUFAs are strongly and positively correlated to acetate, suggesting the breakdown of these FA classes.

The grandsons of HFD_t fed mice presented increased levels of testicular 3HO-But, a ketone body that, in testis, is produced by SCs as a nutritional source for germ cells.[46] In addition, grandsons of HFD_t fed mice had the highest values of FSH, whose release by hypophysis is regulated in negative feedback by Inhibin B, a peptide secreted by SCs.[47] Although we have not found signs of systemic metabolic syndrome, our data suggest that testes of grandsons of mice fed a transient HFD preserve signatures of metabolic syndrome. SC activity is reduced due to insulin resistance,[48] which in turn inhibits the secretion of inhibin B,[49] and decreases the expression of FSH and E2 receptors.[50,51] Thus, decreased SC responsivity to insulin can trigger an increase in serum FSH, due to the disruption of the negative feedback established between SCs and hypothalamus-hypophysis. Therefore, our observations reinforce our previous data describing testicular "inherited metabolic memory" due to ancestral exposure to HFD.[11] Besides, FSH is essential for SC proliferation before sexual maturation, which is a limiting factor for sperm concentration in adulthood.[52] Hence, SC insensitivity to FSH hinders total SC numbers in the testis, consequently limiting sperm counts. Regardless of specific testicular signatures, it is crucial to state that both the grandsons of HFD and HFD_t fed mice (generation F_2) displayed lower sperm counts than the grandsons of CTRL. This finding corroborates evidence from human studies reporting a decline in sperm counts in the last century.[53] Therefore, either life-long or temporary adoption of an HFD by the male has the potential to cause deleterious effects to sperm parameters up to grandsons.

Here we propose two distinct signatures of "inherited metabolic memory," specific to a diet regimen of the ancestor, yet to cause similar results in the progeny. Our analytical approach allowed us to shortlist the molecular candidates underlying the observed phenotypes. The present work expands our previous studies[13,25] and provides a more integrative view on the extent of "inherited metabolic memory" in testes of mice after ancestral paternal adoption of HFDs. Despite having described the "inherited metabolic memory" via paternal lineage, we have not yet addressed the factors responsible for the inheritance of this memory. Epigenetic factors, such as the small non-coding RNAs (sncRNA) carried by spermatozoa have been implicated in the transmission of acquired traits to progeny, including in humans.[54,55] Besides, it has been shown that sperm sncRNA content dynamically changes according to diet[56] and that it drives transgenerational epigenetic inheritance.[55,57] By the action of epigenetic factors during embryo development, the "inherited metabolic memory" of ancestral exposure to HFD will exert a lifelong influence over the testicular function of the offspring. In future work, we intend to explore the epigenetic signature carried by spermatozoa to investigate the molecular mechanisms involved in the inheritance of this inherited metabolic memory. Also, it is unclear whether the inter- and transgenerational effects of ancestral exposures exert a lifelong influence on the progeny. Due to the limited lifespan of our animal model, it was not possible to address this hypothesis, as the effects of senescence would mask the effects of ancestral exposure to HFD. This hypothesis shall be explored in future works, using a similar animal model and collecting tissues at different time points.

Concluding, a paternal life-long HFD causes an intergenerational (father-son) impairment of testicular processes involving choline, mitochondrial activity and antioxidant defenses; and transgenerational (grandfather-grandson) disturbance in testicular $\omega 3/\omega 6$ lipids, promoting a pro-inflammatory environment. The adoption of HFD, even if transient, by the ancestral male is related to a transgenerational signature in mice testes marked by increased 3HO-but levels, with possible implications to tissue-specific insulin resistance. Notably, the adoption of an HFD, even transiently, causes deleterious effects in testicular metabolism and, consequently, sperm parameters, up to two generations.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

L.C., P.F.O., M.G.A., and R.L.B. contributed to study design, analysis, and interpretation of data. L.R., I.J., K.S., T.M., R.A.V., and L.C. performed experimental work. L.C. edited the images and tables, performed the statistics, and contributed to the analysis and interpretation of data. R.L.B., J.F.R., and R.A.V. critically reviewed the manuscript and suggested modifications. All the authors contributed to manuscript writing/editing and approved the final version.

Data Availability Statement

All the data, biological samples and other material used in this work are available upon reasonable request. The R code used for the statistical analysis in this paper is available on GitHub (https://github.com/luis-crisostomo/transgenerational_lipidomics).

Keywords

discriminant analysis, high-fat diet, inherited metabolic memory, lipidomics, paternal inheritance, testis

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