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DOUTORAMENTO
CIÊNCIAS BIOMÉDICAS

Paternally-induced transgenerational effects of high-fat diets

Luís Daniel Machado Crisóstomo

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2022

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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

Luís Daniel Machado Crisóstomo

Paternally-induced transgenerational effects of high-fat diets

Tese de Candidatura ao grau de Doutor em Ciências Biomédicas, submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

Orientador – Doutor Marco G. Alves
Categoria – Professor Afiliado
Afiliação – Instituto de Ciências Biomédicas Abel Salazar (ICBAS), Universidade do Porto

Coorientador – Professor Doutor João Filipe Cancela dos Santos Raposo
Categoria – Professor Auxiliar Convidado
Afiliação – Faculdade de Ciências Médicas, Universidade Nova de Lisboa

Coorientador – Professor Doutor Pedro F. Oliveira
Categoria – Professor Auxiliar
Afiliação – LAQV & QOPNA, Departamento de Química, Universidade de Aveiro

As frases que nunca escreverei, as paisagens que não poderei nunca descrever, com que clareza as dito à minha inércia e as descrevo na minha meditação, quando, recostado, não pertenço, senão longinquamente, à vida. Talho frases inteiras, perfeitas palavra a palavra, contexturas de dramas narram-se-me construídas no espírito, sinto o movimento métrico e verbal de grandes poemas em todos os elementos e um grande entusiasmo, como um escravo que não vejo, segue-me na penumbra. Mas se der um passo, da cadeira, onde jazo estas sensações quase cumpridas, para a mesa onde quereria escrevê-las, as palavras fugiram, os dramas morreram, do nexo vital que uniu o murmúrio rítmico não ficou mais que uma saudade longíqua, um resto de sol sobre montes afastados, um vento que ergueu as folhas ao pé do limiar deserto, um parentesco nunca revelado, os prazeres dos outros, a mulher, que a nossa intuição diz que olharia para trás, e nunca chegou a existir.

Projectos, tenho-os tido todos. A *Ilíada* que compus teve uma lógica de estrutura, uma concatenação orgânica de episódios que Homero não podia conseguir. A perfeição estudada dos meus versos por completar em palavras deixou pobre a precisão de Vergílio e frouxa a força de Milton. As sátiras alegóricas que fiz excederam todas as de Swift na precisão simbólica dos particulares exactamente ligados. E quantos Horácios fui!

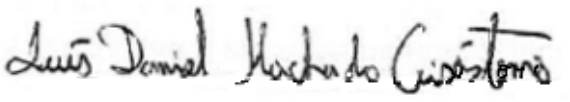
E sempre que me levantei da cadeira onde, na verdade, estas coisas não foram absolutamente sonhadas, tive a dupla tragédia de as saber nulas e de saber que não foram todas sonho, que alguma coisa ficou delas no limiar abstracto em eu pensar e elas serem. Fui génio mais que nos sonhos e menos no que na vida. A minha tragédia é esta. Fui o corredor que caíu quase na meta, sendo até aí o primeiro.

In *"Livro do Desassossego"*

Fernando Pessoa

Declaração de Honra

Declaro que a presente tese é de minha autoria e não foi utilizada previamente noutro curso ou unidade curricular, desta ou de outra instituição. As referências a outros autores (afirmações, ideias, pensamentos) respeitam escrupulosamente as regras da atribuição, e encontram-se devidamente indicadas no texto e nas referências bibliográficas, de acordo com as normas de referenciação. Tenho consciência de que a prática de plágio e auto-plágio constitui um ilícito académico.



(Luís Daniel Machado Crisóstomo)

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Uma tese de Doutoramento está muito longe de ser uma concretização pessoal. Com efeito, o candidato é a pessoa central do Doutoramento, mas o esforço que condensa na sua dissertação transcende a abnegação, a audácia e a habilidade do candidato. Não seria assim justo apresentar o produto de cinco anos de trabalho sem primeiro introduzir e saudar todos aqueles e aquelas que, directa ou indirectamente, contribuíram para a concretização deste breve compêndio.

Em primeiro lugar não poderia deixar de nomear o meu Orientador, o Doutor Marco G. Alves, e o meu Coorientador, Doutor Pedro F. Oliveira, desde logo pela audácia em "resgatar--me" de volta à Ciência. Porventura serão os Professores a prova viva de que é necessária uma dose significativa de loucura para alimentar a criatividade que dita a genialidade. De outra forma será difícil explicar como me recrutaram para o então *Sertoli Cell Biology Lab* após tão desastrada e desastrosa entrevista! Lembro-me bem da minha desolação, em pleno Aeroporto de El Prat, em Barcelona, após a entrevista – sentia que havia perdido a minha última hipótese de regressar à Ciência. Por isso, quando o Prof. Pedro me ligou no dia seguinte, regressado ao escritório numa solarenga manhã de início de Agosto, não esperava outra coisa que não a confirmação de um espetacular falhanço. Não podia ter ficado mais incrédulo quando ele me comunicou que tinha sido seleccionado para o seu projecto. Mas naturalmente não me fiz rogado, e de imediato encetei os preparativos para a mudança de Lisboa para o Porto.

O mês que se seguiu dificilmente será repetível. Dificilmente reunirei novamente os factores que se juntaram naquele Agosto de 2016, e duvido que retenha a energia vital para reviver esses tempos do auge da juventude. E a isso, mas também à constante amizade, devo agradecer ao meu largo grupo de amigos em Lisboa, popularmente conhecido por "Casa do Povo". Certamente teria sido muito mais difícil suportar o meu desvio pela consultoria em tecnologias da informação sem comparsas ao meu nível, prontos para grassar as ruas de Lisboa! Ah, ainda me espanto como as paredes do nº 15 da Avenida Duque d'Ávila ainda se aguentam de pé! Já o ano que se seguiu, por outro lado, penso que estarei sempre em risco

de repetir enquanto prosseguir com a minha carreira académica, que espero duradoura.

Chegado ao Porto para ingressar num inovador programa Doutoral, vejo-me envolvido num tumultuoso embróglio institucional em que os lesados foram, como em todos os casos semelhantes, os alunos. Não tecerei outros comentários acerca deste período conturbado em que afinal a posição para a qual fui seleccionado acabou por não existir. Esta dissertação deve ser um momento de celebração e sinto que não devo nem expressar as minhas cogitações acerca de um processo que estou longe de conhecer em toda a sua dimensão, nem discutir matérias políticas de ciência e educação em Portugal. Porém, não poderia deixar de referir esta situação, até para prestar a justa homenagem à acção do Prof. Pedro e do Prof. Marco, que sempre me apoiaram ao longo desses tempos de incerteza com uma comunicação honesta e clara. Face ao inevitável e triste destino do EndoMet, quero aqui registar a minha gratidão para com o Prof. Marco, que reverteu o prémio monetário da bolsa "Nuno Castelo-Branco", concedida pela SPD em Março de 2017, para me suportar financeiramente durante este período. Talvez o Prof. Marco (ou simplesmente Marco, como ele me pediu) diga que fiz por o merecer ao trabalhar na submissão da candidatura, mas a minha honestidade intelectual não me permite omitir que foi ele a mente criativa deste projecto.

Honestamente, não há nada pelo qual possa estar mais grato ao Prof. Marco do que por me ter envolvido nesse projecto, em Março de 2017. Esse que seria em grande parte o projecto que nesta Dissertação é desenvolvido e que se enquadra num outro ainda maior, financiado pela FCT. É óbvio que encaro esta tese, e este projecto como "os meus projectos de Doutoramento", mas não posso deixar de fazer a devida referência ao Prof. Marco, enquanto Investigador Principal, mas também aos restantes colaboradores e co-autores das várias publicações científicas que dele resultaram. Devo referir o Prof. Pedro F. Oliveira e a Prof. Doutora Rachel L. Batterham que, tal como o Prof. Marco sublinhou por diversas vezes, contribuíram decisivamente para a conceptualização do desenho experimental. Tenho a agradecer ambos os meus co-orientadores, o Prof. Doutor João F. Raposo e o Doutor Pedro F. Oliveira, pelo apoio incondicional e pelos comentários sempre enriquecedores do trabalho agora concluído. Sem dúvida, as suas contribuições incrementaram o valor translaccional e científico deste trabalho, cada qual fazendo uso das suas reconhecidas competências.

Agradeço também ao Doutor Luís Rato, colega de curso que reencontrei no grupo, ainda que não tenhamos partilhado os mesmos espaços senão na altura dos sacrifícios animais. Invariavelmente discutimos vezes e vezes os resultados dos nossos estudos, mas o trabalho rotineiro, meticuloso e longo que leva à sua obtenção é facilmente remetido para segundo plano. Sem dúvida a dedicação do Luís é uma pedra basilar deste projecto; o seu trabalho na

manutenção e monitorização deste complexo modelo animal é por si só notável. Obrigado!

Falando dos modelos animais, não posso esquecer o auxílio impriscindível das agora Doutoras Tânia Dias, Raquel L. Bernardino e Maria João Meneses de Oliveira. Não é de todo justo circunscrever a sua colaboração aos tempos que passámos juntos na Covilhã, na nossa *alma mater*, auxiliando o Luís Rato, e ao tempo que passámos nos laboratórios do ICBAS. O seu papel começa tal como o do Luís e de tantos outros membros do *Sertoli Cell and Gamete Biology Lab* enquanto colegas na Licenciatura (e Mestrado) em Ciências Biomédicas da Universidade da Beira Interior. Sem quaisquer pretenções da minha parte, é notável observar a evolução de cada uma e de mim próprio ao longo deste anos de convivência. Não poderia deixar passar esta oportunidade para dedicar algumas palavras à Maria João, aquela cuja história mais vezes se cruzou com a minha. Haverá sempre um sentimento paradoxal quando aqueles que vemos iniciar depois de nós nos ultrapassam. Se por um lado pensamos que falhámos nalgum momento a vida, por outro sentimos uma alegria enorme por assister à ascensão da nova geração. Hoje parecemos estar mais longe, mas não creio que a Maria João seja uma daquelas pessoas que marcará uma década específica da minha vida. Tenho de assistir o próximo capítulo desta epopeia! À Raquel, que tão douta paciência teve comigo ao longo de todos estes anos, quero reafirmar que não houve uma linha que alguma vez tenha escrito sobre ela da qual me possa arrepender. Reconheço que o valor das minhas palavras possa ser questionável, principalmente as escritas, todavia sempre me limitei a escrever a minha verdade, e não a sonego. Há realidades que nunca caíram do universo das ideias, e ainda bem, porque apenas aí podem ser perfeitas. Muito obrigado a todas!

Ao Doutor Romeu A. Videira quero agradecer muito mais do que as técnicas que me ensinou. Enquanto trabalhei com ele aprendi o Mundo sob o seu olhar crítico, alicerçado numa lógica implacável e sólidas referências. Se há pessoas que levam uma vida de trabalho ímpar embora pouco reconhecido, o Romeu é um desses heróis por cantar. Com ele aprendi História e Política para lá de qualquer organização ou partido. Discutimos temas polémicos, tentei argumentar e desafiar a sua lógica com a minha, mas muito mais vezes senti-me em xeque. E por isso ouvi. Ouvi muito e atentamente. Porque há poucos tão cultos. E assim não posso deixar de referir o anjo do Romeu e de todos nós: a Ana Maria. Se o Romeu é implacável na sua lógica, a Ana Maria é infinita na sua bondade. Ela é o verdadeiro paladino, protectora de todos os que a rodeiam até ao último dos seus esforços. E isto para lá do seu inegável conhecimento... Obrigado a ambos!

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Quero ainda agradecer ao Prof. Doutor Mário de Sousa o qual manteve comigo sempre uma relação profissional e respeitosa. Enquanto foi co-orientador do plano de trabalhos que aqui publico enfim, procurou estar sempre a par da evolução das tarefas e prontificou-se a tecer os comentários necessários. O meu sincero agradecimento.

Embora não seja parte directa deste trabalho, quero ainda agradecer ao Prof. Doutor Pedro N. Oliveira, docente de Estatística do ICBAS e da FCUP, meu mentor principal nesta área crucial deste trabalho. Um dos conselhos que o Prof. Marco sempre me deu, e aos meus colegas, foi o de nos "tornarmos indispensáveis". Talvez meio por acaso, e outro meio por desafio, a Estatística e a Análise de Dados tornaram-se competências basilares do meu perfil científico. Hoje não sinto qualquer pendor negativo ao afirmar que me sinto mais à-vontade na análise e tratamento de dados do que na bancada do laboratório. Curioso, especialmente atendendo às dificuldades que tive quando desenvolvi o tema nas aulas de Matemática do Secundário – provavelmente necessitava de entender a aplicabilidade dos cálculos que então me transmitiam. Esse é o maior feito do Prof. Pedro N. Oliveira, a capacidade de transmitir como cada método, cada parâmetro, afecta as nossas decisões. E com base nesse conhecimento adquiri a confiança e a curiosidade para explorar mais e mais complexos métodos estatísticos. Muito obrigado!

Deixo para o fim o mais difícil e injusto dos agradecimentos. Difícil porque engloba um grande número de pessoas, e injusto porque cada uma delas merecia a sua própria menção. Quero com isto introduzir o meu agradecimento a todos os meus amigos e família. Espero que todos os visados saibam que facilmente seria capaz de lhes dedicar algumas palavras de apreço. Aos meus colegas de laboratório nestes últimos quase 6 (!) anos, quero agradecer a amizade, a entreajuda, a paciência (que teve de ser muita) e os doces e petiscos que energizaram as matérias cinzenta e branca. Confesso que desde que iniciei o caminho deste Doutoramento, sempre tive em mente que a etapa seguinte teria de ser noutro grupo de investigação, noutra instituição, noutro país. Porém, tenho a firme convicção que o *Sertoli Cell and Gamete*

Biology Lab tem o potencial humano para continuar a sua rota ascendente. Contrariamente ao que é popularmente apregoado há milénios, acredito que as gerações futuras irão sempre superar os seus antecessores! Aos meus amigos de sempre, das mais diversas origens, quero deixar um largo abraço que a todos possa embarcar. Esta viagem pelo conhecimento contou com todos eles e elas em alguma etapa, e comigo trago tudo o que aprendi com eles. No entanto, a viagem pelo conhecimento começa pela viagem pela vida; e essa não o era sem a família. Ao longo destes já 32 anos fiz os possíveis para aprender o máximo que cada um deles me tinha para ensinar. E fiz por ensinar o que aprendi aos (poucos) mais novos que foram alargando a família. Com esta tese, penso que serei o primeiro Doutor da família, um feito que sei que a todos enche de orgulho, não só pela minha concretização pessoal, mas pelo progresso incrível que alcançámos em apenas 3 gerações! E a isso devo, e devemos, aos valores que sempre apoiámos, e pelos quais lutámos. O que aqui concluo era impensável no país em que os meu avós nasceram, inalcançável no país em que os meus pais, tios e tias nasceram, mas possível no país em que nasci. E todos nós nascemos em Portugal. Por isso, quero deixar uma homenagem ao meu avô João Machado, que muito lutou para que este progresso fosse possível, mas infelizmente não viveu o suficiente para ver a sua concretização. Obrigado, obrigado a todos!

During the writing of the diverse papers included in this dissertation, I relied on the pivotal role of several international collaborators. Without them, I would not even have the results to write this very dissertation. I am truly grateful for their skills, knowledge, experience and friendship, for I have learnt a lot with every single one of them.

First among the international collaborators, I must thank Prof. Rachel L. Batterham of the University College London (United Kingdom), who acted almost as an unofficial co-supervisor from the beginning of my PhD project. Citing the words of Prof. Marco G. Alves, Prof. Rachel directly contributed to the experimental design that supports this project. She has always been a critical reviewer of all the papers we have published in the past 6 years, in the context of the present work. Her clinical views have significantly enriched the discussion of our results, deepening the translational potential of our research. I am also grateful for the trust she has demonstrated on my own skills, having invited me and Prof. Marco to join research projects led by Prof. Rachel. Thank you very much!

I have learnt with Ivana a broad spectra of things (*pun* definitely intended). Besides all the lessons on Nuclear Magnetic Resonance, I have learnt a lot about the history and culture of the former Yugoslavia - a country that still existed when I was born - and Croatia. From the

moment I joined the *Sertoli Cell and Gamete Biology lab*, I grew fonder of our friendship, the only foreigner member of our research group, despite she was not in our lab often. Of course, her nationality played an important role in our friendship, having lived so many adventures and having kept such beautiful memories of her country (and knowing some gibberish in Croatian). Fun fact, I was in the Balkans, more precisely in Belgrade, Serbia, former capital of Yugoslavia, when I was notified that I have been selected to the next application phase to join our research group. Thanks to Ivana and her recommendations, I have started a personal "Culture list", a list of must-see Books and Films from all sorts of genre and origins (I am still trying to check everything from that list!). We have also spoken long about the scars of wars that broke up the Great South Slavic Nation... I do not know if Ivana will endorse my statement, but after those History lessons I would say that "in war there is no one to blame, but everyone to be ashamed". And yet, we see war in Europe once again... Ivana, my dear friend, to you I say: "Hvala! Vidimo se!".

Thanks to Ivana, I have had the expert support of Doctor Kristina Starčević and Doctor Tomislav Mašek for the mass spectroscopy of fatty acids. Unfortunately, I did not have the opportunity to meet them personally, but I would like to use this opportunity to acknowledge their incredible contributions for this work. Hvala!

I also want to thank the "United Nations of Turku", the community of expats in Turku, Finland, that integrated me immediately upon my arrival, and since then I have also helped to expand beyond the walls of the Academia. I cannot hide how enthusiastic I was about moving abroad, but besides the "romanticism" of experiencing new realities, specially for a Portuguese still today a People of emigrants, there are practical challenges that are never told in any story. Particularly, I must highlight Matthieu Bourgery, who was responsibly for "stepping up my game" in Statistics with Bioinformatics. There are PhD students, and then there's Matthieu - a PhD student with more experience than many Postdocs. He has become my mentor in Linux and R, even if he says that he is not an expert, he just do what is needed to be done. So, I took his approach to learn too, although I would need one more year to learn how to analyse sequencing data without him! With Matthieu I can spend hours and evenings discussing about statistical methods and small RNAs, but also politics with just the same degree of scientific rigour. In the end, we are both truth-seekers. I hope we can soon celebrate Matthieu's PhD, so we can enjoy another wine and cheese session! Merci beaucoup! Also, I must thank Katriina (Katy) Viljanen and Kamila Puławska for their Finnish and Polish translations. This dissertation would not be so diverse without you! Thank you very much!

At last, I would like to address my thanks to all the friends have made along the way to complete this PhD thesis. I was lucky enough to have attended several scientific conferences, meetings and symposia all around Europe (and once in South Korea), where I met fantastic people, both young and acknowledged researchers, but also people that I met by chance in all those locations, who had nothing to do with Science. I am genuinely sorry for all the PhD students during pandemics, because of all the opportunities they had forcefully missed... I will not mention anyone in particular, even if I would like to. I kindly ask your forgiveness, restating that I will always be looking forward to host and guide you through my city, wherever it will be, as you have done for me. When I first wrote this paragraph, I have written that I truly believed in long-lasting peace for Europe. Well, I could not imagine how wrong I was... But I still believe that peace will be possible, and that one day we will have a truly united Europe, sharing the same democratic and humanistic values. I have met Ukrainians and Russians, I have met Croatians, Bosnians and Serbians, I have met people from all over Europe and everyone wanted peace. Unfortunately, when the "Great Powers" are on the move, it will be the people, the common innocent people, to bear the consequences. And war will open old scars, and perpetuate hatred... But the people I have met give me hope. I wish you can share this hope with me, so it may become true. Thank you!

Seuraavaksi, haluaisin osoittaa kunnioitustani arvostettuja suomalaisia työtovereitani kohtaan heidän omalla kielellä.

Aluksi haluaisin kiittää professori Ilpo Huhtaniemeä, sillä hän on ollut päävastuussa tutkimusvierailustani Turun yliopistossa. Olen haaveillut vuosia ulkomaille muutosta ja minulla on ollut mahdollisuus kokea erilainen työkulttuuri sekä arki uudessa ympäristössä. Esimieheni ovat aina kannustaneet tutkimusryhmämme jäseniä hakeutumaan kansainvälisten kokemusten pariin tohtorintutkinnon aikana, ja haluan kiittää heitä siitä. Harkitsimme monien paikkojen välillä ja lopulta Professori Ilpo ehdotti liittymistä Nooran ryhmään. Noora on hänen entinen mentoroitu, josta on tullut johtava asiantuntija omalla erikoistumisalallaan.

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touttamisessani Suomeen ja Turun yliopistoon, sekä hänen kärsivällisyydestä kouluttaa minua laboratoriossa. Tiedän, että niin monia muita suunnitelmia ei koskaan saavutettu pandemian takia. Kiitos!

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Kasiu,

Jak już zapewne wiesz, lubię zostawiać najlepsze na koniec, aby jak najdłużej cieszyć się smakiem. Do tej pory prawdopodobnie wiesz o mnie więcej niż ktokolwiek inny i, nawet po tym, postanowiłaś nie uciec ode mnie! W końcu nie jesteś ambitna, tylko skora do rywalizacji, dlatego potrzebujesz wyzwania takiego jak to od którego to wszystko się zaczęło.

Kompletnie Cię rozumiem. Podbój jest o wiele lepszy niż jakiekolwiek roszczenia. To również płynie w mojej krwi, jak byś powiedziała, w moim kolonialistycznym pochodzeniu. W końcu sam jestem ambitny i zrobiłem to samo. I to jest niesamowite. Długo żyliśmy na krawędzi aby z niej zeskoczyć i ruszyć wspólnie na podbój świata! Jak powiedziałem - a ty się zgodziłaś – jesteśmy produktem okoliczności. Jednak okoliczności się zmieniły. Sami postanowiliśmy je zmienić i jakoś razem to przetrwaliśmy.

Wiele lat temu, moja haniebna reputacja była garstką paskudnych historii. Mój (dość konserwatywny) znajomy szczerze zapytał mnie jak mógłbym kiedyś pogrzebać swoją przeszłość i odpuścić grzechy mojej duszy. Jak mógłbym pewnego dnia znaleźć kobietę która zniosłaby ciężar moich przebiegłych romansów? Obstawiam, że zadaje sobie to pytanie do dziś, jednak moja odpowiedź pozostaje taka sama: po prostu potrzebuję kogoś, kto mnie zrozumie. Ko-

goś, kto jest gotów dzielić ten ciężar i dzielić się ze mną swoim. Teraz czuję, że znalazłem tę kobietę, a nasze ciężary (i nasze błędy) mogą być fundamentem naszego szałasu!

Nie chcę zabrzmieć osądzająco, zwłaszcza wobec Ciebie. Jeśli kiedykolwiek byłem osądzający w tych sprawach, to nigdy nie dotyczyło to Ciebie, a najprawdopodobniej zupełnie odwrotnie! Jestem pewien, że tym razem się ze mną zgodzisz. Chociaż masz prawo się nie zgodzić. Jak mógłbym żyć bez dreszczyku emocji stawiania Ci wyzwań każdego dnia? W końcu każdy dzień jest zwycięstwem. I jesteśmy równi w naszych cudownych różnicach. Równie silnie potrzebuję Twojego ognia jak i Ty mojej cierpliwości. Równie potrzebuję twojej konfrontalności, jak ty potrzebujesz mojej łagodności. Potrzebuję Twojej opieki, jak ty swojej porannej kawusi. Bez tych, i innych, wymian pomiędzy nami, to równanie byłoby niekompletne.

Tak, ta praca doktorska wciąż miałaby wszystkie sekcje, rozdziały i załączniki, i spełniałaby wszelkie wymagania. Mógłbym złożyć gotowy produkt, ale jestem pewien, że brakowałoby mu duszy. Byłoby to po prostu kolejne kompendium, bez żadnego innego praktycznego pożytku oprócz uzyskania mojego tytułu, prawdopodobnie z powierzchowną dedykacją dla kogokolwiek musiałbym zadowolić w tym czasie. Ale Ty nadałaś jej nowego sensu. Teraz, ta praca jest także kluczem do nowego rozdziału życia, pełnego rozdziału po dwóch latach prowizorycznego życia w dziwnej rzeczywistości. Nasze życie dopiero się rozpocznie! Tu i teraz.

Publication list

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Abstract

The prevalence of non-communicable diseases has increased worldwide since the II World War. The improvement of socio-economic conditions and the technological advancements have promoted the adoption of unhealthy lifestyle choices associated with this trend. Among those, excess adiposity and obesity caused by the adoption of High-fat diets (HFDs) and low levels of physical activity is accountable as a major risk factor towards the onset of type 2 diabetes *mellitus* (T2D), one of the most prevalent non-communicable diseases.

Unhealthy lifestyle choices often start early in life, which has led to an increase in the prevalence of childhood obesity, and a decrease in the age of onset of T2D. The earlier onset of T2D has significant economical impact in health care systems as it is a manageable chronic disease, but it is also a major cause of premature death and loss of healthy years. Recent reports rose awareness about the impact of obesity and T2D in fertility, particularly in males. In fact, sperm quality was dwindled while the prevalence of T2D increased. A healthy male reproductive function relies on a tightly regulated nutritional and hormonal balance, which is altered in non-communicable metabolic diseases such as obesity and T2D.

The first objective of the research compiled in this dissertation was to describe the long-term effects of childhood obesity caused by the adoption of HFD from early age to metabolic and reproductive health in males. Within this objective, we have further studied the efficacy of dietary intervention in early adulthood to avert the potential negative effects of HFD and associated excess adiposity during childhood later in life, compared to life-long HFD or life-long balanced diet. The second aim of this project was to describe the inter- and transgenerational effects of ancestral exposure to excess adiposity caused by HFD in the overall metabolic and hormonal homeostasis, testicular metabolism and sperm parameters of the unexposed progeny. The third objective of this work was to study the impact of HFD-related excess adiposity on the sperm small non-coding RNA (sncRNA) content of the exposed subjects and their unexposed progeny. Bringing together the results of those three objectives, we sought after the overarching objective of eliciting molecular mechanisms of paternal epigenetic inheritance of HFD-related changes that could be associated with the

decline of sperm quality.

To do so, we have designed a transgenerational rodent model of ancient exposure to HFD, consisting in three generations of C57BL6/J mice (F_0: exposed founders – F_1: unexposed offspring – F_2: unexposed grand-offspring). Immediately after weaning (21–23 days), the founders (n = 36) generated from normoponderal progenitors were randomly and evenly distributed into three experimental groups (n = 12): standard chow for 200 days (CTRL), high-fat diet for 200 days (HFD), and high-fat diet for 60 days then replaced by standard chow for 140 days (HFD_t). F_0 mice were mated at 120 post-weaning age with same-age, normoponderal females to generate the F_1 unexposed offspring. Mating lasted for 8 days and consisted of placing a male and a female in the same cage for 6 hours each day, without water or food supply. After weaning, the resulting offspring (F_1) were assigned to the same experimental group as their fathers (n = 12 per group), but all animals received the same diet: standard chow for 200 days. The mating scheme was repeated with the unexposed F_1 mice to generate the grand-offspring (F_2), which were assigned to experimental groups and were feed similarly to F_1 mice.

All mice had unrestricted access to water and assigned diet, except during mating. Food and water consumption, and body weight were monitored throughout the experiment. Mice were euthanized by cervical dislocation 200 days post-weaning age, and tissues collected and weighed. Epididymal sperm was collected, and sperm parameters evaluated (concentration, motility, viability and morphology). Glucose homeostasis was evaluated within one week before euthanization. Serum was purified from blood and serum hormones quantified using ELISA. Testis were processed and several quantifications performed: Lipid peroxidation was performed using thiobarbituric acid reactive species (TBARS) assay; mitochondrial complex and antioxidant enzyme activity were measured using colorimetric and fluorometric methods; adenosine nucleotides were quantified using high-performance liquid chromatography (HPLC); metabolites were identified and quantified using ^{1}H-NMR, fatty acids were measured using GC-MS; and sperm sncRNA sequences were counted using Next Generation RNA-sequencing.

The adoption of HFD after weaning rapidly increased the adiposity and the body weight of mice. The reversion of HFD to a balanced diet, in early adulthood, quickly reduced body weight to a similar level of the CTRL mice, exclusively fed with standard chow. This lifestyle intervention was also effective to prevent the onset of metabolic syndrome later in life, evaluated by glucose and insulin stress tests. Also, no changes were found between CTRL and HFD_t mice concerning serum hormones (insulin, follicle-stimulating hormone

(FSH), luteinizing hormone (LH), testosterone and estrogen), testicular antioxidant enzyme activity and mitochondrial function. However, the testicular metabolome and lipidome of mice fed with HFD, even if restricted to childhood, displayed several changes compared to CTRL. The metabolites with most relevant differences were associated with energy-obtaining pathways, antioxidant response, membrane turnover and inflammatory process. Moreover, sperm viability, motility and morphology were irreversibly affected by HFD, and those sperm defects were correlated with the changes found in testicular metabolome and lipidome. In fact, a multivariate analysis combining sperm parameters, testicular metabolites and fatty acids show a clear separation of samples according to diet, evidencing the establishment of a specific testicular metabolic fingerprint in response to diet/adiposity. Interestingly, differences in sperm sncRNAs were only observed between CTRL and HFD_t, and mostly in sncRNAs originating from mitochondria, suggesting a role of this organelle in the reversion of the negative effects of HFD in the male reproductive tract.

Overall, a limited number of differences were found in any of the parameters monitored in the offspring of the exposed mice (Generation F_1). Despite the absence of clear phenotypic traits of HFD, multivariate analysis of testicular metabolites and fatty acids still revealed a testicular metabolic fingerprint of ancestral diet in the unexposed offspring. Besides, sperm sncRNA content was substantially changed between the offspring of mice fed with HFD, HFD_t or CTRL. Among those changes, repeat-derived small RNAs (repRNAs) and piwi-interacting RNAs (piRNAs) were the classes with more differently expressed sequences. Both sncRNA classes are associated with the transcriptional repression of retrotransposons during meiosis, in spermatogenesis. Interestingly, the grand-offspring (Generation F_2) of mice fed with HFD, even if transiently, had lower sperm counts than the grand-offspring of CTRL mice, and the HFD-associated testicular metabolic fingerprint was more evident than in the previous generation. This epigenetic inheritance phenomenon, coined by us as "inherited metabolic memory", transmitted features of pro-inflammatory environment and insulin resistance even two generations after exposure to HFD and associated adiposity. However, sperm sncRNA content had a limited amount of differently expressed sequences, and all of them microRNAs (miRNAs), suggesting that different sncRNAs biotypes have different roles in epigenetic inheritance of acquired traits.

Our results demonstrate that ancestral exposure to HFD-related excess adiposity cause irreversible changes to testicular metabolism and function, which are correlated to sperm quality. We further show that those changes can be inherited by the unexposed offspring and grand-offspring, although the HFD phenotype may not be expressed in terms of metabolic

health or sperm parameters. We also demonstrate that sperm sncRNA content dynamically changes in response to HFD/adiposity, and may play a role in the "inherited metabolic memory" of ancestral HFD. These findings have a significant societal impact, raising awareness about the impact of our own lifestyle to the health of our progeny.

Resumo

A prevalência das doenças não-comunicáveis tem aumentado em todo o mundo desde a 2ª Grande Guerra. A melhoria das condições socioeconómicas aliada aos avanços tecnológicos promoveu a adopção de hábitos e estilos de vida pouco saudáveis que têm sido associados a esta tendência. Entre estes, o excesso de adiposidade e a obesidade causados pela adopção de dietas ricas em gordura (HFD) e por baixos níveis de actividade física são considerados dos maiores factores de risco para o desenvolvimento de diabetes tipo 2 (T2D), uma das doenças não-comunicáveis mais prevalentes no mundo.

Os hábitos pouco saudáveis começam frequentemente na infância, o que tem levado ao aumento da prevalência de obesidade infantil, e ao aparecimento de T2D em idades cada vez mais jovens. O diagnóstico precoce de T2D acarreta um impacto económico acrescido para os sistemas de Saúde, pois trata-se de uma doença crónica mas controlável, para além de ser uma das causas principais para morte e incapacidade prematuras, e para perda de anos de saúde. Estudos recentes alertam ainda para o impacto da T2D na fertilidade humana, em particular nos homens. De facto, a qualidade espermática tem piorado à medida que a prevalência de T2D aumenta. Uma saudável função reprodutiva masculina depende num rigoroso equilíbrio nutricional e hormonal, o qual é perturbado em doenças metabólicas não comunicáveis como é o caso da obesidade e T2D.

O primeiro objectivo da investigação compilada nesta dissertação é a descrição dos efeitos a longo-prazo da obesidade infantil associada à adopção de HFD na infância, na função reprodutiva masculina. Neste contexto, procurámos ainda estudar a eficácia de uma intervenção alimentar no início da idade adulta como forma de prevenção dos potenciais efeitos negativos da HFD e adiposidade excessiva durante infância e adolescência, numa fase posterior da idade adulta, por oposição à adopção de uma HFD ou de uma dieta equilibrada ao longo de toda a vida. Em segundo lugar, procurámos descrever os efeitos inter-e transgeracionais da exposição ancestral a adiposidade excessiva causada por HFD na homeostasia hormonal e metabólica gerais, no metabolismo testicular e nos parâmetros espermáticos da descendência não exposta. O terceiro objectivo deste trabalho foi estudar o impacto da adiposidade ex-

cessiva associada à HFD no conteúdo espermático de *small non-coding* RNA (sncRNA) em indivíduos expostos e na sua descendência não exposta. Partindo destes objectivos, pretendemos desvendar os mecanismos moleculares responsáveis pela herança epigenética paternal de alterações associadas com a adopção HFD, e que podem estar relacionadas com o declínio da qualidade espermática.

Para tal, desenhámos um modelo animal de exposição ancestral à HFD, consistindo em três gerações de ratinhos C57BL6/J (F_0: fundadores expostos – F_1: filhos não-expostos – F_2: netos não-expostos). Imediatamente após o desmame (21–23 dias), os fundadores (n = 36) gerados a partir de progenitores normoponderais foram aleatória e uniformemente distribuídos por três grupos experimentais (n = 12): dieta equilibrada durante 200 dias (CTRL); dieta rica em gordura durante 200 dias (HFD); e dieta rica em gordura durante 60 dias, então substituída por dieta equilibrada durante 140 dias (HFD_t). Os ratinhos F_0 acasalaram com fêmeas da mesma idade e normoponderais aos 120 dias de idade pós-desmame para gerar os filhos não-expostos F_1. O acasalamento foi repetido ao longo de 8 dias, consistindo em colocar um macho e uma fêmea na mesma jaula durante 6 horas por dia, sem qualquer acesso a comida ou bebida. Após desmame, os filhos resultantes (F_1) foram alocados aos mesmos grupos experimentais dos pais (n = 12 por grupo), mas todos os animais foram alimentados com a mesma dieta: dieta equilibrada ao longo de 200 dias. O esquema de acasalamentos foi repetido com os ratinhos não-expostos F_1 para gerar os netos não-expostos (F_2), os quais foram alocados aos grupos experimentais e alimentados de igual forma aos filhos não-expostos F_1.

Com excepção do período de acasalamento, todos os ratinhos gozaram de acesso ilimitado a água e à dieta assignada. O consumo de água e de comida, assim como o peso corporal foram monitorizados ao longo da experiência. Os ratinhos foram eutanasiados por deslocamento cervical 200 dias após desmame, e os tecidos recolhidos e pesados. Recolhemos espermatozoides epididimais e avaliámos os parâmetros espermáticos (concentração, motilidade, viabilidade e morfologia). A homeostase da glicose foi avaliada durante a semana anterior à eutanásia dos ratinhos. Obtivemos soro a partir do sangue total para quantificação hormonal através de ELISA. Os testículos foram processados e usados para diversas quantificações: peroxidação lipídica através do ensaio TBARS; actividade dos complexos mitocondriais isolados e das enzimas antioxidantes através de métodos colorimétricos e fluorimétricos; nucleótidos de adenosina através de HPLC; identificação e quantificação de metabolitos por ^{1}H-NMR, medição de ácidos gordos por GC-MS; e contabilização de sequências de sncRNA espermáticos através de *Next Generation RNA-sequencing*.

A adopção de HFD após desmame promove o aumento rápido da adiposidade e do peso corporal dos ratinhos. A reversão de HFD para uma dieta equilibrada no início da idade adulta depressa reduz o peso corporal ao nível do peso dos ratinhos CTRL, alimentados exclusivamente com dieta padrão. Esta intervenção no estilo de vida é ainda eficaz na prevenção da síndrome metabólica em idade mais avançada, avaliada através de testes de stress à glucose e à insulina. Além disso, não foram verificadas diferenças entre ratinhos CTRL e HFD_t na concentração sérica das hormonas testadas (insulina, FSH, LH, testosterona e estrogénio), na actividade das enzimas antioxidantes testiculares e na função mitocondrial. Contudo, o metaboloma e o lipidoma testiculares de ratinhos alimentados com HFD, mesmo se circunscrita à infância, apresentaram várias diferenças em relação aos ratinhos CTRL. Os metabolitos associados às diferenças mais relevantes estão relacionados com as vias de obtenção de energia, com a resposta antioxidante, com o *turnover* membranar e com o processo inflamatório. Para além disso, a viabilidade, motilidade e morfologia espermáticas foram irreversivelmente afectadas pela exposição a HFD, e estes defeitos correlacionam-se com as alterações observadas no metaboloma e lipidoma testiculares. Na verdade, uma análise multivariada combinando parâmetros espermáticos, e metabolitos e ácidos gordos testiculares demonstra uma clara separação das amostras em função da dieta adoptada, evidenciando o estabelecimento de uma assinatura metabólica testicular específica em resposta à dieta/adiposidade. Curiosamente, apenas foram observadas diferenças no conteúdo espermático de sncRNAs entre CTRL e HFD_t, sobretudo em sncRNAs originários da mitocôndria, o que sugere um papel deste organelo na reversão dos efeitos negativos da HFD no tracto reprodutor masculino.

No geral, as diferenças encontradas entre os filhos dos ratinhos expostos (Generação F_1) foram limitadas, independentemente do parâmetro avaliado neste estudo. Apesar da ausência de traços fenotípicos evidentes relacionados com a exposição a HFD, a análise multivariada dos metabolitos e dos ácidos gordos testiculares revela ainda sinais da assinatura metabólica testicular desta exposição ancestral nos filhos não -expostos. Além disso, o conteúdo espermático de sncRNA foi substancialmente alterado entre os filhos não-expostos dos ratinhos alimentados com HFD, HFD_t ou CTRL. Entre estas diferenças, repRNAs e piRNAs foram as classes o maior número de sequências diferencialmente expressas. Ambas as classes de sncRNA actuam na inibição transcricional dos retrotransposões durante a meiose, no decurso da espermatogénese. Curiosamente, os netos não-expostos (Generação F_2) dos ratinhos alimentados com HFD, mesmo se temporariamente, obtiveram menores contagens espermáticas que os netos dos ratinhos CTRL, e a assinatura metabólica testicular associada à exposição a

HFD foi mais evidente que na geração anterior. Este fenómeno de herança epigenética, o qual denominámos por *"memória metabólica herdada"*, transmitiu características de um ambiente pró-inflamatório e de resistência à insulina mesmo duas gerações após a exposição à HFD e à adiposidade associada. Ainda assim, o conteúdo espermático de sncRNA apresentou um número reduzido de sequências diferencialmente expressas, e todas essas classificadas como miRNAs, sugerindo que diferentes biotipos de sncRNAs desempenham diferentes funções na herança epigenética de traços adquiridos.

Os nossos resultados demonstram que a exposição ancestral a adiposidade excessiva causada pela adopção de HFD conduz a alterações irreversíveis no metabolismo e função testiculares, as quais se correlacionam com a qualidade espermática. Demonstramos ainda que essas alterações podem ser herdadas pela descendência não-exposta (filhos e netos), embora o fenótipo associado à HFD possa não ser expresso em termos de saúde metabólica ou de parâmetros espermáticos. Demonstramos ainda que o conteúdo espermático de sncRNA é alterado dinamicamente em resposta a HFD/adiposidade, e pode ter um papel importante na *"memória metabólica herdada"* pela exposição ancestral à HFD. Estes resultados têm ainda um impacto societal importante, através da sensibilização para as consequências das nossas escolhas de estilos de vida sobre a saúde da nossa descendência.

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List of Abbreviations

Symbole | A | B | C | D | E | F | G | H | I | K | L | M | N | O | P | Q | R | S | T | U | W

Symbole

^{1}H-NMR proton Nuclear Magnetic Resonance

3OHBut 3-hydroxybutyrate

A

a.u. area units

AA amino acid

ACSL6 acyl-CoA synthetase isoform 6

ADP adenosine diphosphate

AMP adenosine monophosphate

AMPK AMP-activated protein kinase

AMPKK AMPK upstream kinase

ANOVA Analysis of Variance

AQGP aquaglyceroporin

AQP aquaporin

ARA arachidonic acid

ARRIVE Animal Research: Reporting of *In Vivo* Experiments

ART assisted reproductive techniques

ATP adenosine triphosphate

AUC Area Under the Curve

B

BAT brown adipose tissue

BCA bicinchoninic acid

BCAA branched-chain amino acid

bFGF basic fibroblast growth factor

BHT 2,6-ditertbutyl-4-methylphenol

BMI Body Mass Index

BPA bisphenol A

BTB blood-testis barrier

C

cAMP cyclic adenosine monophosphate

Casp3 caspase-3

CAT catalase

CFTR Cystic Fibrosis transmembrane conductance regulator

CI mitochondrial complex I

CICS Research Center in Health Sciences

CII mitochondrial complex II

CIII mitochondrial complex III

CIV mitochondrial complex IV

CNT concentrative nucleoside transporter

CPT1 carnitine palmitoyltransferase 1

CS citrate synthase

CTRL Control

CV mitochondrial complex V

CytC cytochrome c

D

D4D Δ4-desaturases

D5D Δ5-desaturases

D6D Δ6-desaturases

DCPIP 2,6-dichlorophenolindo-phenol

DE differential expression

DEPC diethyl pyrocarbonate

DGAV Direcção-Geral da Alimentação e Veterinária

DHA docosahexaenoic acid

DI Disposition Index

DM diabetes *mellitus*

DMT1 divalent metal transporter 1

DNA deoxyribonucleic acid

DPA docosapentaenoic acid

DR diet reversion

DTNB 5,5-dithio-bis-(2-nitrobenzoic acid)

E

E_2 17β-estradiol

EDA Exploratory Data Analysis

EFSD European Foundation for the Study of Diabetes

EGCG epigallocatechin-3-gallate

ELISA enzyme-linked immunosorbent assay

ENT equilibrative nucleoside transporter

EtAm ethanolamine

ETC electron transport chain

F

FA fatty acid

FC Fold Change

FCT Fundação para a Ciência e a Tecnologia

FDR False Discovery Rate

FSH follicle-stimulating hormone

FSHR follicle-stimulating hormone receptor

G

G3P glycerol-3-phosphate

GC-MS Gaseous Chromatography coupled with Mass Spectometry

GLM Generalized Linear Model

GLP-1 glucagon-like peptide 1

GLUT glucose transporter

GnRH gonadotropin-releasing hormone

GO Gene Ontology

GPAT1 glycerol-3-phosphate-acyltransferase 1

GPC glycerophosphocholine

GPx glutathione peroxidase

GSH glutathione

GSI gonadossomatic index

GSR glutathione-disulfide reductase

GSSG glutathione disulfide

H

HBSS Hank's balanced salt solution

HDI Human Development Index

HFD High-fat diet

HFD_t transient High-fat diet

HIF hypoxia-inducible factor

HMDB Human Metabolome Database

HOMA2 Homeostasis Model Assessment 2

HOMA2-%B Homeostasis Model Assessment 2 - β-cell function index

HOMA2-%S Homeostasis Model Assessment 2 - glucose sensitivity index

HOMA2-IR Homeostasis Model Assessment 2 - Insulin resistance index

HPG hypothalamus-pituitary-gonadal

HPLC high-performance liquid chromatography

HRE hypoxia response element

HSD Honest Significant Difference

I

ICBAS Instituto de Ciências Biomédicas Abel Salazar

ICSI Intracytoplasmatic Sperm Injection

IDF International Diabetes Federation

IL-1 interleukin-1

ipGTT intraperitoneal Glucose Tolerance Test

ipGTT intraperitoneal Insulin Tolerance Test

IQR interquartile range

K

KMO Kaiser-Meyer-Olkin Measure of Sample Adequacy

KO knockout

KS Kolmogorov-Smirnoff

L

LC Leydig Cell

LCAD long-chain 3-hydroxyacyl-CoA dehydrogenase

LD lipid droplet

LDH lactate dehydrogenase

LH luteinizing hormone

LINE Long Interspersed Nuclear Element

M

MAPK Mitogen-activated protein kinase

MC myoid cell

MCAD medium-chain 3-hydroxyacyl-CoA dehydrogenase

MCT monocarboxylate transporter

MDA malondialdehyde

MeSH Medical Subject Headings

miRNA microRNA

MMPC NIH Mouse Metabolic Phenotyping Center

mRNA messenger RNA

MSI Metabolomics Standards Initiative

mtRNA mitochondrial tRNA-derived small RNA

MUFA monounsaturated fatty acid

N

NADH nicotinamide adenine dinucleotide

NADPH nicotinamide adenine dinucleotide phosphate

NAFLD non-alcoholic fatty liver disease

NBT nitro blue tetrazolium

NGS Next Generation Sequencing

NIH The US National Institutes of Health

O

ORBEA Órgão Responsável pelo Bem-estar dos Animais

OXPHOS Oxidative Phosphorylation

P

PC principal component

PCA principal components analysis

PChol phosphocholine

PERMANOVA Permutational Multivariate Analysis of Variance

PEtAm phosphoethanolamine

PI Pathway Impact

piRNA piwi-interacting RNA

PLS-DA Projection of Latent Structures with Discriminant Analysis

PPAR peroxisome proliferator-activated receptor

PPP pentose phosphate pathway

PUFA polyunsaturated fatty acid

Q

qRT-PCR quantitative Reverse Transcriptase Polymerase Chain Reaction

R

RA retinoic acid

repRNA repeat-derived small RNA

RIN RNA integrity number

RNA ribonucleic acid

ROS reactive oxygen species

RPM Reads per Million

rRNA ribossomal RNA

rRNA transfer RNA

rsRNA rRNA-derived small RNA

S

SC Sertoli Cell

SD standard deviation

SEM standard error of the mean

SeT seminiferous tubules

SFA saturated fatty acid

SGP slow growth period

SHBG sexual hormone binding globulin

SINE Short Interspersed Nuclear Element

SLC9 solute carrier 9 family members

SMPDB The Small Molecule Pathway Database

sncRNA small non-coding RNA

snRNA spliceosomal small nuclear RNA

SOD copper-zinc superoxide dismutase

SPD Sociedade Portuguesa de Diabetologia

sPLS Projection of Latent Structures

sPLS sparse Projection of Latent Structures

sPLS-DA sparse Projection of Latent Structures with Discriminant Analysis

sprRNA signal recognition particle RNA

SR-BI class B scavenger receptor type I

T

T1D type 1 diabetes *mellitus*

T2D type 2 diabetes *mellitus*

TAG triacylglyceride

TBA thiobarbituric acid

TBARS thiobarbituric acid reactive species

TCA tricarboxylic acid

TCAA trichloroacetic acid

TFA trans fatty acid

TfR1 transferrin receptor 1

Thr threonine

tiRNA transcription initiation RNA

tRF tRNA-derived fragment

tsRNA tRNA-derived small RNA

U

UCSC University of California Santa Cruz

UMIB Unidade Multidisciplinar de Investigação em Biomedicina

W

WAT white adipose tissue

WHO World Health Organization

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Chapter 1

Introduction

1.1 Molecular mechanisms and signalling pathways involved in the nutritional support of spermatogenesis by Sertoli cells

This section was partially reproduced from the published work:

Crisóstomo, **L.**, Alves, M. G., Gorga, A., Sousa, M., Riera, M. F., Galardo, M. N., Meroni, S. B., & Oliveira, P. F. (2018). Molecular mechanisms and signalling pathways involved in the nutritional support of spermatogenesis by Sertoli cells. In M. G. Alves & P. F. Oliveira (Eds.), *Sertoli cells - methods and protocols* (1st, pp. 129–155). Humana Press. https://doi.org/10.1007/978-1-4939-7698-0

The integral text is available in the Annex A of this Dissertation.

Abstract

Sertoli Cells (SCs) play a central role in spermatogenesis. They maintain the blood-testis barrier, an essential feature of seminiferous tubules which creates the proper environment for the occurrence of the spermatogenesis. However, this confinement renders germ cells almost exclusively dependent on SCs' nursing function and support. Throughout spermatogenesis, differentiating sperm cells become more specialized, and their biochemical machinery is insufficient to meet their metabolic demands. Although the needs are not the same at all differentiation stages, SCs are able to satisfy their needs. In order to maintain the seminiferous tubule energetic homeostasis, SCs react in response to several metabolic stimuli, through signaling cascades. The AMP-activated kinase, sensitive to the global energetic status; the hypoxia-inducible factors, sensitive to oxygen concentration; and the peroxisome proliferator-activated receptors, sensitive to fatty acid availability, are pathways already described in SCs. These cells' metabolism also reflects the whole-body metabolic dynamics. Metabolic diseases, including obesity and T2D, induce changes that, both directly and indirectly, affect Sertoli cell function and, ultimately, (dys)function in male reproductive health. Insulin resistance, increased estrogen synthesis, vascular disease, and pubic fat accumulation are examples of metabolic-related conditions that affect male fertility potential. On the other hand, malnutrition can also induce negative effects on male sexual function. In this chapter, we review the molecular mechanisms associated with the nutritional state and male sexual (dys)function and the central role played by the SCs.

1.1.1 Introduction

The reproductive system is the only system unnecessary for the survival of an individual of any species. Nevertheless, a considerable amount of energy is directed toward the reproductive functions. Under an evolutionary perspective, this fact is easy to understand, since (sexual) reproduction is the engine for any species to prevail and succeed, inducing genetic variance and the natural selection of the most advantageous characteristics

Known as "nurse cells" (Rato, Alves, Socorro, Duarte, et al., 2012), the Sertoli Cells play a decisive role on the nutritional support of the male germline during the processes of differentiation, particularly to fulfill their energetic needs. To achieve this, SC metabolism is tightly regulated by a complex network of endocrine and paracrine stimuli, as well as by environmental cues. In addition, the role of SCs is not restricted to the nutritional support of spermatogenesis; they also provide physical support for the differentiating germ cells, regulate and maintain the blood-testis barrier (BTB), and ensure the homeostasis of the tubular fluid in the lumen of the seminiferous tubules (SeT).

The SCs were firstly described by Enrico Sertoli in 1865, when he had just graduated in Medicine at the University of Pavia, Italy (Griswold, 1998). On his original work, Enrico Sertoli named his newly identified cells as "branched cells" due to their peculiar appearance, with many cytoplasmic projections surrounding germ cells (Sertoli, 1865). Sertoli was able to notice with a regular optic microscope and a limited number of samples (check original again) several other histological details and cellular ultrastructure, such as the large nucleus (Sertoli, 1865). SCs would only get their current designation 22 years later, by the Austrian histologist Victor von Ebner (Ebner, 1887). In spite of the immediate recognition of SC research potential, bibliography about them is scarce until the mid-1950s, when electronic microscopy and new biochemistry techniques, such as immunohistochemistry and immunocytochemistry, became available and after the discovery of DNA and RNA structure (França et al., 2016). Since then, the number of journal articles has been steadily increasing, to the present 400 articles per year.

This chapter focuses on the molecular mechanisms and the signaling pathways responsible for SC metabolic regulation and, thus, regulation of the environment for proper germ cell development and differentiation.

1.1.2 Relevance of Sertoli Cells to Spermatogenesis

There is still some debate concerning the specific roles for SCs, but they are usually reported as the main supporters of spermatogenesis (Griswold, 1998; P. F. Oliveira & Alves, 2015a; Rato, Alves, Socorro, Duarte, et al., 2012). This evidence is supported by three experimental premises. Firstly, SCs are always present in the testis, whereas there is the SC-only condition where the testis does not present any germ cells (Rebourcet et al., 2014). When SC population suffers significant loss by any cause, germ cells are quickly lost, too (Rebourcet et al., 2014). This observation is further confirmed *in vitro*, where germ cells struggle to develop and thrive in the absence of SCs, and spermatogenesis has not been fully achieved in culture, while the closest attempts to mimic it are always based on co-cultures with SCs (França et al., 2016; Griswold, 1998; Nagano et al., 1998; Xie et al., 2015; Zanganeh et al., 2013).

Secondly, there is evidence that the number of germ cells supported by a single SC is limited and species-dependent; hence, the number of germ cells is directly proportional to the number of SCs (França et al., 2016; Griswold, 1998; L. Johnson et al., 2008; Schlatt & Ehmcke, 2014). Experimental evidence comes from studies where the number of SCs was controlled and the spermatogenic output quantified (Griswold, 1998; L. Johnson et al., 2008; Orth et al., 1988; Orth et al., 1984; Simorangkir et al., 1995). When SC numbers were

restricted by inhibition of proliferation during testicular development, either by hormonal or chemical action, spermatogenic output and testicular volume decreased, also illustrating that the number of SCs significantly contributes to a normal testicular volume (Orth et al., 1988). When the procedure was hemicastration (ablation of a testis) during the development phase, the remaining testis had bigger volume and spermatogenic output (Orth et al., 1984; Simorangkir et al., 1995). However, in both experimental approaches, the SC-spermatid ratio remains constant (Griswold, 1998). The direct relation between spermatogenic output and SC number is further supported by evidence that, in some species, adult SC proliferation occurs at the breeding season to increase their spermatogenic output, but, in humans, SCs have limited mitotic capacity *in vivo* (L. Johnson et al., 2008).

Lastly, it is observed that spermatogenesis is highly dependent upon hormonal regulation from testosterone and FSH, and those hormones only exert their action directly over SCs, not germ cells (França et al., 2016; Griswold, 1998). Experimental evidence relies in several *in vitro* and *in vivo* models. FSH and thyroid hormones mediate SC proliferation during testis development, thus affecting testis size, weight, and spermatogenic output (Schlatt & Ehmcke, 2014). In addition, there were several gene mutations identified in the subunit of follicle-stimulating hormone receptor (FSHR) of SCs, which resulted in dysfunctional protein and infertility in men (Layman et al., 2002; Schlatt & Ehmcke, 2014). The affected individuals presented small testicular size and azoospermia (Layman et al., 2002; Schlatt & Ehmcke, 2014). It is evidenced *in vivo* that the expansion of germ cell reserve is FSH-dependent rather than androgen-dependent but always primarily mediated by SC action (Arslan et al., 1981; Griswold, 1998). Furthermore, differentiation from germ cells to round spermatids by FSH stimulation is possible, but its yield is only about 5 % of the normal, SC-mediated, yield (Schlatt & Ehmcke, 2014).

The advances on SC research now elicit that their critical role on spermatogenesis is related, but not limited, to germ cell physical support, environmental control, BTB maintenance and modulation, tubular fluid dynamics, endocrine and paracrine regulation, biochemical stimulation, and germ cell nutritional support (Alves, Martins, Rato, et al., 2013; Dimitriadis et al., 2015; Griswold, 1998; P. F. Oliveira & Alves, 2015a; P. F. Oliveira et al., 2015; Rato, Alves, Socorro, Duarte, et al., 2012; Rato et al., 2016).

SCs ensure the physical support to male germline cells due to a very distinguishable feature: long and thin cytoplasmic arms, similar to branches (as observed by Enrico Sertoli) (Sertoli, 1865). These elongations create a cuplike form to hold germ cells, dramatically increasing the membrane surface area and creating stage-specific microenvironments for germ

cells throughout their differentiation (França et al., 2016). However, germ cells are not only stabilized by it but also gain protection and other important features. Another significant and singular characteristic is the apical ectoplasmic specialization, a cell-cell adherent junction based on actin filaments, responsible for the germ cell movement and docking along the SC's membrane (França et al., 2016). In a larger extent, SCs are the very nature of the seminiferous tubule epithelium, since their polarization and side-by-side placement create a tubular structure and two distinct mediums (Wong & Cheng, 2005).

SCs are responsible for the maintenance of the two-compartment system found in the testis, which is a critical condition for spermatogenesis (P. F. Oliveira & Alves, 2015a). The BTB separates the interstitial fluid, which surrounds peripheral cells, and the tubular fluid, which fills the lumen of the seminiferous tubules (P. F. Oliveira & Alves, 2015a). *In vitro* studies have demonstrated that human stem cells are able to differentiate into spermatogonia in a SC-conditioned culture medium (Geens et al., 2011; Xie et al., 2015), a strong evidence of the environment importance for spermatogenesis but also of a significant contribution from SCs, rather than from germ cells, in this process. The SCs maintain tubular fluid homeostasis by guaranteeing the integrity of BTB and regulating its composition (Bernardino et al., 2016; Bernardino et al., 2013; Martins et al., 2014; Rato, Alves, Socorro, Duarte, et al., 2012). Due to the fact that SC is highly polarized, SCs are the most important structure of the BTB, since its basal membrane is directed toward the interstitial fluid, while its apical membrane is directed toward the seminiferous tubule lumen (Janecki & Steinberger, 1986). Tubulobulbar complexes are another SC peculiar feature. They are another anchoring system between cells at the BTB that works similarly to a puzzle: cytoplasmic elongations of a SC, ending on a spherical structure containing a cistern-like complex related to the endoplasmic reticulum, fit into corresponding invaginations on the adjacent cell (França et al., 2016; Vogl et al., 2013). This cell junction is further reinforced by the action of other anchoring systems present in these cells (Cheng & Mruk, 2002; Wong & Cheng, 2005). The tubular complexes at the basal region, tight junctions, gap junctions, and desmosome-like junctions, between SCs are responsible for making this blood-tissue barrier even more impermeable (Cheng & Mruk, 2002; Wong & Cheng, 2005). SCs dock themselves through hemidesmosomes to the basal lamina, the physical border of seminiferous tubules consisting in the basement membrane, a collagen network, and a layer of peritubular myoid cells (MCs) (P. F. Oliveira & Alves, 2015a; Rato, Alves, Socorro, Duarte, et al., 2012). The apical Tubulobulbar complexes mainly promote interaction with germ cells, in late maturation stages (spermatid), and are thought to be crucial for spermiation in the cytoplasmic reduction and mature spermatozoa

release processes (Wong & Cheng, 2005).

The most important endocrine mechanism mediated by SCs is its role on the reproductive axis which is the central regulator for the reproductive function. It starts at the beginning of puberty, with the pulsatile release of gonadotropin-releasing hormone (GnRH). GnRH neurons secreting GnRH are derived from the nasal placode and migrate into the brain during prenatal development. Once within the brain, GnRH neurons become integral components of the hypothalamic-pituitary-gonadal axis, essential for reproductive maturation and maintenance of reproductive function in adults. GnRH induces adenohypophysis production and release of gonadotropins, FSH, and LH. In male reproductive system, LH stimulates steroidogenesis in Leydig Cells (LCs), namely, androgens such as testosterone, while FSH stimulates SC activity, metabolism, and endocrine function, as inhibin source. The axis is under negative feedback control; therefore, inhibin and androgens inhibit GnRH and gonadotropin release when their bloodstream concentration overcomes its feedback threshold (Alves, Rato, et al., 2013; Cardoso et al., 2017; Schlatt & Ehmcke, 2014). In males, this cycle lasts for life but can be disrupted by any abnormality in any of the players. When it happens, spermatogenesis might be seriously affected (Alves, Rato, et al., 2013; J. E. Anderson & Thliveris, 1986; Cardoso et al., 2017). Spermatogenesis depends on paracrine regulation from SCs too, as they provide several differentiation factors (Alves, Rato, et al., 2013; Rato et al., 2016). Nevertheless, SC endocrine and paracrine roles on spermatogenesis may also be indirect. LCs, the most important steroidogenic cell of the testis and the main testosterone source, suffer dramatic population losses, and peritubular MCs, BTB is responsible for the immune-privilege, also suffer a significant drop in their activity in the absence of SCs (Rebourcet et al., 2014).

The critical role of SCs in spermatogenesis begins long before sexual maturation and at the beginning of regular spermatogenesis. Their influence starts as soon as on testicular embryonic development (França et al., 2016; Rebourcet et al., 2014). In addition to their stimulation by FSH to a normal testis development, SCs also secrete the anti-Mullerian hormone, a critical endocrine modulator involved on the correct development of the primary sexual characteristics (Alves, Rato, et al., 2013; Griswold, 1998; P. F. Oliveira & Alves, 2015a). Thus, these cells are involved in virtually all steps required for a normal spermatogenesis.

1.1.3 Germ Cell Dependence of Sertoli Cell Metabolism

Germ cells cross several differentiation stages during spermatogenesis. Their reliance on SCss are not the same at all stages, but it is a pivotal feature at all steps (P. F. Oliveira & Alves, 2015a). The least differentiated cells in this line are the spermatogonia, diploid

germ cells that constitute the germline reserve. Within spermatogonia there is a low differentiation degree between those which are committed to mitotic division for germ cell reserve preservation (type A) and those which are committed to meiotic division and undergo spermatogenesis (type B) (Griswold, 1998; P. F. Oliveira & Alves, 2015a; Schlatt & Ehmcke, 2014). The nomenclature for spermatogenesis stages differs from author to author, but here we will consider the following: spermatogonium (2n), primary spermatocyte (4n), secondary spermatocyte (n), round spermatid (n), elongated spermatid (n), and spermatozoon (n).

As mentioned, the two-compartment system, delimitating two distinctive environments, the interstitial fluid and the tubular fluid, is of the utmost importance for germ cell survival and differentiation (Schlatt & Ehmcke, 2014; Wong & Cheng, 2005). SC metabolism controls the metabolic microenvironment needed for each stage of germ cell differentiation. This is evident on the onset of the first meiotic division, when a type B spermatogonium enters phase S on its cell cycle and doubles its genetic charge (4n). Some authors define this phase as two distinct stages, the preleptotene and leptotene spermatocyte (França et al., 2016; Schlatt & Ehmcke, 2014). However, the interstitial environment is not suitable for meiosis, and in order to complete the first meiotic division, the cell crosses the BTB into the adluminal environment, where it readies meiosis (primary spermatocyte stage) and finally completes the first division to originate two secondary spermatocytes. The conversion of type A to type B spermatogonium seems to be modulated by retinoic acid (RA), a vitamin A derivate that cannot pass the BTB and is locally secreted by SCs, so it acts as a signal for meiosis initiation and thus spermatogenesis (Whitmore & Ye, 2015).

Overall, testes are oxygen-deprived organs; hence, germ cells, independently from its maturation stage, mainly rely on glycolytic metabolism to obtain energy (Alves, Rato, et al., 2013; Boussouar & Benahmed, 2004). The importance of glycolytic pathways is such that germ cells present, while undergoing spermatogenesis, unique enzyme isoforms related to it (P. F. Oliveira et al., 2015). Glycolytic potential decreases along the differentiation stages of spermatogenesis. Spermatozoon is the illustrious exception. It is even the most glycolytic cell of the male germline family, but has the lowest tricarboxylic acid (TCA) cycle activity, and relies almost exclusively on hexoses as substrate (Miki, 2006; Rato, Alves, Socorro, Duarte, et al., 2012). Lactate and pyruvate resulting from SC metabolism is an important energy substrate for germ cells (Alves, Martins, Rato, et al., 2013). SCs export both to the tubular fluid via monocarboxylate transporter (MCT) membrane transporter family members, while germ cells also import those substrates via MCTs (Alves, Martins, Rato, et al., 2013; Martins et al., 2013; P. F. Oliveira et al., 2012). Therefore, lactate

availability for germ cells depends on its production and release rate from SCs, which in turn is dependent upon MCT protein expression and glycolytic flux (P. F. Oliveira et al., 2015; Rato, Alves, Socorro, Duarte, et al., 2012). Nevertheless, SCs' glycolytic activity depends on substrate availability, *i.e.*, glucose import from the extracellular, interstitial environment, mediated by glucose transporter (GLUT) family member (P. F. Oliveira et al., 2015; Rato, Alves, Socorro, Duarte, et al., 2012). Summarily, germ cell substrate availability is intimately related to SCs' own substrate availability and metabolic rate. Fortunately for germ cells, albeit oxygen deprivation promotes the glycolytic metabolism rather than more oxidative metabolic pathways, SCs are competent metabolic "factories" able to metabolize a large spectra of substrates, including fatty acids and amino acids, in order to sustain the production of lactate and pyruvate (P. F. Oliveira & Alves, 2015a; Rato, Alves, Socorro, Duarte, et al., 2012). SCs direct three quarters of all their own pyruvate production to germ cells, either as lactate or pyruvate, whereas β-oxidation is suggested to be used only to satisfy their own energy needs (Grootegoed et al., 1986). Interestingly, SC metabolism resembles a cancer cell metabolism, heavily relying on glycolytic metabolism and glutaminolysis; however, contrarily to cancer cells which direct such energy for cell proliferation, SCs seem to direct it for production of substrates for developing germ cells (Alves, Rato, et al., 2013; P. F. Oliveira et al., 2015).

Besides being the preferred energy substrate, a novel evidence for a role of lactate as a signaling molecule in the seminiferous tubule has been provided, and lactate might be considered a paracrine factor secreted by SCs. In this context, lactate, taken up by germ cells, becomes oxidized to pyruvate with the resultant increase in reactive oxygen species (ROS) which act as second messengers regulating signal transduction pathways and gene expression (Galardo et al., 2014). In addition, lactate has also anti-apoptotic properties on germ cells and promotes RNA and protein synthesis on spermatids, by promoting NADPH oxidation for pentose phosphate pathway (PPP) and ATP production for protein synthesis (Erkkilä et al., 2002). On the other hand, SCs are relevant for germ cell apoptosis through other mechanisms (Murphy & Richburg, 2014). The division rate of spermatogonia is higher than the spermatozoa release rate. In mammals, germ cell death is conspicuous during spermatogenesis and occurs spontaneously at various phases of germ cell development such that seminiferous epithelium yields fewer spermatozoa than might be anticipated from spermatogonial proliferations. Although most of the apoptotic cells are type B spermatogonia and primary spermatocytes, programmed cell death can occur at any differentiation stage (Murphy & Richburg, 2014). SCs quickly absorb their apoptotic bodies by phagocytosis, because they

express class B scavenger receptor type I (SR-BI), a receptor sensitive to the apoptosis-marker phospholipid phosphatidylserine (Murphy & Richburg, 2014). Thus, apoptotic cells are rarely spotted on histological preparations (Murphy & Richburg, 2014). Notably, SCs are reported to even use the apoptotic bodies as energy source (Xiong et al., 2009) illustrating their high metabolic capacity to metabolize substrates and convert them into relevant metabolites for spermatogenesis.

SCs are responsible for ionic balance and pH control in tubular fluid, due to their metabolic and secretory activity (Bernardino et al., 2016; Bernardino et al., 2013; Martins et al., 2014). The hydrocarbonate ion plays a decisive role on pH balance, and its concentration depends on SC protein expression of Cystic Fibrosis transmembrane conductance regulator (CFTR) (Bernardino et al., 2016; Bernardino et al., 2013). Another SC-related pH regulation mechanism is the solute carrier 9 family members (SLC9) Na^+/H^+ exchangers (Martins et al., 2014). Aquaporins (AQPs) expressed by SCs are another important transporter family for tubular fluid homeostasis and are reported to be co-expressed with CFTR (Jesus et al., 2014a; 2014b). Germ cells heavily rely on this regulation, as they are very fragile against environmental aggression. Indeed, oxidative stress is a major external aggression for germ cells, as they lack antioxidant mechanisms. Oxidative stress induces germ cell apoptosis due to lipid peroxidation of the germ cell membrane's lipids (Rato, Alves, Socorro, Duarte, et al., 2012). The physical supportive role of SCs is a key factor for germ cell survival as shown before the end of spermiation when a premature germ cell detachment causes apoptosis on detached germ cells (Cheng & Mruk, 2002). In the occurrence of SC death, the previously nursed germ cells, which became freely suspended in the lumen, rapidly engage programmed cell death (Cheng & Mruk, 2002). This might be caused by the germ cell stage-specific environmental needs, only maintainable at the microenvironments created by the SC's membrane invaginations and by the necessity of growth factors synthesized by these cells (Cheng & Mruk, 2002; Vogl et al., 2013). Figure 1.1.1 summarily illustrates the most important roles of SCs in the testicular tissue.

Within paracrine mediators and growth factors in the cross talk between SCs and germ cells, perhaps the most described players are basic fibroblast growth factor (bFGF) and interleukin-1 (IL-1). bFGF is expressed by Sertoli and germ cells, but only SCs express its receptor, where it acts as a major metabolic modulator. It modulates transferrin, 17β-estradiol (E_2) and lactate secretion, glucose uptake, and FSHR expression (Alves, Rato, et al., 2013). Transferrin is an iron-binding plasmatic glycoprotein that, due to BTB impermeability, cannot transfer their ferric ions to the adluminal compartment where it is needed

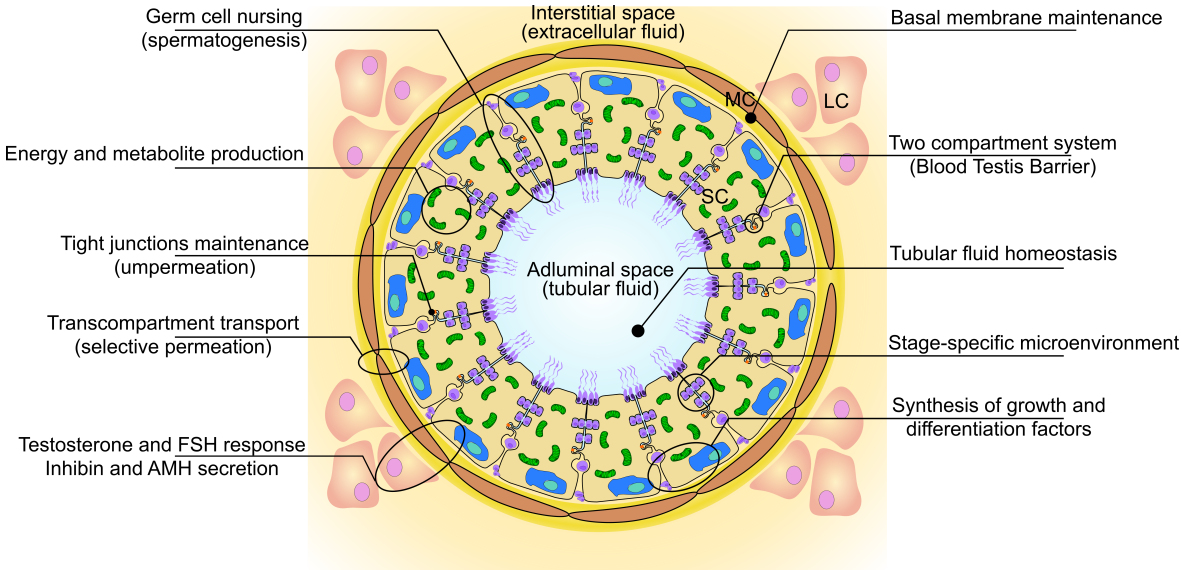


Figure 1.1.1: The central role of Sertoli cells in testicular tissue environment and organization. Sertoli cells play several roles in testicular tissue's physiology. Besides their critical role on spermatogenesis, granting physical and nutritional support to developing germ cells, they also show paracrine and endocrine properties and are the main component of the blood-testis barrier, one of the tightest blood-tissue barriers of the human body.

by the differentiating germ cells. Once again, SCs mediate the ferric ion transport from the interstitial fluid to the tubular fluid. Many proteins have been demonstrated to be implicated in iron transport to germ cells. Iron import to the seminiferous tubule by transferrin was demonstrated, and it was hypothesized that SC could import iron basolaterally, transport it across the BTB, and supply iron to the developing male germ cells. In this way, the entry of iron to the germ cells is thought to be mediated by transferrin secreted by SC (Griswold, 1998). Furthermore, ferroportin transcript levels were detected in SC and can contribute with this transport. In addition to transferrin and transferrin receptor 1 (TfR1), other proteins involved in iron transport, such as divalent metal transporter 1 (DMT1) and glycosyl phosphatidylinositol-anchored ceruloplasmin, and iron storage, such as cytosolic and mitochondrial ferritin, were found in the testis. IL-1 is a multifunctional cytokine, having two isoforms: IL-1α and IL-1β. In the testis, IL-1α is produced by SCs and germ cells, while IL-1β is produced by LCs and interstitial macrophages (Rato et al., 2016). Surprisingly, IL-1α causes antispermatogenic effects over SCs, while IL-1β promotes pro-spermatogenic responses from them (Alves, Rato, et al., 2013). On the other hand, IL-1 is specially involved in meiosis initiation, promoting DNA synthesis and differentiation of type B spermatogonium into preleptotene spermatocytes (Alves, Rato, et al., 2013). Nevertheless, we have to take into account that the majority of the studies of paracrine and growth factor regulation of spermatogenesis were performed *in vitro*; hence, differences in *in vivo* behavior must be considered (Alves, Rato, et al., 2013).

1.1.4 Impact of Whole-Body Metabolic Dysfunction in Sertoli Cell Metabolism and Spermatogenesis

In the last decades, we have been witnessing a steady increase in male fertility dysfunction and a decrease on fertility rates worldwide but particularly significant on high Human Development Index (HDI) countries (Hamilton & Ventura, 2006). Simultaneously, the incidence of metabolic disorders, particularly obesity and T2D, significantly increased worldwide and again with special impact in developed countries (World Health Organization [WHO], 2000). The overlapping nature of both premises led to the hypothesis that a relation between metabolic dysfunction and male infertility may occur (**Crisóstomo**, Sousa, et al., 2017). In fact, clinical studies seem to confirm this association: obese and overweight men have poorer fertility outcomes than lean men, and thus, couples with a man obese or overweight will have problems to conceive (**Crisóstomo**, Sousa, et al., 2017). Although some causes for those fertility problems were not difficult to associate to excess of weight, the same is not true for other metabolic disorders such as T2D. Obese and overweight men often have their fertility compromised by mechanic factors, such as testicular overheat due to adipose tissue accumulation or even coitus difficulties and erectile dysfunction linked to cardiovascular complications (Chambers & Richard, 2015). Diabetic men also present fertility dysfunction linked to microvascular disease, reflected as lower tubule diameter, abnormal testicular capillary and lymphatic endothelia, SC-only tubules, and ultrastructural damage in SC apical cytoplasm (Alves, Martins, Cavaco, et al., 2013). It is also reported that microvascular disease increases vascular density and cellular matrix thickening, simultaneously promoting germ cell apoptosis due to increased scrotal temperature and hypoxiainduced cellular damage (Alves, Martins, Cavaco, et al., 2013; J. E. Anderson & Thliveris, 1986; Cai et al., 2000). Moreover, T2D patients often present ejaculatory defects, such as erectile dysfunction and retrograde ejaculation (Alves, Martins, Cavaco, et al., 2013). But those factors do not reflect most of infertility's etiology.

One of the first molecular mechanisms explaining male infertility due to obesity was based on the steroidogenic potential of adipocytes (Belanger et al., 2002). Obese men present higher E_2 levels than the average male population, because they have more fat cells, and those cells present p450 aromatase, an enzyme that converts testosterone to E_2 (Roumaud & Martin, 2015). Estrogens in men have similar effects over the pituitary as in women, exerting a negative feedback over LH and FSH release (Cardoso et al., 2017) and a consequent decrease of testosterone secretion and SC activity. Ultimately, the reproductive axis disruption leads to spermatogenesis defects or even arrest (Alves, Rato, et al., 2013; Cardoso et al., 2017; Rato,

Alves, Duarte, et al., 2015). Furthermore, E_2 impairs ionic transport via CFTR on SCs, resulting in less ionic exchanges between lumen and intracellular environment, cytoplasmic acidification, and possibly spermatogenic defects (Bernardino et al., 2016; Bernardino et al., 2013). On the opposite side, fasting suppresses GnRH pulses, thus perturbing reproductive axis, first by inhibiting LH release, which in turn inhibits the release of gonadotropins, and lastly by decreasing androgen secretion by LCs and SC activity (P. F. Oliveira, Sousa, et al., 2017; Rato, Alves, Socorro, Duarte, et al., 2012).

The SC is the central target of environmental toxicants responsible to male reproductive defects, as evidenced by *in vitro* and *in vivo* models (Reis et al., 2015). The first compromising action of those toxicants is the negative effect on junction between SCs and Sertoli-germ cells (Reis et al., 2015). Chemotherapeutic drugs (W. H. B. Wallace et al., 1989), gossypol (Monsees et al., 2000), glycerol (Wiebe et al., 2000), alkylphenols (Raychoudhury et al., 1999), pesticides (Alves, Neuhaus-Oliveira, et al., 2013), and heavy metals (Bizarro et al., 2003) are just examples of compounds known for interfering with cells' adhesion. Furthermore, some pesticides, phthalates, and obesogens can also work as endocrine disruptors, since their chemical structure is either similar to steroid hormones or competitively inhibits gonadotropin's receptors in SCs (Cardoso et al., 2017; Reis et al., 2015). Those compounds are known as endocrine disruptors because their action causes imbalances on the tightly balanced reproductive axis, disrupting the expected negative feedback mechanisms between the hypothalamus and hypophysis complex and the gonads (Cardoso et al., 2017; Reis et al., 2015). Obesogens aggravate their disrupting effect by promoting adipose tissue accumulation, which causes (1) increasing storage of environmental toxicants, since most of them are fat-soluble, and (2) a self-endocrine disruptor, since adipocytes present significant activity levels of aromatase, an enzyme that converts testosterone to E_2 (Cardoso et al., 2017).

However, the nefarious effects of metabolic disorders are not restricted to systemic defects; they are also reflected *in loco*. Metabolic hormones affect whole-body cell metabolism, and testes are no exception (Alves et al., 2016; Martins et al., 2015; Martins et al., 2016; P. F. Oliveira et al., 2012; P. F. Oliveira, Sousa, et al., 2017; Rato, Alves, Socorro, Duarte, et al., 2012). One of the main metabolites secreted by SC is acetate, which is suggested to be involved in germ cell membrane remodeling. Interestingly, in the absence of insulin, SCs totally inhibit acetate production (Alves, Martins, Rato, et al., 2013), illustrating that insulin dysfunction may cause serious defects on the metabolic support of spermatogenesis by SCs. The close metabolic cooperation between Sertoli and germ cells is based on their proximity, maintained by the numerous cell junctions, which are threatened by changes on

environment composition, and metabolic by-products capable to disrupt those junctions (Reis et al., 2015). Glycerol is a suitable example. Its antispermatogenic properties when directly injected in testes, at high concentrations, are caused by BTB disassembly due to tight junctions' cleavage and overall SeT disorganization (Wiebe & Barr, 1984; Wiebe et al., 2000). There are several examples of hormones linked to whole-body metabolic status that also exert direct and indirect effects on SC metabolism (Alves et al., 2016; Martins et al., 2015; P. F. Oliveira et al., 2012; P. F. Oliveira, Sousa, et al., 2017; Rato, Alves, Socorro, Duarte, et al., 2012; Rato et al., 2016).

Leptin increases fat mobilization from adipocytes so it can be used as fuel by cells. Liver is the main destiny of adipocyte-derived triacylglycerols, where they suffer lipolysis. The resulting fatty acids are used for energy production, mainly through β-oxidation (Rodríguez et al., 2011). Resulting glycerol is phosphorylated into glycerol-3-phosphate (G3P) and enters the G3P shuttle, a cyclic reaction group that links lipidic and carbohydrate metabolism, TCA cycle, and electron electron transport chain (ETC) (Lin, 1977). Obese and diabetic men present higher serum glycerol concentrations resulting from triacylglycerol breakdown (Hagström-Toft et al., 1997). The combination of insulin and leptin also promotes lipid mobilization in SCs for energy production, and they express several aquaglyceroporins (AQGPs), a AQP subfamily capable of transporting small solutes such as glycerol, in addition to water (Jesus et al., 2014b; Rodríguez et al., 2011; Yeung et al., 2010). However, glycerol has antispermatogenic properties, since it destabilizes the actin filaments that allow the BTB and the germ cell attachment to SCs (Wiebe et al., 2000). Leptin also promotes GLUT expression and lactate dehydrogenase (LDH) activity in SCs but decreases acetate production (Martins et al., 2015).

Ghrelin is another metabolic hormone whose receptor is present in SCs. Its whole-body metabolic effect is glucose blood and interstitial concentration decrease, due to increased insulin sensitivity of the cells (Vestergaard et al., 2013). Ghrelin concentration is inversely proportional to nutritional state, so obese individuals show the lowest blood concentration (and higher insulin resistance), while undernourished present the highest blood concentration (and lowest insulin resistance) (Martins et al., 2016). In cultured SCs, ghrelin media concentration seems to be inversely proportional to lactate production (Martins et al., 2016), which indicates that fasting may be worse than overeating in what concerns the effects of ghrelin on the nutritional support of spermatogenesisby SCs. However, the ghrelin concentration corresponding to the average in lean man increases the efficiency of glycolytic metabolism, *i.e.*, redirects the cell metabolism to obtain a better lactate-to-glucose ratio, increasing LDH

activity while decreasing glucose consumption (Martins et al., 2016). Since reproductive function requires a proper nutritional state, and according to ghrelin concentration variation in function of the nutritional state of the individual, it is legitimate to declare that ghrelin acts as a nutritional state sensor (Lorenzi et al., 2009). Ghrelin inhibits LH release by pituitary in dose-dependent manner. This is especially important for puberty onset, as it needs proper energy store, although this has only been observed in males (Lorenzi et al., 2009). Yet, this effect is valid for adulthood as well; thus, ghrelin may impair the reproductive axis due to LH release inhibition and consequent decrease in testosterone (Lorenzi et al., 2009). In Figure 1.1.2, all the hormonal mechanisms which influence SC function mentioned in this chapter are depicted.

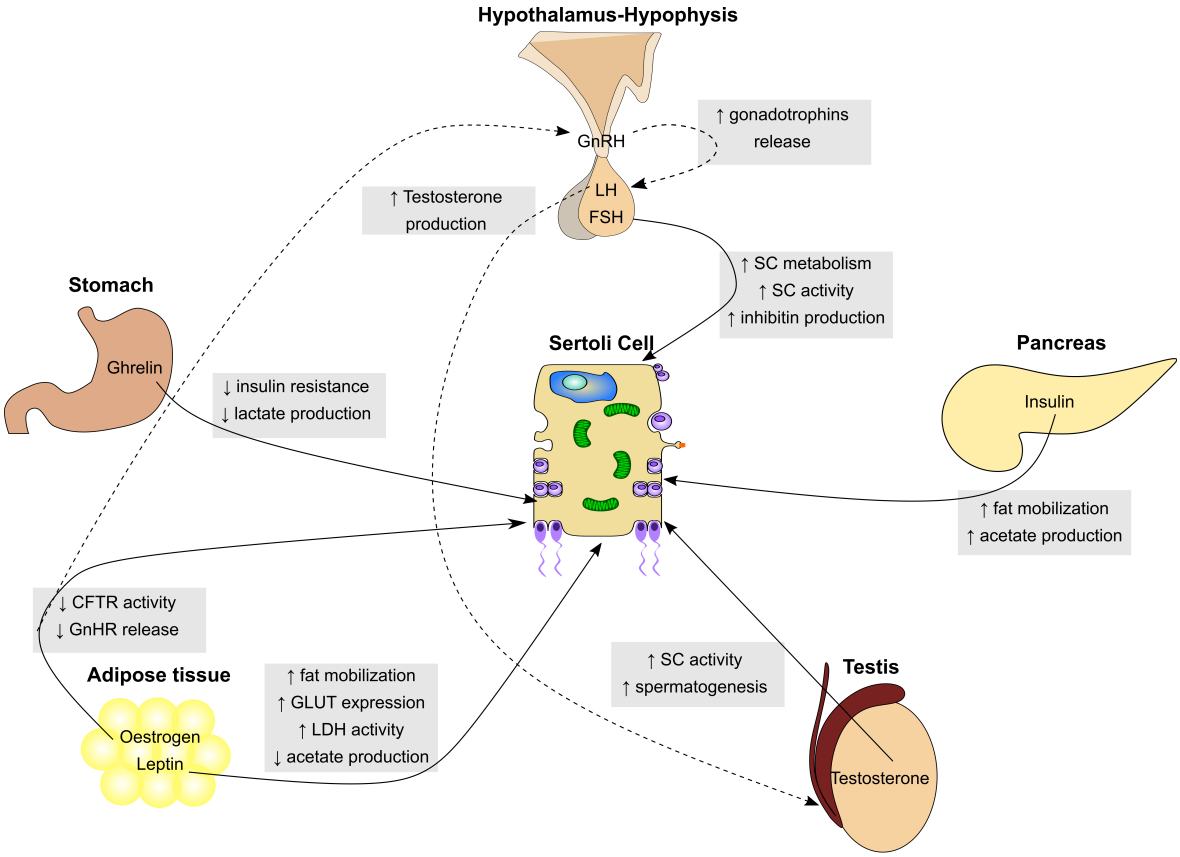


Figure 1.1.2: Hormonal regulation of Sertoli cells. Sertoli cells are directly targeted and react to several hormones. Due to their central role on testicular physiology, those responses potentially affect spermatogenesis outcomes, either positively or negatively. Moreover, the tightly regulated hormonal circuits mean that there are hormones that can indirectly affect Sertoli cell function.

1.1.5 Conclusions

The importance of SCs in spermatogenesis is undeniable. They are extremely specialized cells and had developed unique characteristics to accomplish their function effectively. We must not forget that germ cells are also unique cells in the human body, thus requiring unparalleled

conditions. SCs reflect their unique needs and are responsible for creating the suitable environment for germ cell differentiation and survival throughout spermatogenesis, acting as an interface between them and the extracellular environment. Knowing this principle, it becomes easier to understand all the actions promoted by SCs to achieve a successful spermatogenesis. They create the BTB, by the tight junctions, hemidesmosomes and tubulobulbar complexes between SCs, creating the two-compartment system. This barrier impermeates the adluminal space, where germ cells differentiate, while granting it immunological immunity; otherwise, germ cells would be regarded as targets of the immune system. However, this insulation makes difficult the diffusion of substrates for germ cells. Once more, SCs mediate their diffusion, importing the substrates from the extracellular medium and then providing them to the germ cells or exporting them to the tubular fluid while maintaining the right pH and ionic balance of this fluid.

The regulation of the abovementioned functions is complex. SCs must respond to tubular fluid and extracellular medium composition, to the cooperation of the neighboring cells, and to hormonal regulation. In this chapter, we had shed light over the molecular mechanisms which regulate SC response to the various stimuli. Understanding those processes is pivotal on male fertility research. Any alteration of the metabolites, hormones, and messengers on these processes can deeply impact male fertility. Although there are already plenty bibliographies on the subject, many questions arise from the actual knowledge. The molecular mechanisms which regulate SC is a fertile research ground. Advances on the research on this subject may lead us to new therapeutical approaches against the ever-increasing number of male infertility cases.

1.1.6 Author contributions

Luís Crisóstomo, Agostina Gorga performed the bibliography search, screened the selected publications and wrote the manuscript. Marco G. Alves, Silvina B. Meroni and Pedro F. Oliveira have critically reviewed the manuscript and conceptualized the chapter. Mário Sousa, María F. Riera and María N. Galardo have critically reviewed the manuscript. All the authors contributed to manuscript writing/editing and approved the final version.

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1.2 Metabolic diseases affect male reproduction and induce signatures in gametes that may compromise the offspring health

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Abstract

The most prevalent diseases worldwide are non-communicable such as obesity and type 2 diabetes. Noteworthy, the prevalence of obesity and type 2 diabetes is expected to steadily increase in the next decades, mostly fueled by bad feeding habits, stress, and sedentarism. The reproductive function of individuals is severely affected by abnormal metabolic environments, both at mechanical and biochemical levels. Along with mechanical dysfunctions, and decreased sperm quality (promoted both directly and indirectly by metabolic abnormalities), several studies have already reported the potentially harmful effects of metabolic disorders in the genetic and epigenetic cargo of spermatozoa, and the epigenetic inheritance of molecular signatures induced by metabolic profile (paternal diet, obesity, and diabetes). The inheritance of epigenetic factors towards the development of metabolic abnormalities means that more people in reproductive age can potentially suffer from these disorders and for longer periods. In its turn, these individuals can also transmit this (epi)genetic information to future generations, creating a vicious cycle. In this review, we collect the reported harmful effects related to acquired metabolic disorders and diet in sperm parameters and male reproductive potential. Besides, we will discuss the novel findings regarding paternal epigenetic inheritance, particularly the ones induced by paternal diet rich in fats, obesity, and type 2 diabetes. We analyze the data attained with *in vitro* and animal models as well as in long-term transgenerational populational studies. Although the findings on this topic are very recent, epigenetic inheritance of metabolic disease has a huge societal impact, which may be crucial to tackle the 'fat epidemic' efficiently.

1.2.1 Introduction

The prevalence of metabolic diseases has soared globally in recent decades (Figure 1.2.1). As non-communicable diseases, they are largely caused by lifestyle, mainly diet and physical (in)activity. The fast pace characteristic of our more developed societies pushes populations toward unhealthy dietary choices highlighted by current epidemiologic data presented by the World Health Organization (WHO) rendering obesity as a global pandemic (WHO, 2000). However, obesity is a multifactorial disease associated with several co-morbidities, including T2D. Taken together with their precursors, particularly overweight and prediabetes, T2D accounts for one of the main reasons for years of poor health and premature retirement worldwide. Besides, along with the negative impact on productivity, the patients suffering from those diseases also pose a significant expense for health systems highlighting metabolic diseases as a preeminent economic issue that should be taken into consideration. Several other silent co-morbidities are usually overlooked, even with new research showing that people living with metabolic diseases for part of their lives may pass metabolic signatures to the

next generation. Indeed, as the prevalence of obesity and T2D is expected to increase in the upcoming decades, and more people will live with the disease for most of their active life, it is becoming very relevant to discuss how their reproduction may also contribute to this pandemic. The relationship between mother's obesity and the metabolic phenotype of the offspring has been somewhat characterized (Berglund et al., 2016) but the same is not as true for males.

Interestingly, as we witness a rise in the prevalence of metabolic diseases, fertility rates and sperm parameters are reported to decline concomitantly (Levine et al., 2017; Skakkebaek et al., 2016). Although the specific biochemical mechanism(s) that associate metabolic disease with subfertility and infertility remains unknown, it must be highlighted that the reproductive function relies on a tightly regulated hormonal and nutritional balance (P. F. Oliveira & Alves, 2015b; P. F. Oliveira, Sousa, et al., 2017; Rato, Alves, Socorro, Duarte, et al., 2012; Rato et al., 2016). Therefore, it is not surprising that reproductive function may suffer changes accordingly with the metabolic status of the individual. More recently, it has been reported that predisposition to metabolic disease can be inherited via the paternal lineage. Interestingly, some of those reports seem like a revival of Lamarck's theories, almost two centuries later, showing that offspring acquires adaptive and pathological features in response to environmental conditions and pressure. The truth is, those findings clearly show an interaction between environment and phenotype.

Herein, we discuss recent literature on the emerging role of metabolic status and the information conveyed in the male gamete. We have included some older works which were deemed unavoidable for the comprehension of the subject or works which presented a hallmark to the present knowledge. Our bibliographical review spanned for the first trimester of 2020, using PubMed, Scopus, and Google Scholar specialized search engines. Key search terms included: 'spermatogenesis', 'obesity', 'mechanical dysfunctions', 'spermatic quality', 'western-diet', 'weight loss', 'bariatric surgery', 'obesity-related genes', 'epigenetic', 'sncRNA', 'sperm RNA', 'sperm epigenome', 'transgenerational study', 'epigenetic inheritance', 'metabolic signatures', 'diabetes miRNA', 'male gamete'. MeSH terms were applied to our search whenever possible.

1.2.2 Overweight and Obesity: Etiology and Acquired Profile

Obesity results from a disturbance between energy intake and expenditure, inducing excessive fat accumulation in the adipose tissue. In humans, there are two major types of adipose tissue: white and brown adipose tissue. Brown adipose tissue uses energy to maintain body

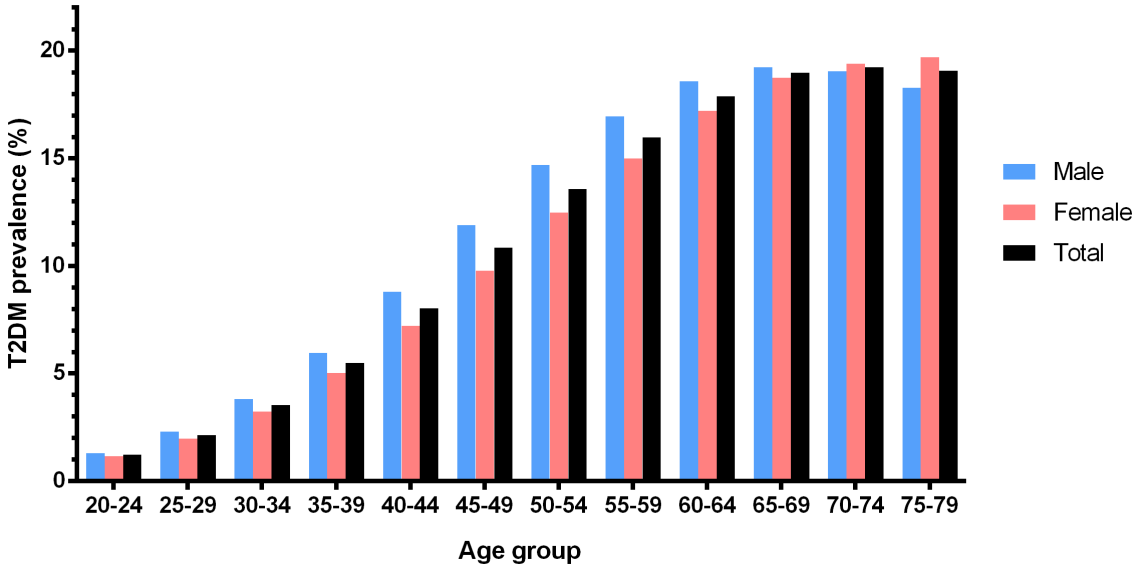


Figure 1.2.1: Present figures for global diabetes prevalence in adults, per gender and age group. Results are expressed in percentage of the total age subjects in the age group/gender group.

temperature, in a process called thermogenesis. This tissue is very abundant in newborn babies and though there is a decrease in its deposition with aging, it is still possible to find in adults (Santhanam et al., 2015). The white adipocytes are the most abundant adipocytes in humans and its main function is to store energy. There are many types of white adipose tissue, classified by its location, but the white intra-abdominal adipose tissue seems to be the most relevant to the development of metabolic abnormalities (Foster et al., 2011). Furthermore, the white adipocyte tissue also provides mechanical protection and thermal isolation to the body.

Fat accumulation is determined by the balance between fat synthesis, a process known as lipogenesis; and fat breakdown, a process known as lipolysis, which also includes fatty acid oxidation. Lipogenesis is a process responsive to diet and stimulated when high levels of free fatty acid and glucose can be found in the bloodstream (Kersten, 2001). Similarly, a state of lack of energy induces lipolysis and fatty acids oxidation (Torchon et al., 2017). There is no linear correlation between the Body Mass Index (BMI), fat mass and adipocyte size. Furthermore, different adipose depots respond to weight-gain differently, mainly through white adipose tissue hyperplasia (increase in cell number), and/or hypertrophy (enlargement of individuals cells) (Arner et al., 2010; Karpe & Pinnick, 2015). When enlarged adipocytes are not able to expand anymore due to triglyceride saturation, rupture of the cells occurs and there is macrophage invasion, increased release of pro-inflammatory adipokines, and decrease in anti-inflammatory adipokines. Thus, obesity is usually associated with a chronic inflammation state (Kennedy et al., 2009). Furthermore, the saturation of adipocytes leads

to the deposition of triglycerides in undesirable places, such as vital organs, which can lead to serious complications.

The excessive accumulation of fat and obesity is socially discussed as the result of lack of exercise, a sedentary lifestyle, and diets rich in fats. However, this phenomenon is not exclusively behavioral: recent studies show that the development of obesity has an environmental contribution. Indeed, some environmental compounds are even classified as obesogens, which describe a class of chemical compounds that can enhance adipogenesis and promote lipid accumulation. The exposure to these compounds, usually through diet, impairs energy metabolic pathways, and disrupts signaling pathways (Grün & Blumberg, 2009). Obesogens seem to have great stability and a large portion of these compounds are lipophilic. Furthermore, the chemical structure of these compounds is similar to that of several physiological regulators, including hormones. This means that obesogens have the potential to be powerful endocrine disruptors. These effects are reflected in the reproductive systems of both, males and females (Cardoso et al., 2017; Rato et al., 2017). Studies in families involving twins and adopted children have provided compelling evidence that adiposity is highly heritable and that the development of obesity has a strong genetic contribution (Hasselbalch, 2010; Sørensen & Stunkard, 1993). Those reports showed that children born from obese parents have a genetic predisposition to develop metabolic disorders. Later, obesity was found to be strongly related to genetics and epigenetics causes (Herrera et al., 2011) illustrating that the metabolic profile associated with obesity and overweight can be inherited.

1.2.3 Obesity and the Mechanical Dysfunctions That Hamper Sexual Activity

Overweight and obese individuals (both male and female) have a high frequency of problems related to the performance during sexual intercourse, including lack of sexual desire and poorer sexual performance (Kolotkin et al., 2006). Also, obese men are commonly associated with an increased risk of developing erectile dysfunction and penile vascular impairment. By definition, these mechanical and psychic dysfunctions are associated with an abnormal function of the penile vasculature, due to aging or comorbid diseases (Shamloul & Ghanem, 2013). Naturally, risk factors for the development of cardiovascular diseases are also associated with erectile dysfunction. These risk factors include lipids abnormalities, hypertension, diabetes *mellitus* (DM), and obesity (Demir et al., 2009). As expected, metabolic disorders have been inversely correlated with penile arterial flow rates (Fonseca & Jawa, 2005). This finding suggests that penile arterial smooth muscle relaxation capacity is altered by the metabolic profile. More specifically, it impairs endothelium-dependent vasorelaxant substances production and

decreases distensibility (Demir et al., 2009), which is reflected in erectile dysfunction. When erectile dysfunction becomes too severe, the development of other mechanical disorders, such as premature ejaculation, can occur. However, it has not yet been settled the degree of inter-relation between erectile dysfunction and premature ejaculation (El-Sakka, 2008). To further stimulate the discussion, premature ejaculation was reported to have an inverse relationship with obesity (Gökçe & Ekmekcioglu, 2010). These findings highlight the complex relationship between sexual and metabolic disorders.

Lifestyle changes and weight loss are associated with an improvement in sexual function in about one-third of the obese men with erectile dysfunction, as reported by Esposito et al. (2004). This study was conducted in 55 obese men which achieved a loss of 10 % of their initial weight by exercise and healthy food choices. Also, obesity is often associated with impaired emotional health and negative body image. Along with the social stigmatization associated with erectile dysfunction, obese/overweight individuals feel discouraged to seek medical help (Kolotkin et al., 2006).

1.2.4 Multifactorial Effect of Obesity in Sperm Quality

There are several studies focused on obesity and classic sperm parameters (namely, concentration, motility, and morphology) with mixed results (summarized in Table 1.2.1). While several studies showed that excess weight is associated with low semen volume and sperm motility as well as aberrant sperm morphology (Jensen et al., 2004; Luque et al., 2017; Tsao et al., 2015), other studies failed to show such a clear correlation (Chavarro et al., 2010; M. Al-Ali et al., 2014; Wen-Hao et al., 2015). The observed discrepancies in the literature are likely caused by the several limitations associated with studies performed in humans. In addition, sperm function can be altered by innumerable lifestyle factors, such as smoking, alcohol consumption, and recreational drugs, which are confusing factors difficult to control in human studies. Furthermore, the use of BMI as an adiposity calculator has also been challenged (Eisenberg et al., 2013). Obesity is also associated with the development of other complications, such as DM and oxidative stress, which can also impair sperm parameters (Aitken et al., 2014; Ding et al., 2015), promote sperm DNA fragmentation (Chavarro et al., 2010; Fariello et al., 2009), and induce aberrant sperm mitochondrial function (Fariello et al., 2009; J. B. A. Oliveira et al., 2018). Other comorbidities associated with obesity have also been pinpointed as responsible for the detrimental effects of obesity in sperm quality. Beyond weight gain and adiposity, there are other relevant factors associated with obesity impact in sperm quality, including hormonal dysfunction, fat accumulation in reproductive

organs, accumulation of environmental toxic substances, or even inflammation and oxidative stress. Below we briefly discuss each of these factors (Figure 1.2.2).

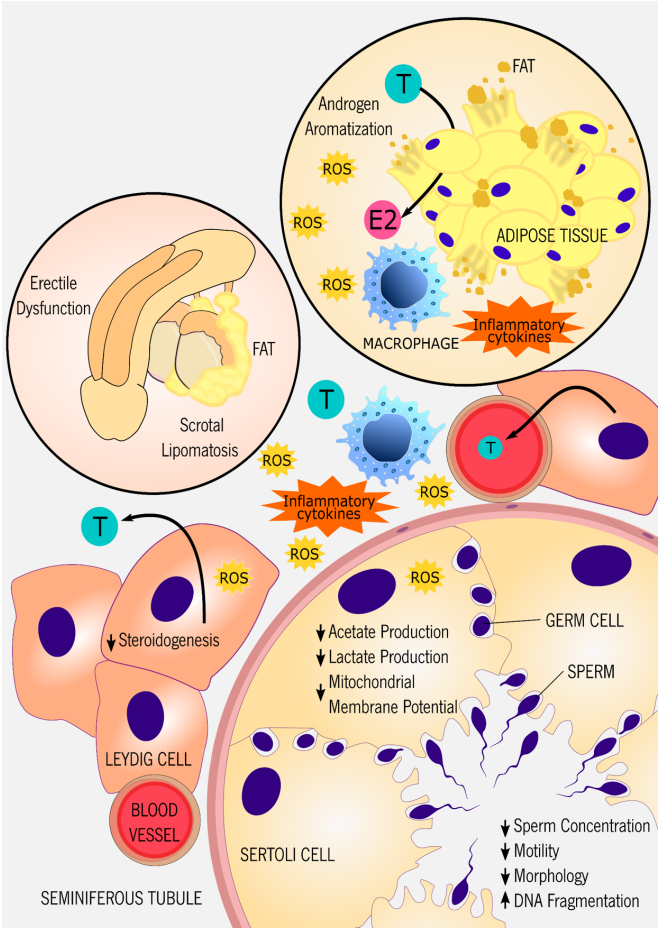


Figure 1.2.2: The excessive white fat deposition, a typical feature of obesity, is a threat to male reproductive function by several mechanisms. Obese men are commonly associated with an increased risk of developing erectile dysfunction and penile vascular impairment. Moreover, fat deposition on the scrotal area, a condition called scrotal lipomatosis, promotes the rise of scrotal temperature, inducing germinal atrophy and spermatogenic arrest. Testosterone aromatization into estradiol by adipocytes can induce hypogonadism (a condition characterized by low levels of androgens). The excessive lipid accumulation leads to adipocytes rupture, trigging a pro-inflammatory state. Due to its location, LCs are exposed to the ROS and inflammatory cytokines produced by the macrophages. Consequently, the steroidogenesis machinery is compromised, and the production of testosterone drops, further promoting hypogonadism. In response to the abnormal metabolic situation, SC metabolism is remodeled, decreasing both the production of lactate and acetate. Meanwhile, the mitochondrial membrane potential is also affected, threatening the 'nursing' of developing germ cells by SCs. E2, estradiol; ROS, reactive oxygen species; T, testosterone.

Hormonal Dysregulation

Obesity causes whole body hormonal dysregulation. Hormones related to energy homeostasis — insulin, glucagon, leptin, glucagon-like peptide 1 (GLP-1), ghrelin — have also been implicated in male reproductive dysfunction. Due to the essential role of hormones in the maintenance of glucose homeostasis, hormonal disruption affects not only signaling pathways associated with food intake control but also functions intimately related to energy homeostasis, such as reproduction (Alves et al., 2016). Leptin is a peptide hormone mainly produced by white adipocytes, which is related to adipose tissue mass and decreased food intake. In humans, leptin and sex hormones blood concentrations appear to be strongly related (Moschos et al., 2002). Usually, androgens suppress leptin production (Moschos et al., 2002), supporting that testosterone is an important regulator of leptin. Concurrently, leptin has been found to exert important effects on gonadal organs through leptin receptors that were reported in testicular cells, including in the surface of LCs (Moschos et al., 2002; Pasquali, 2006) and

Table 1.2.1: Summary of studies investigating the effects of obesity on classic sperm parameters.

Author(s), year	Concentration	Motility	Normal morphology	Population
Jensen et al. (2004)	⇓	No correlation	⇓	1558 young men (average: 19 years)
Pauli et al. (2008)	No correlation	No correlation	No correlation	87 adult men
Chavarro et al. (2010)	No correlation	No correlation	No correlation	483 men from subfertile couples
Hofny et al. (2010)	⇓	⇓	⇓	42 fertile obese men and 80 infertile obese men
Martini et al. (2010)	No correlation	⇓	No correlation	794 adult men
Paasch et al. (2010)	⇓	No correlation	⇓	2157 men (17–67 years)
Sekhavat and Moein (2010)	⇓	⇓	No correlation	852 men (25–50 years)
Wegner et al. (2010)	No correlation	No correlation	No correlation	107 men attending an infertility clinic
Rybář et al. (2011)	No correlation	No correlation	No correlation	153 men attending an infertility clinic
Shayeb et al. (2011)	No correlation	No correlation	⇓	2035 men from subfertile couples
Hammiche et al. (2012)	⇓	⇓	n/a	450 men from subfertile couples
Anifandis et al. (2013)	No correlation	⇓	n/a	301 men from subfertile couples
MacDonald et al. (2013)	No correlation	No correlation	⇑	511 men attending an infertility clinic
Belloc et al. (2014)	⇓	⇓	No correlation	10 665 men consulting for a semen analysis
M. Al-Ali et al. (2014)	No correlation	No correlation	No correlation	2110 men attending an andrology unit
Thomsen et al. (2014)	No correlation	No correlation	n/a	612 men from infertile couples undergoing ART
Luque et al. (2017)	⇓	⇓	⇓	4860 adult men (18–65 years)
Wen-Hao et al. (2015)	No correlation	⇓	No correlation	617 male infertility patients
Tsao et al. (2015)	⇓	No correlation	⇓	7630 adult men
Calderón et al. (2018)	⇓	⇓	No correlation	30 obese men and 10 lean men
J. B. A. Oliveira et al. (2018)	⇓	⇓	⇓	1824 men from subfertile couples

Sperm quality worsening is represented by (⇓), and sperm quality improvement is represented by (⇑). Parameters not addressed in the study are represented by n/a.

SCs (Martins et al., 2015). The excess of circulating leptin found in obese men contribute to reducing testosterone concentrations due to impairment of LCs (Isidori et al., 1999). High levels of leptin are also known to modulate SCs metabolism, decreasing lactate dehydrogenase activity (Martins et al., 2015), which has the potential to compromise male fertility. Concordantly, higher serum leptin levels found in obese infertile men were found to be correlated with abnormal sperm morphology (Hofny et al., 2010).

Contrarily to leptin, circulating ghrelin levels are lower in obese than in lean men (Tschöp et al., 2001). Recent *in vitro* studies have reported that ghrelin can inhibit testicular secretion of testosterone (Ishikawa et al., 2007) while suppressing the LH and FSH secretion by the pituitary (Dupont et al., 2010). Ghrelin appears to carry anti-proliferative action in several cell lines, which led to the hypothesis that ghrelin can inhibit the proliferative activity of immature LCs (Barreiro & Tena-Sempere, 2004). Furthermore, it avoids excess build-up of germ cells, which appears to be a crucial spermatogonia survival factor (Kheradmand et al., 2012). Martins et al. (2016) reported that glucose consumption and mitochondrial membrane potential of human SCs are sensitive to ghrelin levels. Those results suggested that ghrelin acts as an energy sensor for human SCs in a dose-dependent manner, modulating the nutritional support of spermatogenesis (Martins et al., 2016). Thus, it appears that ghrelin balance can contribute to the metabolic dynamics within the male reproductive axis in situations of energy deficit (Alves et al., 2016; Dupont et al., 2010).

It is also known that the incretin effect, a process that describes the increase in insulin plasma levels along with the decrease in glucagon levels, after a meal, is impaired in obese individuals (Muscelli et al., 2008). This evidence suggests that incretins, such as GLP-1, may be altered in obese subjects. However, the findings regarding GLP-1 levels in obesity were inconsistent and its variations have not been conclusively determined (Muscelli et al., 2008). Yet, some evidence suggested that leptin may exert a regulatory effect in GLP-1 secretion and that impairment of leptin regulation associated with obesity may also be associated with impairment of GLP-1 regulation (Anini & Brubaker, 2003). Although the effects of GLP-1 in male reproduction remain overlooked, its contribution to glucose homeostasis suggests that it may be important for the regulation of spermatogenesis. The expression of the GLP-1 receptor was first identified in isolated human SCs by Martins et al. (2019). The authors also reported that exposure to GLP-1 decreased glucose consumption while increasing lactate production, in those cells. Furthermore, GLP-1 modulates glucose metabolism of SCs and is suggested to have an anti-apoptotic effect in developing germ cells (Martins et al., 2019). In addition, it decreases testosterone secretion (Jeibmann et al., 2005), highlighting a possible

effect in the hypothalamus-pituitary-gonadal (HPG) axis. Furthermore, this hormone seems to increase GnRH secretion, which suggests that it may have an important role in puberty development (MacLusky et al., 2000).

Hyperglycemia, which is usually found in obese men, seems to cause a decrease in sexual hormone binding globulin (SHBG) production by the liver (Le et al., 2012). SHBG is responsible for the transport of sex hormones through the blood to the target tissue. Concurrently, with a decrease in SHBG concentration, testosterone availability will also decrease. Hyperglycemia per se has long been associated with compromised male fertility. The frequency and duration of hyper- and hypoglycemic events, the degree of glucose control, age, and other factors are important to evaluate the incidence of infertility (Holstein et al., 2012). However, it has been reported that poor glucose control, such as in diabetic men, is associated with impairment of sperm motility (la Vignera et al., 2009), sperm DNA fragmentation (Agbaje et al., 2007), and hormone dysregulation (Maric et al., 2010). In addition, gonadotropin secretion tends to progressively decrease in obese men (Pasquali, 2006). Overweight is also associated with increased androgen aromatization by the adipose tissue. This may result in a decrease in total and free testosterone in serum, associated with hypogonadism (Roth et al., 2008).

Fat Accumulation on the Scrotum

Obese individuals have an excess and abnormally distributed fat in the testis, a condition known as scrotal lipomatosis, and associated with infertility (Shafik & Olfat, 1981). The abnormal distribution of scrotal fat can form a diffuse sheet of fat with variable thickness and diffuse fat covers the cord veins and the spermatic cord. In addition, a lobular pattern of fat distribution can be found in the internal spermatic fascial tube (Shafik & Olfat, 1981). One of the main reasons why lipomatosis induces infertility is the impairment of thermoregulation in the testis of those individuals. Ideally, the scrotal sac is composed of thin skin with minimal subcutaneous fat, dense sweat glands, and scant hair distribution. These characteristics are essential for testicular aeration and heat radiation dispersion. Furthermore, vasodilation of the scrotal vessels and activation of the sweat glands are crucial for the maintenance of the testicular temperature 2–4 °C lower than the body temperature (Durairajanayagam et al., 2015). Excess scrotal fat increases insulation, rising scrotal temperature, and promoting testicular germinal atrophy and spermatogenic arrest. The reason behind germ cell vulnerability to heat stress lies in their higher mitotic activity. Specifically, hyperthermic testis induces germ cell apoptosis (Lue et al., 1999), autophagy (Zhang et al., 2012), DNA dam-

age, and generation of ROS (Ikeda et al., 1999). The excess of fat can compress the cord veins of the testis, being the cause of testicular ischemia, a condition characterized by the increased tension within the testis. Furthermore, the compromised blood pumping results in a venous status of the testis and testicular congestion (Shafik & Olfat, 1981) which, translates in metabolic impairment of the gonads.

Accumulation of Environmental Toxic Substances

Some environmental toxins, usually agriculture pesticides or industrial compounds, act as endocrine disruptors (Oliva et al., 2001). Indeed, obese individuals are at higher risk to suffer from the action of these disruptors since a large portion of them are liposoluble (Muñoz-de-Toro et al., 2006) and tend to accumulate in fat. However, the interaction among adipose tissue, environmental toxins, and male fertility remains to be elucidated. Still, the presence of organochlorines, a class of toxins usually found in pesticides (Jayaraj et al., 2017), has already been reported in the seminal fluid of men. Some studies have also evaluated the impact of these compounds on sperm quality parameters. The exposure to some organochlorines is associated with decreased sperm counts (Glass et al., 1979), while aromatic hydrocarbons, another class of toxins, are reportedly described as major contributors for the dysfunction of sperm parameters (De Celis et al., 2000). Although the presence of liposoluble toxins in the testis is likely one of the ways by which obesity induces infertility in males, this topic has been overlooked over the years (Cardoso et al., 2018).

1.2.5 Inflammation and Oxidative Stress

The balance between death/regeneration of developing germ cells is essential for the maintenance of spermatogenesis. ROS are important modulators of several apoptotic signaling pathways, including the p38 Mitogen-activated protein kinases (MAPKs) pathway. Damaged germ cells produce ROS, which can then activate the p38 MAPK pathway thus starting the apoptotic process (Ranawat & Bansal, 2009). This process highlights the importance of ROS in the regulation of testicular germ cell populations, under stress conditions. ROS produced by testicular macrophages also mediates steroidogenesis. The close physical association between this cell and LCs suggests that they are functionally related. Indeed, ROS inhibit cholesterol mobilization to mitochondria in LCs, which is a crucial step for steroidogenesis. Concurrently, the degradation of the steroidogenic machinery is promoted by ROS and other inflammatory cytokines (Diemer et al., 2003). Nevertheless, mature spermatozoa are highly susceptible to oxidative stress due to their limited amount of antioxidant machinery and cy-

toplasm (Asadi et al., 2017). Obese individuals have a high metabolic rate, to restore the energetic homeostasis of the body. As a result, oxygen consumption rates increase, leading to ROS overproduction through the mitochondrial respiratory chain (Turrens, 1997). This situation is aggravated by the permanent state of inflammation caused by the rupture of adipocytes due to triglyceride saturation (Kennedy et al., 2009). Consequently, macrophages invade the tissues, where proinflammatory cytokines are released (Olusi, 2002). In the testicular environment, ROS is a major contributor to sperm cell dysfunction, inducing DNA damage and compromising cell membrane integrity in spermatozoa (Asadi et al., 2017). Also, it has been observed that a higher amount of ROS in obese men causes altered DNA methylation, meaning that methylation changes in sperm may alter the embryo development and phenotype of the offspring (Tunç & Tremellen, 2009). This close relationship between inflammation and oxidative stress in the testis deserves special merit, particularly to understand the subfertility and infertility associated with overweight and obesity.

1.2.6 Direct Effects of Western Diet in Spermatic Quality

As human populations became more urban, fiber-rich diets were replaced by sugar and fat-rich diets. This change in food habits is often explained as those diets are affordable, readily available, and palatable. Along with that, our social environment invites to the consumption of large-sized meals while also promoting a sedentary lifestyle (Drewnowski, 2000). Nevertheless, the exact role of fat-rich diets on the development of obesity is still controversial. It is tempting to summarize it as fat-rich diets, along with a lack of exercise, ultimately leading to a positive energy balance, since energy intake is higher than energy expenditure, and thus weight gain. However, the metabolic significance of that weight gain depends on several factors, including the special distribution of fat, the type of fat, and the dysregulation associated with it. Depending on all those factors, distinct health effects can be induced. For instance, unhealthy diets rich in trans and saturated fats, red processed meat, refined grains, and sweets are associated with poor semen quality (Afeiche, Gaskins, et al., 2014). Meanwhile, healthy diets, rich in vegetables, fruits, whole grains, and seafood have been associated with better sperm parameters and a lower likelihood of having abnormal sperm concentrations, total sperm count, and motility (Karayiannis et al., 2016). However, this may be more complex than initially thought as some papers report that overweight and obesity are not associated with loss of sperm quality (M. Al-Ali et al., 2014; Rybář et al., 2011) Curiously, the hormonal disruption of the HPG axis was reported by the large majority of these studies (M. Al-Ali et al., 2014; Pauli et al., 2008; Rybář et al., 2011). This hypothesis proposes that

the main effect of obesity on male fertility is on libido and sexual performance rather than sperm quality parameters in itself. Furthermore, it is proposed that the detrimental effect of obesity on spermatic quality can only be observed in morbid-obese individuals (Pauli et al., 2008). Notwithstanding, spermatogenesis requires a large variety of building blocks during the formation of spermatozoa. This means that, along with the metabolic impairments associated with overweight/obesity, the food that individuals consume will have a direct impact on sperm production and will be reflected in the spermatic quality.

The Impact of Sugar-Rich Diets on Sperm Parameters

Some centuries ago, sugar was a delicacy only available for the wealthiest. The globalization of sugar started when the Portuguese began the exploitation of sugarcane and sugar refinery, in Brazil, through the implantation of a slave-based economy, during the late 15th century. The consumption of sugar raised to mass consumption around the mid-17th century due to the industrial revolution and, nowadays, sugar is one of the cheapest foods in the world, costing, on average, <10 cents per pound (≈450 g), or 1800 calories of sugar (Afeiche, Gaskins, et al., 2014). Furthermore, sugar is a powerful flavor booster, meaning that sugar-rich foods tend to be more palatable. The relationship among sugar consumption, body weight gain, and the incidence of obesity is widely acknowledged (WHO, 2000; 2014). Moreover, the consumption of high doses of sugar is associated with the development of complications normally associated with obesity, such as metabolic dysregulation, cardiovascular diseases, and T2D (WHO, 2014; 2016a). Until now, it remains unclear whether the damage to sperm is attributed to local effects from hyperglycemia or to disruption of the HPG axis. Uncontrolled hyperglycemia was associated with lower leptin secretion, promoting a decrease in LH secretion (Schoeller, Schon, et al., 2012). Both hormones play crucial roles in the integration of fat cells signals in the reproductive system. Indeed, the drop in leptin levels has been associated with reducing numbers of sperm within the seminiferous tubules and shrunken LCs in male mice (Fujikawa et al., 2010). Another study performed in mice revealed that long-term hyperglycemia leads to significant testicular dysfunction and decreased fertility potential (Cameron et al., 1990). After six months of induced hyperglycemia, it was observed fewer LCs. At the same time, it was also detected fewer LH receptors, leading to an accentuated fall of testosterone levels in diabetic animals. As a consequence, the seminiferous tubules from these animals presented depletion of germ cells and, structural abnormalities between Sertoli–Sertoli junctions were found (Cameron et al., 1990). In humans, hyperglycemia has been associated with an increase in sperm DNA damage (Agbaje et al., 2007). This factor may be related to poorer embryo

quality and implantation success in couples where the male suffers from DM (Mulholland et al., 2011). Alternatively, some contaminants, such as bisphenol A (BPA) and phthalates, were identified in processed sweets and sugarsweetened beverages. The presence of phthalates metabolites in the urine was significantly associated with decreased sperm motility, sperm DNA damage, and sperm aneuploidy (Jurewicz et al., 2013). These studies highlight that beyond the direct effect of sugarrich diets, we must take into consideration the hormonal dysregulation induced and the presence of contaminants that can mediate deleterious effects on the reproductive system and/or spermatic quality.

The Impact of Fat-Rich Diets on Sperm Parameters

Almost all dietary fats are triacylglycerols (composed by three fatty acids molecules bound by a glycerol molecule) which can be categorized into four distinctive classes: saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), polyunsaturated fatty acids (PUFAs), and trans fatty acids (TFAs). This classification is based on the presence, absence, number, and type of double bonds which alter the biochemical proprieties of the fatty acid molecule (Vannice & Rasmussen, 2014).

The first animal products consumed by men were derived from wild animals, whose fat content and quality in meat vary with species, age, sex, and season. In wild animals, MUFA and PUFA constitute the large majority of triacylglycerol present in the edible meat, since the SFA reservoirs are depleted during most of the year. Because of the seasonal depletion of SFA, pre-Neolithic diets were rich in MUFA and PUFA, which are known to prevent the onset of T2D and cardiovascular diseases (Cordain et al., 2005). Some centuries later, cattle started to be fed with grains and seasonal depletion of SFA stopped. This significantly decreased the animal life expectancy (only 24 months until slaughter), while excessively increased SFA content in the edible meat (Cordain et al., 2005). Ruminant animals are also natural producers of TFA, due to intestinal bacterial enzymes which catalyze the partial hydrogenation and/or isomerization of cis-unsaturated fatty acids. Thus, dairy products are also rich in TFA (Aro et al., 1998). Along with this, western-diet is known for the high consumption of processed products, rich in TFA due to the hydrogenation vegetable oils (Roe et al., 2013).

Spermatozoa fatty acids are predominately composed of SFA (around 60 %), however, their plasma membranes are predominantly composed of phospholipids and a significant amount of PUFA. TFA account, on average, for <1 % of the total fatty acids content of sperm (Chavarro et al., 2011). The authors further reported that total sperm TFA content was inversely correlated with sperm concentration while total sperm PUFA content was

positively correlated with that parameter (Chavarro et al., 2011). The intake of TFA was also inversely associated with sperm concentration. A proposed hypothesis is that TFA can interfere with the incorporation of PUFA into sperm membranes, consequently affecting spermatogenesis (Chavarro et al., 2014). Furthermore, the intake of TFA was reported to decrease total testosterone concentration and lower testicular volume, affecting, therefore, the HPG axis (Mínguez-Alarcón et al., 2017). In boars, the SFA content of sperm was negatively correlated with sperm quality. In opposition, PUFA sperm content was positively correlated with motility, viability, and normal morphology (Am-in et al., 2011). In humans, the intake of SFA has also been inversely correlated with sperm concentration and total sperm count (Jensen et al., 2012). Furthermore, the high intake of SFA and TFA represents a risk factor for asthenozoospermia (the medical term for reduced sperm motility) (Eslamian et al., 2015). Alternatively, it is relevant to note that anabolic sex steroids are administrated to cattle for growth promotion. Although the European Union banned this practice in 1989, it is still practiced in other parts of the world (Sundlof, 1989). The hormone levels in edible tissues are higher in treated animals than non-treated animals (Afeiche, Williams, et al., 2014; Andersson & Skakkebæk, 1999). Afeiche, Williams, et al. (2014) reported that intake of processed red meat was associated with a decreased sperm count in young males. Another study revealed that the consumption of processed meat during pregnancy is associated with lower sperm concentration among the male offspring (Swan et al., 2007). Furthermore, men who consume highly processed red meat were associated with a higher risk of asthenozoospermia (Eslamian et al., 2012). However, due to the paucity of data about this topic, further studies are needed to establish a solid correlation between meat ingestion and sperm parameters, particularly concerning meat origin and how it can induce metabolic signatures in sperm.

Metabolic Signatures Induced by Diabetes and Prediabetes in Testis

T2D is often associated with overweight and obesity, although it is not limited by it. In fact, it is reported as early as the 1980s that there are individuals with normal weight, considering BMI, who are 'metabolically obese' (Ruderman et al., 1981). Despite that, overweight and obesity, associated with increasing adiposity, are risk factors for the development of a phenotype coined as prediabetes and that can rapidly progress to T2D (International Diabetes Federation [IDF], 2017; WHO, 2016a). Prediabetes and T2D are characterized by persistent hyperglycemia and are caused by either an increasing degree of cellular insulin resistance or insufficient secretion of insulin in response to glycemia, by the pancreatic β-islets. The dysregulation of glucose homeostasis, also known as glucose metabolism or whole-body

metabolism, is the main driving force toward metabolic reprograming in different tissues. The persistent elevated glycemia levels are also related to T2D comorbidities, particularly microvascular (retinopathy, nephropathy, neuropathy) and macrovascular (coronary artery disease, myocardial infarction, stroke, congestive heart failure, and peripheral vascular disease) disorders (Long & Dagogo-Jack, 2011). Those disorders are further responsible for other typical complications found in diabetic patients, such as glaucoma, chronic kidney disease, poor wound healing, and increased infection risk (which often leads to amputation) (Díaz-López et al., 2015). The reproductive system is also affected by T2D-related comorbidities. Besides microvascular disorder associated with erectile dysfunction, diabetic men often suffer from retrograde ejaculation and subfertility. This is not surprising, as reproductive function requires proper nutritional support, and thus is sensitive to nutritional status and whole-body energy balance (**Crisóstomo** et al., 2018; P. F. Oliveira, Sousa, et al., 2017; Rato, Alves, Socorro, Duarte, et al., 2012). Recently, more evidence has been published regarding the metabolic changes induced by a hyperglycemic state in male reproductive function (Alves et al., 2015; **Crisóstomo** et al., 2018; Dias et al., 2014; P. F. Oliveira et al., 2015; Rato et al., 2013). Besides, there is a growing concern about the metabolic signatures of hyperglycemia in the male gamete, as a potential epigenetic factor capable of modulating the predisposition for the onset of metabolic disease on the offspring (Baxter & Drake, 2019; Perez & Lehner, 2019).

Glucose Homeodynamics and Spermatogenesis

In mammals, spermatogenesis is initiated during puberty. By the onset of this developmental phase, specialized neurons in the hypothalamus increase the frequency of the pulsatile secretion of GnRH, which is transported via the hypothalamic-hypophyseal portal system to the anterior hypophysis (also known as adenohypophysis), where it is recognized by GnRH receptors expressed by gonadotropes. In response, these cells synthesize and secrete into the bloodstream the gonadotrophic hormones, FSH and LH. In males, LH stimulates the production and secretion of androgens (*e.g.* testosterone and dihydrotestosterone) by LCs, which in turn are responsible for secondary sexual characteristics (Emerson, 2000). The main target of FSH is the SC (P. F. Oliveira, Cheng, et al., 2017), which acts as a central mediator in the differentiation process of germ cells, known as spermatogenesis (Nieschlag et al., 1999).

The onset of the GnRH pulse is one of the earliest known effects of metabolic variables. In humans, the onset of puberty varies in a board range in the function of climate, race, and gender (Karaolis-Danckert et al., 2009). However, regardless of any other factor, under-

nutrition delays the onset of puberty, while overweight and childhood obesity anticipate it (Martos-Moreno et al., 2010). Although this effect is not directly related to gamete epigenetic signature, in males, an early onset of puberty caused by overnutrition will mean that the first spermatogenic cycles will be completed in a high energy environment, capable of inducing irreversible changes in testicular metabolism (**Crisóstomo** et al., 2019). Even though spermatogenesis is the differentiation process of spermatogonia into mature spermatozoa, SCs are the main players in this process, providing nutritional, material, and structural support to differentiating germ cells. In fact, due to the blood-testis barrier, differentiating germ cells are secluded within the adluminal space of seminiferous tubules and confined to microenvironments created by SC cytoplasmatic protrusions. Therefore, the metabolic needs of germ cells depend on SC function, which in turn is sensitive to the metabolic status of the subject or animal. For instance, in SCs, the Ins2 (insulin-2) gene expression is regulated by Rhox family members, with a direct impact of insulin signaling (MacLean et al., 2013). Rhox is a homeobox gene sequence, which codes for a transcription factor that modulates the expression of target genes encoding proteins involved in processes relevant to spermatogenesis. Insulin has also an important regulatory role over the reproductive axis. Akita rats, a model for type 1 diabetes *mellitus* (T1D), were demonstrated to resume spermatogenesis after injection of external insulin doses, by the restoration of the normal blood concentrations of gonadotropins (Schoeller, Albanna, et al., 2012). High-energy diets were shown to induce a pre-diabetic state in rodents causing shifts in the glycolytic metabolic profile of SCs (Rato et al., 2013). The authors further elicited that this effect is linked to testosterone deficiency, caused by progressive stages of DM, ultimately translated into metabolic reprograming of SCs (Rato, Alves, Duarte, et al., 2015). Taken together, those studies report the effects of SC metabolic reprograming over sperm parameters in rodents (Rato et al., 2013; Rato, Alves, Duarte, et al., 2015). In sum, glucose homeodynamics during spermatogenesis causes metabolic signatures that can be further imprinted in sperm and open the possibility of changing the (epi)genetic information that is passed to the next generations.

Glucose Dysfunction, Sperm, and Reproductive Parameters

The impairment of sexual function is not regarded as one of the most immediate comorbidities of DM. Nevertheless, as the global prevalence of DM is expected to increase in the upcoming years, and, in the case of T2D, at progressively lower ages, there is a growing concern about the prevalence of the disease in men on reproductive age (IDF, 2017; WHO, 2016a). Moreover, as the reproductive function is dependent upon the individual's nutritional state, the

symptomatic loss of glycemic control observed in DM is expected to produce negative outcomes in sperm and reproductive parameters (**Crisóstomo** et al., 2018; P. F. Oliveira, Sousa, et al., 2017; Rato et al., 2013). Indeed, ejaculated sperm from men suffering from T1D and T2D presents poorer quality parameters (lower motility, viability, and DNA integrity) than normoponderal men (Roessner et al., 2012). In their study, Roessner et al. relate the deterioration of sperm parameters with an overactivation of apoptotic signaling in sperm cells, such as the caspase-3 (Casp3) programed cell death pathway. In couples followed due to fertility problems, the success rate of embryo transfers resulting from ART is lower when the male is diagnosed with DM (Mulholland et al., 2011). This effect is also reported in couples who did not seek medical advice toward ART: in couples where the male partner was affected by DM, the time-to-pregnancy was longer, independently of sexual frequency (Eisenberg et al., 2016b). The molecular mechanisms remain somewhat unknown, but several animal models support those observations and allow the study of some mechanisms. In one of the earliest studies on this phenomenon, Seethalakshmi et al. (1987) observed lower sperm counts and motility in streptozotocin diabetes-induced rats, concomitantly with elevated seminal fructose, lower gonadal weight and lower levels testosterone, LH, and FSH. After insulin treatment, all those parameters were recovered to control-like values (Seethalakshmi et al., 1987) illustrating that glucose dysfunction was responsible for the detected reproductive problems. Rats fed with a fat-rich diet develop a diabetic or pre-diabetic etiology (increase adiposity and hyperglycemia) which induces lower sperm quality but improvements in metabolic function and sperm parameters can be achieved after diet, exercise or a combined diet/exercise plan (Palmer, Bakos, Owens, et al., 2012). Exercise has been prescribed as a tool to improve glycemic control in humans, by improving overall glucose utilization at the cellular level with positive results (Rönn et al., 2013; Way et al., 2016). However, volume, intensity, and frequency of physical exercise must be dosed accordingly to the specificities of the diabetic patient (Colberg et al., 2016). Therefore, other metabolic solutions have been explored, such as nutritional control and functional foods. For instance, vitexin, and apigenin flavone glucoside present in some herbal infusions was shown to improve reproductive behavior, sperm and fertility parameters in streptozotocin-induced diabetic mice (Z.-M. Li et al., 2019). Similarly, the substitution of water by white tea has been demonstrated to improve sperm parameters in pre-diabetic mice (Dias et al., 2016). This effect was further explored *in vitro*, with human SCs, supplemented with epigallocatechin-3-gallate (EGCG), the most abundant tea catechin (Dias et al., 2017). At a concentration of 50 µM, EGCG increased glucose and pyruvate, maintained lactate production whilst decreasing oxidative damage and mitochondrial membrane potential of human

SC (Dias et al., 2017). These examples illustrate how nutritional variables can reprogram SC metabolism, which will ultimately affect spermatogenesis.

Small non-coding RNAs and Epigenetic Mechanisms May Also Be Affected by Metabolic Dysregulation

In recent years there has been increasing awareness regarding the epigenetic contents carried by spermatozoa. Notably, there is a growing number of published papers on sperm epigenetic regulation by the action of sncRNAs (piRNAs, tRNA-derived small RNAs (tsRNAs), miRNAs), DNA methylation, and chromatin dynamics (post-translational histone modification and protamination) (Baxter & Drake, 2019; Donkin & Barrès, 2018; Schagdarsurengin & Steger, 2016). piRNAs is the largest class of sncRNAs. They form RNA–protein complexes through interactions with Piwi-subfamily Argonaute (RNA-induced silencing complex) proteins. They are involved in epigenetic silencing, post-transcriptional silencing, and regulation of gene expression in germ cells. tsRNAs have similar functions, but those functions vary according to the length and the region from they are are cleaved from the mature transfer RNA (rRNA). There is also extensive literature supporting the essential role of RNA species for germline progression, as extensively reviewed by Lehtiniemi and Kotaja (2018). Presently, there are a plethora of sncRNA reportedly found in mammalian testes. Some of them, as the miR-34 and let-7 miRNA families (Frost & Olson, 2011) are sensitive to metabolic variables, namely T2D (Baldeón R. et al., 2015). Another study, in mice, showed that piRNAs were crucial spermatogenic regulators in meiotic cells. Herein, the authors reported the meiotic piRNA population can silencing specific mRNAs, regulating by this way the progression of meiosis (Goh et al., 2015). DNA methylation in spermatozoa is also affected by the metabolic profile. The DNA methylation pattern (which included FTO, MC4R, and other metabolic genes) found in sperm from men who underwent bariatric Roux-en-Y gastric bypass surgery was different from the pattern found in sperm from preintervention cohorts (Donkin et al., 2016). In addition, the differences were observed as early as one week after surgery, but even more evident after one year (Donkin et al., 2016). The Roux-en-Y is often called bariatric metabolic surgery because it not only acts to reduce weight but it is also effective in dampening insulin resistance (Dirksen et al., 2012). Despite this, it is not possible to unquestionably postulate whether the effects observed by Donkin et al. (2016) are a result of improved glycemic or weight/adiposity reduction. Sperm chromatin dynamics are also related to methylation, as both mechanisms regulate the accessibility to DNA sequences (genes) for further transcription. Besides, the enzymes responsible for DNA (de)methylation and histone

modification are sensitive to metabolic variables, and local availability of energy substrates and cofactors (Kaelin & McKnight, 2013). Herein, it is possible to include the sirtuin family of deacetylases, which nuclear members are known to control the epigenetic state of chromatin in an NAD^+-regulated manner (Kaelin & McKnight, 2013). Although chromatin is majorly condensed around protamines, the portions of histone-bound DNA in germline cells seem to bear essential information, notably imprinting genes, for adequate spermatogenesis (Marques et al., 2008), fertilization (van der Heijden et al., 2008) and early embryo development (Brykczynska et al., 2010). These changes, induced by metabolic dysfunction, can be further passed to the next generations (Figure 1.2.3).

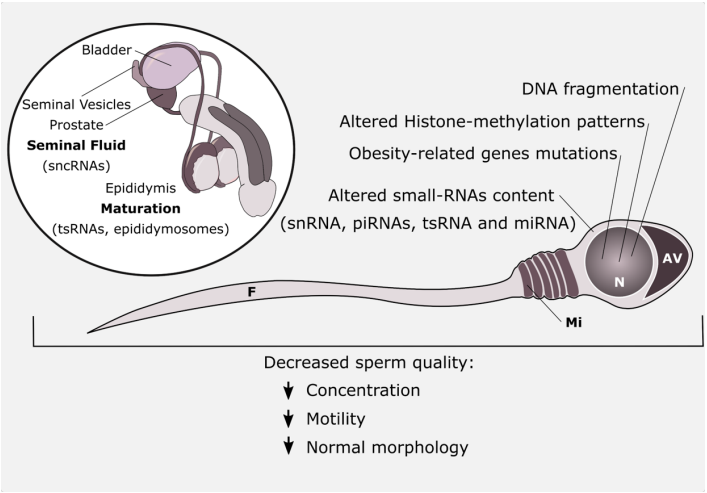


Figure 1.2.3: Epigenetic signatures carried by sperm that are affected by metabolic profile. Even after completing spermatogenesis, sperm epigenetic signatures are modulated through its transit in the male reproductive tract. For instance, the maturation process at the epididymides and the seminal fluid resulting from seminal and prostate secretions modulate the overall epigenetic cargo of spermatozoa. Obesity and T2D interfere with the relative abundance of different epigenetic factors, which are later expressed in phenotypic alterations such as lower sperm concentration, lower motility, and increased prevalence of abnormal spermatozoa. AV, acrosomal vesicle; F, flagellum; Mi, mitochondria; N, nucleus.

1.2.7 Paternal Transgenerational Effects of Metabolic Dysfunction

The epigenetic signatures carried by the spermatozoon that manages to fuse with the oocyte are inherited by the offspring (Gapp et al., 2014). The extent of epigenetic signatures of spermatozoa that influence the outcome of the offspring metabolic status can start from the early developmental phase to birth and even later into adulthood (Perez & Lehner, 2019). The findings in this field have shaken the very basis of Evolutionism, prompting a new glance of the role of Physiology over it, as emphasized by Noble et al. (2014) in their Editorial for 'The Journal of Physiology'. Epigenetic inheritance was a controversial theory because it was considered that epigenetic signatures were reset after gamete fusion to permit zygote's totipotency (Mayer et al., 2000).

For a long time, it was assumed that sperm could not carry potential epigenetic factors into the oocyte, especially because of protamination, demethylation, and the small cyto-

plasmatic volume. However, over the last decades, this perspective was drastically changed. Protamination replaces canonical histones with specific sperm protamine proteins after meiosis, an essential set for DNA compaction in mammalians (Braun, 2001). Protamines can compact paternal DNA to a millionth of its original volume, or six times denser than in somatic cells (Miller et al., 2010), which is crucial for sperm head size and dynamics (Gillies et al., 2009). It was thought that this process would remove all epigenetic modification from the sperm, hence epigenetic inheritance was primarily attributed to the mother (Wykes & Krawetz, 2003). Nowadays, it is known that ≈5 to 15 % of sperm chromatin remains nucleosome-bound (van der Heijden et al., 2008; Wykes & Krawetz, 2003). S. S. Hammoud et al. (2009) reported that the retained nucleosomes are significantly enriched in imprinted gene clusters, miRNA and other signaling factors. Therefore, it was postulated that retained histones could contain modifications that play an epigenetic role in embryonic regulation (S. S. Hammoud et al., 2009). Further studies consolidated this concept. A gene ontology analysis revealed that genes carried by histones were associated with metabolic and development processes (Arpanahi et al., 2009). Furthermore, even though the position of the histone is unaltered in both spermatozoa from lean or obese men (Arpanahi et al., 2009), DNA methylation patterns are markedly different (Donkin et al., 2016).

Methylation, notably on CpG islands (repetitive DNA sequences containing C-G nucleotides regulating gene expression) is erased in the primordial germ cells during mammal embryonic development (Messerschmidt et al., 2014). There is evidence now that this demethylation is not total: it is reduced by 70 percent on the first phase (Tang et al., 2016) and up to a minimum of 8 percent in humans after a second demethylation cycle (Gkountela et al., 2015). In fact, paternal obesity was correlated with altered methylation status of multiple imprint regulatory regions from the offspring genome. The authors measured the methylation percentage in the extracted DNA from the umbilical cord blood leucocytes of 92 newborns, born from parents defined as obese through the BMI scale. These results highlight how imprinted instabilities may be transmitted to the next generation, although the molecular mechanisms by which it occurs were not fully elucidated (Soubry et al., 2015). Genes involved in neurological diseases and metabolic regulation are epigenetic hotspots in gametes (Donkin et al., 2016). Interestingly, among the genes reported to be differently methylated in spermatozoa from obese and lean individuals, there is a group of genes that are potential carriers of polymorphisms associated with onset-obesity. Some genes from this group are the fat mass and obesity (FTO), melanocortin-4 receptor (MC4R), and the transmembrane protein 18 (TMEM18) (Donkin et al., 2016).

Paternal epigenetic inheritance was also considered unlikely as the spermatozoon was believed to simply carry genomic information, and it would not even contribute with mitochondria to the zygote. Moreover, even if it carried any other inheritance factors on its cytoplasm, the scale difference between it and the oocyte's cytoplasm would render them insignificant for the zygote's outcome. For some decades now, it is known that sperm is completely incorporated in most mammalian oocytes (Ankel-Simons & Cummins, 1996), and the first cases of mitochondrial heteroplasmy in humans were recently reported (S. Luo et al., 2018). Moreover, some epigenetic inheritance mechanisms have been exclusively identified in mammalian male gametes. For instance, contrarily to protamine regions of the sperm genome, sperm histones are not replaced by maternal nucleosomes after gamete fusion and contribute to zygote's chromatin (Arpanahi et al., 2009).

Although the mechanisms involved are not fully understood yet, the offspring of obese individuals are at greater risk toward the development of metabolic disorders, later in life. For instance, the mortality rate is increased in adult offspring of obese mothers, especially from cardiovascular diseases (Reynolds et al., 2013). Some studies have also demonstrated the potentially harmful effects of paternal food habits in the offspring health. The Överkalix study (Kaati et al., 2002) and the 'The Dutch Famine Birth Cohort Study' (Stein & Susser, 1975), both highlight the influence of parental food habits in the development of metabolic diseases in the offspring. Yet, animal models have provided the most evidence on the mechanisms underlying paternal transgenerational effects. The increase in metabolic diseases is expected to be accompanied by an increase in infertility. More people will be seeking fertility treatments and the levels of anxiety and depression associated with involuntary childlessness are expected to rise in the coming years (Hendriks et al., 2014). Therefore, it is essential to understand the molecular pathways by which metabolic disorders are inherited by the offspring of overweight/obese parents, and how the development of metabolic diseases could be prevented in predisposed individuals.

Evidence from Animal Studies

Animal studies are the best suited experimental model to study the transgenerational effects of the signatures induced by metabolic profile in male gametes. It is simply not possible timewise, nor is it ethical, to study those effects in a human population. Table 1.2.2 synthesizes several examples of studies where mammalian models were used. One of the earliest works on the function of mammalian sperm miRNAs revealed the role of miR-34c on the first zygote division, in mice (W.-M. Liu et al., 2012). Rats fed by a fat-rich diet overexpress let-7c miRNA

on sperm cells, a phenotype also observed in the adipose tissue of their offspring (de Castro Barbosa et al., 2016). Moreover, the female offspring showed a metabolic reprograming, related to shifts in let-7c expression and subsequent transcriptomic shift of its targets, causing glucose intolerance and resistance to diet-associated weight gain (de Castro Barbosa et al., 2016). Another breakthrough study, by Grandjean et al. (2016), showed how a miRNA can induce a metabolic disease phenotype *per se*. In this work, miRNA miR19b was injected in naïve mice zygotes, inducing a metabolic phenotype in resulting mice similar to the changes verified after diet-induced metabolic disease (Grandjean et al., 2016). These results need further confirmation in humans but are the basis of a novel concept regarding the inheritance of metabolic diseases.

Human Studies

Transgenerational studies in human populations present significant challenges due to contingencies of time and variable control. Nevertheless, there are two special cases where a human population and its offspring were described thoroughly, providing up to this day new data for retrospective studies on the effects of metabolic conditioning on the health outcomes of the offspring. Data from the late 19th and early 20th centuries, from the famous Överkalix study, revealed the consequences of the opposite stimuli, *i.e.* excess food supply, in a Swedish male population (Kaati et al., 2002). This study was based on a cohort of 320 individuals born in 1890, 1905, and 1920 in the parish of Överkalix in northern Sweden. Thanks to the record of local communities, it is known the food availability of each year during the 19th and early 20th centuries. This allowed the authors to estimate food availability during children slow growth period (SGP) (the period before the prepubertal peak in growth, 8–10 years for girls and 9–12 years for boys). Interestingly, sons of fathers who experience poor food availability during SGP were protected against cardiovascular death. This tendency was also found for grandsons of paternal grandmothers who were exposed to crop failure during their SGP. Grandsons of paternal grandfathers who were exposed to crop failure during their SGP had lower risk to have diabetes as a cause of death. As the authors highlight, the development of cardiovascular diseases and diabetes are also related to children's and grandchildren own nutrition, social environment, and adult life circumstances, which none were taken into consideration in this study. However, these results highlight the sensitivity of the SGP for the capture of environmental information, and gene expression alteration, which can then be transmitted to the next generation through the male line (Kaati et al., 2002). Another study, based in the same period and parish of Överkalix, revealed that food availability during grandfathers and

Table 1.2.2: Mammalian studies on paternal transgenerational inheritance of phenotypes associated with altered metabolic profiles (diet, metabolic disease, exercise).

Author(s), year	Metabolic trigger	Species, generations	Effects on offspring	Proposed mechanism
Carone et al. (2010)	Paternal low-protein diet (F0)	*Mus Musculus*, F0 + F1	Increased expression of genes related to lipid and cholesterol biosynthesis in liver, reduced cholesterol esters	Hypermethylation of Pparα gene
Ng et al. (2010)	Paternal fat-rich diet (F0)	*Rattus Norvegicus*, F0 + F1 (Female)	Pancreatic β-cell dysfunction	Inhibition of gene expression in pancreatic islet cells, hypomethylation of Il13ra2 gene
Guth et al. (2013)	Parental physical activity (F0)	*Mus Musculus*, F0 + F1 + F2	Changes in expression of metabolism-related genes. F1 females: Lower body height, decreased omental fat. F2 females: Increased glycaemia.	Germline inheritance of gene expression patterns
Wei et al. (2014)	Paternal prediabetes (F0)	*Mus Musculus*, F0 + F1	Glucose intolerance and insulin resistance	Changes to sperm methylation pattern
Grandjean et al. (2016)	Western-like diet (F0), RNA injection (F1)	*Mus Musculus*, F0 + F1, F1 (one-cell embryos)	Glucose intolerance and insulin resistance	Cytoplasmatic injection of sperm-borne miR19b into zygotes
Q. Chen et al. (2016)	Paternal fat-rich diet (F0)	*Mus Musculus*, F0 + F1	Impaired glucose tolerance and insulin resistance. Changes in gene expression of metabolic pathways in embryos and pancreatic islets	tsRNA delivery by epididymosomes to spermatozoa
Cropley et al. (2016)	Paternal obesity and prediabetes (F0)	*Mus Musculus*, F1 + F2 + F3	Impaired glucose and lipid metabolism. (F1: after dietary challenge; F2: without dietary challenge)	Changes to sperm content in sncRNA, notably tsRNA
de Castro Barbosa et al. (2016)	Paternal fat-rich diet (F0)	*Rattus Norvegicus*, F0 + F1 + F2	F1: Reduced body weight and pancreatic β-cell mass. F1 and F2 females: glucose intolerance and resistant to weight gain by fat-rich diet.	Alterations in methylation pattern of spermatozoa and in the expression of let-7c miRNA
Murashov et al. (2016)	Paternal physical activity (F0)	*Mus Musculus*, F0 + F1	Greater susceptibility for negative effects of fat-rich diets.	Changes in methylation profile and miRNA content of spermatozoa
Sharma et al. (2016)	Paternal low-protein diet (F0)	*Mus Musculus* and *Bos Taurus*, F0 + F1	Overexpression of squalene epoxidase gene (cholesterol biosynthesis) in liver.	rRNA fragments delivery by epididymosomes to spermatozoa, decreased miRNA let-7c expression

grandmothers' SGP was related to grandsons and granddaughters mortality, in a sex-specific matter (Pembrey et al., 2006). Grandsons relative mortality increased ($P = 0.009$) when paternal grandfathers had experienced a good food supply during SGP. Similarly, granddaughters relative mortality was two-fold higher when paternal grandmothers experience a good food supply during SGP. The opposite effect was found for grandparents who experienced a poor food supply during SGP (Pembrey et al., 2006). The same study also evaluated the effects of father's midchildhood smoking on the offspring BMI. For this, a questionnaire was done to soon-to-be fathers (April 1991 to December 1992) about their tobacco habits. The authors found that fathers with earlier onset smoking habits were more likely to smoke during conception, which was associated with increased BMI in sons at the age of nine (Northstone et al., 2014). These results highlight a sex-specific transgenerational effect of paternal grandparents to the relative mortality of grandsons and granddaughters, specifically. Similarly, the transgenerational effects of father mid-childhood smoking habits on his son BMI at nine years old reflect the importance of SGP for the capture of environmental information (Pembrey et al., 2006). Most importantly, these studies highlight that a male-line transgenerational response system exists in humans.

The second grand case report was provided by 'The Dutch Famine Birth Cohort Study' (Stein & Susser, 1975), as a result of observations collected from the Dutch population after World War II Dutch Famine. Indeed, this is a rare case where an advanced (high developmental index) society, where medical care is readily available and proper records are made, was affected by great stress. Thanks to medical records from this time and their descendants, it is now possible to relate epigenetic traits modulated by dietary behavior (nutritional restriction) and inheritable characteristics. Pregnant, malnourished women during this period, gave birth to underweight children who would develop metabolic complications later in life (Painter et al., 2008; T. Roseboom et al., 2006; T. J. Roseboom et al., 2001). Notably, the authors of those studies report a higher incidence of obesity and T2D (and cardiovascular disease) in this population comparing to cohort populations. These studies, analyzed nowadays with current knowledge, clearly support that there are metabolic signatures induced by diet.

None of these human studies can provide any data regarding the molecular mechanism involved in the transgenerational effects of paternal habits and offspring health, highlighting the importance of animal studies to explore these molecular mechanisms.

1.2.8 Conclusions

The health costs of our societal success are becoming progressively clear. In developed countries, independently of its geographical context, disease outbreaks caused by contagious diseases are rare nowadays and are accounted for by a low death-toll every year. However, this success demands a fast pace which often pushes individuals in adopting unhealthy lifestyles, such as poor food habits and sedentarism. Therefore, a new pandemic rose: the fat pandemic. Obesity, overweight and associated comorbidities (most notably DM) have promoted noncommunicable diseases to the main cause of death and, years of health loss, in developed countries (WHO, 2014). Despite several efforts to tackle the increasing prevalence of obesity, overweight, and DM worldwide, the WHO predicts its prevalence to keep this trend for the upcoming decades (IDF, 2017; WHO, 2016a). The reproductive function relies on a tight nutritional and hormonal balance; therefore, it is sensitive to diet variables and global nutritional status. Fat-rich diets can affect the reproductive outcomes of men as shown in recently published clinical and biochemical evidence. Several studies even have gone further to point toward a metabolic reprograming via transgenerational effects in the male lineage, which might trigger a predisposition for the onset of obesity and related comorbidities on the offspring.

The present data shows the urge for further investment in the biochemical mechanisms correlating fat-rich diets and male fertility outcomes. From all reported cases of infertility, one-third of them is still considered idiopathic, but it is reported that the success rate is lower if the male is overweight (Palmer, Bakos, Fullston, et al., 2012). The nutritional interventions have retrieved contradictory results so far, thus it is important to address which individual characteristics promote a positive reproductive response to those methodologies. The interaction of metabolic variables, as homeostasis but also at the reproductive tissue, need further description and are likely influenced and influencers of the endocrine balance. Yet, one of the key aspects in future research on this field is the transgenerational effects of nutritional variables, via the male gamete, and the inheritance of non-genetic factors.

Recent research on the inheritance mechanisms reveals a new level of complexity. Rather than considering each gamete fusion as a full biological 'reset' that would virtually guarantee the ideal survival chances for the offspring (and disregarding genetic inheritance), there is now evidence that environmental and behavioral variables may also be determinant in the health outcome of the next generations. These recently unknown mechanisms have been critical in evolutionary processes, but in our present, resourceful environment, they may pose a threat to our future as a species. The more is known about the impact of metabolic profile over

the signatures carried by (male) gametes, and their effects on the health of descendants, the more awareness about healthy food habits grows. Generally, individuals give more primacy to their offspring health than to their own health. Thus, research on this subject rises as a new hope to curb the 'fat epidemic'.

1.2.9 Author contributions

Sara C. Pereira and **Luís Crisóstomo** performed the bibliography search, screened the selected publications and wrote the manuscript. Pedro F. Oliveira and Marco G. Alves have critically reviewed the manuscript and conceptualized the review. Mário Sousa has critically reviewed the manuscript. All the authors contributed to manuscript writing/editing and approved the final version.

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1.3 Sperm, metabolic memory and echoes from Lamarck

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Abstract

- There is a widespread misconception that only maternal variables affect *in utero* development.
- Epigenetic markers carried by the spermatozoon are transmitted to the zygote.
- Sperm-born epigenetic factors influence *in utero* development, for various generations.
- Acquired traits of metabolic disease can be inherited by the offspring via the male gamete.
- Health assessment of future fathers is essential to predict the offspring's health.

1.3.1 Introduction

The importance of environmental variables in utero has been acknowledged for several decades. Clinical evidence regarding the negative impact of maternal alcohol and drug consumption, tobacco and prescription drug use, unhealthy diet and sedentarism, obesity and diabetes, and pollutant and chemical exposures has originated guidelines for pregnant women worldwide to reduce newborn mortality and morbidity rates sharply (WHO, 2016b). However, this evidence also created an incorrect assumption of the uterus as an "isolated system", whose conditions are uniquely dependent on the woman. However, recent reports highlight that the spermatozoon carries more than just genomic information that will influence the embryo development in the uterus. Notably, epigenetic signatures, such as DNA methylation and sncRNAs, are also carried by spermatozoa and encode acquired traits that may be inherited, hence programming the offspring.

1.3.2 The long way of epigenetic markers from testis to uterus

The role of the male gamete in embryonic development has long been neglected, mostly due to a lack of knowledge on the epigenetic markers that can "survive" in spermatozoon and the zygote. The advances made in the description of the epigenetic mechanisms created theoretical "dead ends" for the transgenerational inheritance of epigenetic signatures via the male lineage. Among these critical epigenetic points, probably the greatest obstacles were the theories of the "epigenetic reset" and the "bottleneck" (Figure 1.3.1). After the discovery of the importance of DNA methylation as an epigenetic mechanism to control gene expression, and especially considering that methylation marks are preserved in the semi-conservative replication of DNA, it was thought that germline cells could transmit those methylation patterns to

the offspring (Felsenfeld, 2014). However, it was previously demonstrated that germline cells go through several demethylation events to recover totipotency, even after gamete fusion. Moreover, it was known that histones were replaced by protamines during spermatogenesis, thereby erasing any histone modification that could alter the condensation state of chromatin (Puwaravutipanich & Panyim, 1975). Besides, according to the "bottleneck" theory, the huge difference in cytoplasmic volume between the spermatozoon and the oocyte was suggested to hinder any factors carried by the male gamete since it would be too diluted to have any significant impact on the developing embryo (Hoekstra, 2000).

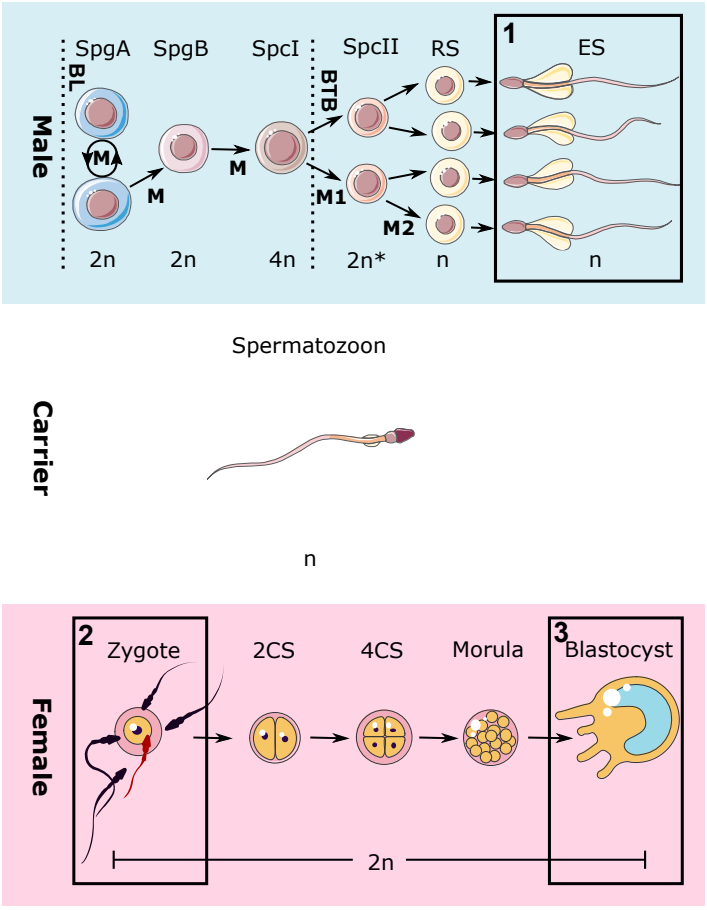


Figure 1.3.1: Critical barriers to paternal transgenerational epigenetic inheritance. Epigenetic remodelling events constantly modify the epigenetic cargo of male germline cells in the male body, in transit (spermatozoa), and in the female body. However, three major epigenetic events were thought to completely erase all potential epigenetic markers carried by spermatozoa. 1 — Histone removal and extensive cytoplasmic reduction. 2 — After fertilization: demethylation of paternal DNA and "bottleneck" effect. 3 — Extensive DNA remethylation. Abbreviations: BL — basal lamina; BTB — blood-testis barrier; M — mitosis; M1 — meiosis 1; M2 — meiosis 2; SpgA — spermatogonia A; SpgB — spermatogonia B; SpcI — primary spermatocyte; SpcI — secondary spermatocyte; RS — round spermatid; ES — elongated spermatid; 2CS — two-cell stage; 4CS — four-cell stage. *Although the secondary spermatocyte carries 2n chromosomes, this is the first haploid cell in the male gametogenesis.

It was known that environmental variables could affect health outcomes on the offspring, even by the male lineage. Probably the earliest scientific evidence of this phenomenon dates to 2002, to the Överkalix study in northern Sweden (Kaati et al., 2002). This study, based on historical records of three cohorts (born in 1890, 1905, and 1920) of the remote community, demonstrated that food availability during the SGP of boys influences the risk for the onset of obesity and diabetes in their grandsons. This work caused controversy due to the absence of a known mechanism that could support the inheritance of acquired traits via the male lineage, and also due to the weight of cultural factors that hampered the scientific relevance of the findings.

Following this study, several human and animal studies further supported the hypothesis that acquired traits could be equally transmitted via maternal and paternal lineage, but the mechanisms involved in the paternal epigenetic inheritance remained largely unknown. It was not before 2008 that a mechanism supporting transgenerational inheritance of acquired traits by sperm was described. One of the first advancements was the discovery that not all histones in sperm are replaced by protamines (van der Heijden et al., 2008). van der Heiden and colleagues further expanded the relevance of the retained histones by injecting human sperm into murine oocytes and showing that the retained histones of sperm chromatin contributed to paternal zygote chromatin (van der Heijden et al., 2008). This work highlighted that the epigenetic markers added to retained histones may be inherited by the offspring. However, and contrary to previous studies, it was not clear which traits could be transmitted. More complete evidence emerged from animal models of in utero exposure to different stimuli. In 2010, two research groups have independently reported that the administration of the fungicide vinclozolin to pregnant mice changed the methylation pattern of imprinted genes carried by spermatozoa up to two generations (Guerrero-Bosagna et al., 2010; Stouder & Paoloni-Giacobino, 2010). Their findings proved not only that the methylation pattern of sperm-carried DNA was not completely erased during spermatogenesis and fecundation, which can be inherited by the offspring, but also that environmental factors can change the localization and extension of methylation areas in sperm DNA. Soon other mechanisms of paternal transgenerational epigenetic inheritance were described. In 2014, Gapp et al. demonstrated that the intracytoplasmic injection of RNA extracted from sperm of traumatized mice into fertilized eggs induced the same behavioural changes in the offspring as those observed in fathers Gapp et al. One year later, another group published similar results, but using a rodent high-fat diet model (Grandjean et al., 2016). Besides, these studies identified sncRNAs as responsible for coding this epigenetic inheritance. Although the amount of injected RNA was manyfold greater than the volume carried by a spermatozoon, these works clearly demonstrated that sncRNA content carried by sperm cells can also be responsible for the transgenerational-acquired phenotypes observed in the male lineage in mammals. Surprisingly, despite the small cytoplasmic volume of the spermatozoon, biparental inheritance of mitochondrial DNA was recently described in humans (S. Luo et al., 2018), which further supports the role of paternal transmission of acquired traits.

1.3.3 Male epigenetic signatures influence *in utero* development

Once several mechanisms underlying the transgenerational inheritance of acquired traits via the male gamete started to be unfolded, new questions arose. When do the epigenetic signatures exert their action? To what extent do they affect health outcomes in the offspring?

Following the study published in 2010, Guerrero-Bosagna and colleagues have linked the changes in sperm DNA methylation pattern with sperm, testicular, prostate and kidney abnormalities (Guerrero-Bosagna et al., 2012). According to the authors, the methylation pattern was inherited during gonadal sex determination, when male germline cells are remethylated according to the original methylation pattern carried by sperm DNA (Skinner et al., 2010). Histones can also be methylated causing chromatin condensation, hence inhibiting gene expression. This mechanism is particularly crucial in sperm DNA because retained histones are associated with critical genes for embryo development (S. S. Hammoud et al., 2009). The demethylation of a histone subunit (H3K4me2) in mouse sperm, by action of a transgene, causes severe developmental impairment and decreases survival in the male offspring (Siklenka et al., 2015). Additionally, although the offspring had not been exposed to the transgene, sperm histone demethylation persisted for up to two generations and was identified even in the grand-grandsons of the mice exposed to the transgene. This study also elicits a crosstalk between diverse epigenetic mechanisms during embryonic development. At the two-cell stage, embryos of mice obtained from demethylated sperm had a reduced amount of RNAs, particularly sncRNAs (Siklenka et al., 2015). This effect is diluted in subsequent generations, but considering that spermatozoa are transcriptionally silent, this means that chromatin condensation state may regulate gene expression of both coding and noncoding genes from the early embryo.

Considering the crosstalk between different epigenetic inheritance mechanisms, Donkin et al. described a comprehensive profile of the epigenetic changes caused by obesity in human sperm (Donkin et al., 2016). They also compared the sperm epigenome of obese men before and after bariatric surgery. Additionally, the authors simulated the impact of those epigenetic markers in embryo development, using a bioinformatics analysis based on Gene Ontology (GO) terms. They found differences in the amount and distribution of DNA methylation in motile sperm between lean and obese men. Notably, protamine-bound regions contained more methylation changes than histone-bound DNA regions. Interestingly, most methylation changes occurred in genes related to embryonic development of the central nervous system and metabolism. Motile sperm from obese men presented higher amounts of sncRNAs, especially piRNAs related to chromatin condensation and food intake regulation (Donkin et al., 2016).

1.3.4 Neo-Lamarckism, metabolic memory and human evolution

The advances in the knowledge of the mechanisms underlying the transgenerational transmission of epigenetic markers have proven that the inheritance of acquired traits is not stochastic or, in humans, it is not limited to socio-cultural factors. These findings have provided molecular evidence supporting to some extent the first theory on the evolution of species, postulated by Jean-Baptiste de Lamarck in the XIXth century. This rediscovering of Lamarck's evolution theory based on the transmission of acquired traits for generations has even been coined as neo-Lamarckism (Gissis et al., 2011). Indeed, recent data based on animal and human studies have demonstrated that epigenetic markers in spermatozoa dynamically respond to environmental factors, with long-lasting effects for several generations (Table 1.3.1).

These findings have a critical importance for public health, as humankind fights against

Table 1.3.1: Selected studies eliciting a role of sperm epigenetic content in embryo development, and the associated developmental outcome.

Study	Species	Ancestral exposure	Epigenetic mechanism	Outcome in the offspring
Stouder and Paoloni-Giacobino (2010)	Mouse	Vinclozolin	Hypermethylation	Abnormal methylation pattern (somatic and germline cells)
Guerrero-Bosagna et al. (2012), Guerrero-Bosagna et al. (2010)[a]	Mouse	Vinclozolin	Abnormal methylation pattern	Abnormal methylation pattern (somatic and germline cells), sperm and testicular abnormalities
Gapp et al. (2014)	Mouse	Trauma/stress	Sperm-borne RNA (sncRNA)	Traumatic behaviour
Grandjean et al. (2016)	Mouse	High-fat diet	Sperm-borne RNA (mmu-miR-19)	Increased insulin resistance and body weight
Siklenka et al. (2015)	Mouse	Transgene (overexpression of hKDM1A)	Hypomethylation of retained histones (H3K4me2), reduced sncRNA	Impaired development, increased mortality (for 3 generations)
Donkin et al. (2016)	Human	Obesity, diabetes, high-fat diet, sudden weight loss	Abnormal methylation pattern (protamine-bound DNA regions)	Abnormal neuronal development, metabolic changes (theoretical)
Nätt et al. (2019)	Human	Short-term high-sugar diet	Overexpression of tsRNAs and mitochondrial sncRNAs in sperm	Abnormal tsRNA biogenesis, altered sperm mitochondria/cytosol compartmentalization (theoretical)

[a] Complementary studies.

the epidemic proportions of non-communicable diseases, caused by lifestyle choices. However, studies involving lifestyle interventions such as dietary change and/or adiposity loss have provided inconclusive outcomes on the transmission of acquired traits to the offspring. Particularly, the development phase of the subject seems to be of critical importance to revert acquired traits. Recently, **Crisóstomo** et al. demonstrated that mice fed with a high-fat diet from weaning to early adulthood, although they do not show long-lasting symptoms of metabolic syndrome (contrary to mice fed with lifelong high-fat diet), have poorer sperm parameters and altered testicular lipidome and metabolome at adulthood, comparing with mice fed with standard chow (**Crisóstomo** et al., 2019; **Crisóstomo** et al., 2020). These studies evidence that the SGP is critical to define the "metabolic memory" of testicular metabolism, and consequently sperm quality later in life. The findings further corroborate the results of the earlier Överkalix study, which refers to the nutritional status during the SGP as a critical factor to influence health outcomes in the offspring (Kaati et al., 2002). Although this study does not report data on the sexual health of the cohorts, it is likely that the "metabolic memory" is inherited by the offspring via spermatozoa and that it is coded by the epigenetic markers described in other studies (Donkin et al., 2016; Guerrero-Bosagna et al., 2012; Siklenka et al., 2015).

The epigenetic signatures carried by spermatozoa can also react to acute changes. Donkin et al. have observed an extensive epigenetic remodelling of motile sperm of men as soon as one week after bariatric surgery, especially in the number of methylated genes (Donkin et al., 2016). Another group showed that nuclear tsRNAs, and mitochondria-derived rRNAs and tsRNAs are overexpressed in sperm of men exposed to an acute highsugar diet (Nätt et al., 2019). It is likely that the changes observed in sperm epigenome, in response to nutritional status, affect embryo development, as demonstrated by earlier studies (Grandjean et al., 2016; Guerrero-Bosagna et al., 2012; Siklenka et al., 2015).

The human species have freed itself from the laws of neo-Darwinism, but the views of neo-Lamarckism show that our success can be our most significant threat. Lifestyle is becoming a crucial element of transgenerational health, a driving force of human evolution. Therefore, it is essential that better health policies promoting healthy lifestyle choices are designed to prevent an escalade in the prevalence of morbidity attributed to noncommunicable diseases.

1.3.5 Concluding remarks

Modern Medicine has pushed for a more inclusive perspective of pregnancy, where mother and father equally share responsibilities towards the best health outcomes of the neonate. This

effort has even been transcribed to the law in several countries, in different continents, where now fathers and mothers can enjoy parental leave to follow the baby for the first months of life. It is now time to push for more equality in the health follow-up of parents. The success of a pregnancy cannot be just attributed to the lifestyle, health status or simply physical "inability" of the woman to bear a child. It is time to acknowledge the influence of the man, his lifestyle and environmental exposures, and his global health status at conception, in the development of progeny. During the XXth century, more educated and healthy women led to a dramatic decrease in child mortality and morbidity worldwide. It is time to invest also in more informed and healthy fathers. Healthier fathers will contribute to healthier children and healthier grandchildren.

However, we are just taking the first steps to understand the mechanisms of paternal transgenerational epigenetic inheritance. New research must clarify the signalling pathways involved in the epigenetic regulation in response to environmental stimuli. Single-cell omics can contribute to unravel specific epigenetic signatures responsible for different outcomes in response to the same stimuli. It is also largely unknown the relationship between acute or chronic environmental exposures and the extent and nature of the resulting epigenetic signatures. From a clinical perspective, it is crucial to integrate this knowledge in terms of "health capital", that is to what extent epigenetic signatures are correlated with clinically significant phenotypes and how can we promote beneficial epigenetic changes in individuals.

1.3.6 Author contributions

Luís Crisóstomo performed the bibliography search, screened the selected publications, performed the analyses, and wrote the manuscript. Pedro F. Oliveira and Marco G. Alves have critically reviewed the manuscript and conceptualized the review. All the authors contributed to manuscript writing/editing and approved the final version.

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Chapter 2

Aims

The prevalence of overweight and obesity has significantly raised worldwide in the last decades. Projections predict a steady increase in this number, particularly in emerging economies, despite global efforts to curb this trend. Lifestyle is a major factor of this equation, notably the adoption of poor dietary habits and low levels of physical activity. Still, excess adiposity, a broad definition of overweight and obesity, is a multifactorial condition resulting from genetic predisposition, dietary habits, physical activity levels, health policies and social-economical-cultural context.

Recent reports add a new layer of complexity to this equation: the epigenetic inheritance of ancestral exposure to obesogenic environments. Data from the "Överkalix Study" and from "The Dutch Famine Cohort" showed that food supply has a significant role in the health outcomes of the descendants, up to two generations, even in humans. Additionally, adiposity reduction and diet are suggested to change epigenetic markers of the sperm, prompting the hypothesis that sperm can also be a vector of this inheritance of acquired traits. Other reports have further demonstrated a causal relationship between sperm epigenetic fingerprints and pathologies in the offspring.

Yet, it is unclear the impact of high-fat diets in testicular metabolism and how it correlates with sperm parameters and overall testicular function. Also, the role of the male in the transgenerational inheritance of environmental adaptations has been overshadowed by the female factor. Finally, the mechanisms underlying the transgenerational epigenetic inheritance via the male gamete are poorly understood, especially in the context of ancestral exposure to high-fat diets all life or even during childhood and adolescence.

Based on these premises, we have postulated three main hypotheses:

- **The adoption of high-fat diets, even if transiently, alters testicular metabolism and physiology.** A normal reproductive function strongly depends on energy homeostasis.

Hence, high-fat diets, due to their high caloric value per mass unit, disturb this balance with consequences to the reproductive function. In males, this high-fat-induced dysfunction may occur due to increased glucose intolerance and insulin resistance in testicular cells, but also due to endocrine disruption of the HPG axis. Moreover, Sertoli cells and mature spermatozoa have unique, extremely specialized cell membranes whose phospholipids have a critical role in the maintenance of their properties. In turn, these properties depend on the fatty acids that are incorporated in the apolar tails of the phospholipids. Hence, dietary fatty acids may affect the testicular content of different classes of fatty acids, with an impact on the properties and functions of cell membranes. Eventually, testicular cells metabolism adapts to the energy and nutrient intake of a high-fat diet, which may cause changes in testicular function and impact sperm parameters.

- **Ancestral exposure to high-fat diets affects testicular metabolism and function in non-exposed offspring.** "Metabolic memory" was coined with the maintenance of metabolic changes in the daughter-cells resulting from mitosis, notably in the context of the characteristic metabolic changes in diabetes. However, several studies described the inheritance of the metabolic state of the progenitor (including the male progenitor at the moment of conception) by the offspring. Considering the increase in the prevalence of obesity and the simultaneous decrease in human fertility and sperm quality, it is plausible that the adoption of high-fat diets is related to a deleterious testicular metabolic signature that is inherited by the offspring's testicular cells, causing similar effects. This "inherited metabolic memory" of ancestral exposure to high-fat diets may be transmitted via the male gamete and persist for several generations, despite the small cytoplasm contribution of the spermatozoon to the developing embryo. Besides, there is conflicting evidence in current literature concerning the transgenerational effects of high-fat diets on the metabolic and reproductive health of male offspring.

- **Sperm epigenetic fingerprints of ancestral exposure to high-fat diets.** To claim an "inherited metabolic memory" of ancestral exposure to a high-fat diet in testicular cells, an inheritance mechanism must be described. Long-term responses to environmental conditions are often regulated via epigenetic mechanisms. Nevertheless, epigenetic markers are almost completely erased during gametogenesis and embryogenesis. The erasure of epigenetic markers used to be one of the major barriers to Neo-Lamarckian theories based on the transgenerational epigenetic inheritance of acquired traits, but this view has been recently challenged. Sperm sncRNA content depends upon nutritional state,

adiposity and diet, and has been linked with health outcomes on the offspring. Hence, sperm sncRNA content is a potential epigenetic inheritance mechanism of testicular metabolic changes caused by direct and ancestral exposure to high-fat diets.

To test our hypotheses, we have designed a transgenerational animal model (*Mus musculus*, strain C57BL/6), extensively described in the following chapters. This experimental model permits the formulation of secondary objectives on which our main hypotheses rest upon:

- **The efficacy of diet reversion.** Although couples seeking medical advice due to fertility problems are often requested to lose weight, there is scarce scientific evidence supporting the efficacy of lifestyle intervention to rescue fertility rates. A link has been traced between male obesity and T2D, and decreased sperm quality and lower success rate of ART techniques. Notwithstanding, available literature on this association is conflicting and the real impact on fertility rates is not evident. Hereby we simulate a dietary intervention using a group of mice whose high-fat diet is switched to a standard diet in early adulthood. Comparing this group with mice fed exclusively with a high-fat diet or standard diet will clarify to what extent body adiposity, glucose homeostasis and testicular metabolism and function can be recovered by switching from a high-fat to a balanced diet.

- **Childhood obesity and future reproductive potential.** The reproductive system continues to develop post-partum, through childhood and adolescence until reaching sexual maturity. Therefore, environmental exposures during this period are potentially critical for a healthy reproductive function and to define reproductive potential later in life. Indeed, results from the Överkalix study highlight the importance of the nutritional status during the slow growth period for health outcomes later in life. Due to the inclusion of a transient high-fat diet group, we will demonstrate which tissues and organs present metabolic plasticity after sexual maturity.

- **The importance of duration of ancestral exposure.** The transgenerational effects of an ancestral exposure must vary according to the intensity and duration of exposure. This dose- and time-dependent effect has been previously reported in the scope of ancestral exposure to environmental toxicants and pollutants, but a similar effect is unclear in the context of lifestyle factors, like diet. With two groups exposed to high-fat diet for different durations, we can observe distinct responses in the progeny and even identify critical periods of exposure to high-fat diet.

- **Distinct diets, distinct testicular metabolic signatures.** According to the previous point, if the effects are distinct, then it is likely that the metabolic signatures associated with the altered or inherited phenotypes are also distinct. Any phenotypic difference found between the offspring of diet-challenged mice shall reflect a distinct metabolic signature. Considering sperm parameters, these signatures must be found in testes, whose primary function is to produce mature sperm. This is also valid for groups whose ancestors were exposed to high-fat diets for different durations.

- **Distinct metabolic signatures, distinct sperm sncRNA content.** Similarly, if ancestral exposures, inherited phenotypes and underlying metabolic fingerprints are distinct, then the sperm sncRNA must also be different. However, even if no changes are found in testicular metabolism and function, the analysis of sperm sncRNA content may provide hints of other target organs and systems in the progeny, in response to high-fat diet, especially during embryo development. The role of sperm sncRNAs in the transgenerational epigenetic inheritance of adaptions to high-fat diets provides meaningful information about past and future: it reflects the testicular environment in which sperm has gone through spermatogenesis; and it indicates target organs, cells and metabolic pathways of the progeny.

Chapter 3

A switch from high-fat to normal diet does not restore sperm quality but prevents metabolic syndrome

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Crisóstomo, **L.**, Rato, L., Jarak, I., Silva, B. M., Raposo, J. F., Batterham, R. L., Oliveira, P. F., & Alves, M. G. (2019). A switch from high-fat to normal diet does not restore sperm quality but prevents metabolic syndrome. *Reproduction*, *158*(4), 377–387. https://doi.org/10.1530/REP-19-0259

Abstract

In recent decades, the prevalence of metabolic diseases has concomitantly increased with a decline on fertility rates and sperm quality. HFDs are seldom considered part of the problem, but the molecular mechanisms underlying its effects on male fertility remain poorly understood. Herein we postulated that HFD alter sperm quality. We evaluated the effects of switching from a HFD to a normal diet in early adulthood on metabolic disease onset, testicular metabolism and sperm quality. Thirty-six male C57BL6/J mice were divided in: a control group fed with standard chow; a group fed with HFD for 200 days; and a group fed with HFD for 60 days and then with standard chow (HFD_t). Biometric data and whole-body metabolism were assessed. Epididymal sperm was studied for concentration, motility, viability and morphology. ^{1}H-NMR metabolomics approach was performed on testicular extracts to trace the metabolic changes. Diet switch reduced body weight and fat mass, preventing metabolic syndrome onset. However, sperm viability, motility and morphology were deteriorated by HFD consumption and not restored by diet switch. HFD induced irreversible changes in pyruvate and glutamate metabolism, ethanol degradation and ammonia recycling in testis. Furthermore, HFD_t changed purine and cysteine metabolism, urea cycle, and glutathione content. Overall, HFD caused irreversible changes in testicular metabolism even after switching to normal diet. HFD feeding until early adulthood decreases sperm quality, which cannot be restored by diet switch or weight loss, even when development of metabolic syndrome is avoided.

3.1 Introduction

Since the second half of the 20th century, the prevalence of metabolic disease has increased worldwide. Obesity and T2D are the most prevalent metabolic diseases, and are regarded as global epidemics by the WHO (2000). Obesity and T2D, are largely caused by lifestyle, particularly sedentary behaviours and poor dietary habits, such as high-fat diets (HFD). Interestingly, during the same period, several studies report a decline in human fertility rates, and particularly in male reproductive parameters (Hamilton & Ventura, 2006; Skakkebaek et al., 2016).

Indeed, couples with overweight and obesity attending fertility clinics have worse reproductive outcomes. Energy balance is crucial for spermatogenesis and overweight men are reported to present poorer sperm quality amongst other reproductive problems (P. F. Oliveira, Sousa et al., 2017). Similarly, men with T2D suffer from several reproductive problems, such as microvascular disease. Men attending fertility clinics are often requested to change their eating habits and lose weight to improve their sperm parameters. Nevertheless, few studies report the molecular basis linking food habits, overweight and poor male fertility. So,

the efficiency of dietary intervention such as reduced intake of dietary fats and weight loss upon male fertility is unknown.

Male reproductive function demands a considerable amount of energy to be sustained. Thus, metabolic regulation of testicular cells is crucial to maintain spermatogenesis (Rato, Alves, Socorro, Duarte et al., 2012). The elevated energy content of HFD (and hypercaloric diets) registers a metabolic signature in the body. The first visible effect of hypercaloric diets is the storage of excess energy as fat in white adipose tissue (WAT) pads. This accumulation promotes the onset of metabolic disease, translated into glucose intolerance, insulin resistance and insulin deficiency. The rodent model has been long used to carry out experimental designs and test hypotheses that would be impossible to test or take too long in humans, and to avoid the typical confounding factors of human subjects (Dutta & Sengupta, 2016).

In this work, we propose to study the molecular mechanisms by which HFD and HFD_t can alter male reproductive health, such as glucose homeostasis and metabolic changes in testes that compromise sperm quality. We used a C57BL6/J mice model fed with HFD or HFD_t and compared it with mice fed with a standard diet. The biometric data and the whole-body metabolic function were characterised. In addition, epididymal sperm parameters and testicular metabolic fingerprints were studied. The effect of switching from HFD to standard chow was given special attention. We integrated the data under a metabolomics based approach to establish a testicular metabolic profile and determine how this profile correlates with sperm quality, in animals fed with HFD and HFD_t.

3.2 Materials and methods

Animal model

Mus musculus C57BL6/J mice (n = 36) were randomly divided into three groups after weaning (21–23 days): control (CTRL; n = 12), HFD (n = 12) and transient HFD (HFD_t; n = 12). All mice were generated from normoponderal males and females, and they were subjected to the same random in utero stimuli. Mice from the CTRL group were fed with a standard chow (#F4031, BioServ, USA – carbohydrate: 61.6 %, protein: 20.5 %, fat: 7.2 %). Mice from the HFD group received a fat-enriched diet (#F3282, BioServ – carbohydrate: 35.7 %, protein: 20.5 %, fat: 36.0 %) during 200 days after weaning. The mice from HFD_t group were fed with a transient high-fat diet (#F3282, BioServ) for 60 days after weaning, then switched to standard chow (#F4031, BioServ). At 120 days post weaning, mice were randomly assigned to a normoponderal female, in mating pairs, 6 h per day, for seven consecutive days, for

breeding. Reproductive success rate, litter size and male ratio were assessed. Animals 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 is compliant with the ARRIVE guidelines and was licensed by the Portuguese Veterinarian and Food Department - DGAV (0421 /000 /000 /2016).

Glucose tolerance test and insulin resistance test

One week before killing (190–193 days after weaning), animals were subjected to intraperitoneal Glucose Tolerance Test (ipGTT) and intraperitoneal Insulin Tolerance Test (ipGTT) as described by the NIH Mouse Metabolic Phenotyping Center (MMPC) Consortium guidelines (Ayala et al., 2010). Briefly, 16–18 h before the test, access to food was removed from the animals (overnight fasting). For the ipGTT, an intraperitoneal injection with 6 mL glucose 30 $\%\frac{m}{v}$ per kg of body weight was given to each animal and blood glucose levels were measured every 30 min during 2 h. For the ipGTT, an intraperitoneal insulin injection (0.75 U per kg of body weight) was given to each animal (McGuinness et al., 2009) and blood glucose levels were measured every 30 min during 2 h.

Evaluation of epididymal sperm parameters

Epididymis 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 (Rato et al., 2013).

NMR spectroscopy

A combined extraction of polar and nonpolar metabolites from testicular tissue was performed as previously described (Alves et al., 2011). The aqueous phase containing polar water-soluble metabolites was lyophilised and analysed by NMR spectroscopy as described (Jarak et al., 2018). Metabolites were identified by comparing recorded spectra with reference spectra and the Human Metabolome Database (HMDB) (Wishart et al., 2007) and according to Metabolomics Standards Initiative (MSI) guidelines for metabolite identification (Sumner et al., 2007). Identification levels are indicated in Supplementary Table 3.S1 (see section on supplementary data given at the end of this article). After processing, ^{1}H spectra were processed using previously described methods (Jarak et al., 2018). Obtained peak areas were normalised by total spectral area and expressed in area units (a.u.) for multivariate and univariate analysis.

Metabolomics integration

Metabolites found to have differences between groups, or to have strong and significant correlations against tested parameters, were selected for metabolomics integration using MetaboAnalyst 4.0 tools (Chong et al., 2018). Pathway enrichment and topology analysis were used for metabolic pathway identification and evaluation of metabolic impact and significance. For Pathway Topology Analysis, we used the The Small Molecule Pathway Database (SMPDB) *Mus Musculus* database, relative-betweenness centrality and Fisher's exact test. Pathways with Pathway Impact (PI) > 0.1 and one-tailed unadjusted $P < 0.05$ were considered affected.

Statistical and correlation analysis

Different statistical methods were applied depending on the objective and the nature of data under analysis. One-way ANOVA with Tukey's *post hoc* test was used to compare biometric data, glucose metabolism and reproductive data between experimental groups. Normalised areas of testicular metabolite signals were also analysed by ordinary one-way ANOVA followed by Tukey's multiple comparisons test. Two-way ANOVA with Tukey's Honest Significant Difference (HSD) *post hoc* test was used to compare body weight, ipGTT and ipGTT. Mixed factorial ANOVA with Tukey's *post hoc* test was applied to compare sperm morphology, in respect to within-subject variance of the counts. Multivariate analysis based on Spearman's rank correlation (Spearman's r) was used to correlate the different sets of variables in study (testicular metabolite content, sperm parameters, global metabolic state and biometric data). The correlation strength was classified according to ranks (Taylor, 1990). Exact P value was calculated when 16 or less pairs were correlated. An approximate P value was obtained when more than 16 pairs were correlated. All methods were performed using GraphPad Prism 7.04 for Windows (GraphPad Software). Independently of the statistical method used, significance was considered whenever $P < 0.05$.

3.3 Results

Dietary switch from HFD to standard chow normalises biometric data and whole-body metabolic function

Body weight was monitored throughout the experiment (Figure 3.1a). Mice from CTRL group were the heaviest after weaning (CTRL: 13.09 ± 1.31 g; HFD: 6.64 ± 0.23 g; HFD_t: 7.38 ± 0.29 g), but that difference faded in 7 days. By the 35th day after weaning, mice from the HFD and HFD_t groups were heavier than mice from the CTRL group (CTRL:

23.0 ± 0.7 g; HFD: 24.0 ± 0.6 g; HFD_t: 26.0 ± 1.0 g). This difference increased up to 60 days after weaning, when mice fed with HFD and HFD_t weighed, approximately, 20 % more than CTRL mice. At this time point, HFD in mice from the HFD_t group was replaced by standard chow. Fourteen days later, HFD_t mice had lost weight and reached the mean values of mice from the control (CTRL: 27.0 ± 0.5 g; HFD_t: 28.0 ± 0.9 g), while mice fed with the HFD continued to gain weight (34 ± 1 g). During killing, HFD mice were 50 % heavier than CTRL and HFD_t mice.

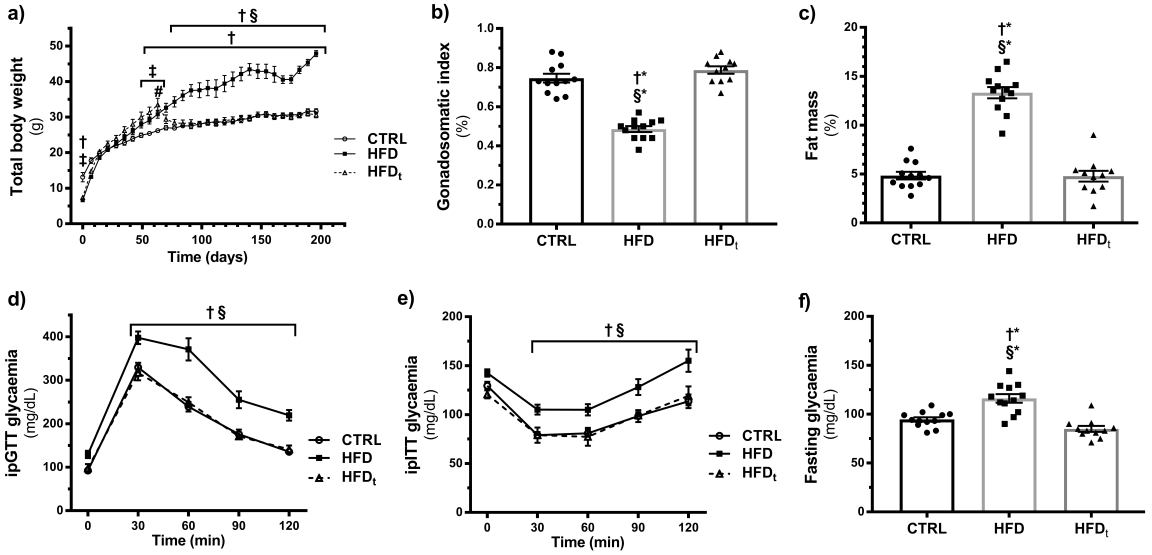


Figure 3.1: Biometric and metabolic characterisation of mice in this experiment, per group. Results are expressed as mean ± SEM (n = 12 for each group). a) Total body weight (g) evolution throughout the experiment (days). Weight was evaluated individually every 7 days. Results were tested by a repeated-measures (RM) two-way ANOVA with Tukey's multicomparison test. b) and c) Show the comparison of Gonadosomatic Index (GSI) and fat mass mean between experimental groups, respectively. Results are expressed as the mean (%) ± SEM (n = 12 for each group). Results were tested by one-way ANOVA with Tukey's multicomparison test. d) Mice glycaemia (mg dL^{-1}) during the ipGTT – intraperitoneal glucose tolerance test and e) during the ipITT – intraperitoneal insulin tolerance test. Results for ipGTT and ipITT were tested by RM two-way ANOVA with Tukey's multicomparison test. f) Data for fasting glucose before sacrifice was compared using ordinary one-way ANOVA with Tukey's multicomparison test. $^{\#}$Dietary switch; †CTRL vs HFD; ‡CTRL vs HFD_t; §HFD vs HFD_t. *P < 0.05; **P < 0.005; ***P < 0.0005; ****P < 0.0001.

Gonadossomatic index (GSI) (Figure 3.1b) was determined as the relative size of gonads to total body mass. Similarly, body fat (Figure 3.1c) was calculated as the ratio of adipose tissue to total body weight. GSI of mice fed with a HFD was lower than that in mice from CTRL and HFD_t group. Weight gain as WAT is the main source for body weight increase and lower GSI in HFD (Supplementary table 3.S2). Liver was 20 % heavier in mice fed with HFD (1.40 ± 0.09 g) than in CTRL mice (1.20 ± 0.02 g) and 27 % heavier than in mice from the HFD_t group (1.10 ± 0.03 g). Finally, mice fed with a HFD presented higher glycaemia levels during ipGTT (Figure 3.1d), during ipGTT (Figure 3.1e) and after overnight fast (CTRL:

$95 \pm 2\,\mathrm{mg\,dL^{-1}}$; HFD: $116 \pm 4\,\mathrm{mg\,dL^{-1}}$; $\mathrm{HFD_t}$: $85 \pm 3\,\mathrm{mg\,dL^{-1}}$) (Figure 3.1f). $\mathrm{HFD_t}$ mice showed fasting glycaemia levels in line with CTRL group.

Diet switch in mice does not restore the decreased sperm quality induced by consumption of a HFD

We collected sperm from mice epididymis immediately after killing and characterised sperm quality by analysing sperm count, viability, motility and morphology. We found no differences in sperm count in mice from the different groups (Figure 3.2a). However, mice fed with a HFD, even transiently, had approximately 12 % and 15 % less motile spermatozoa than mice from the control group (CTRL: $82 \pm 1\,\%$; HFD: $69 \pm 2\,\%$; $\mathrm{HFD_t}$: $66 \pm 4\,\%$ (Figure 3.2b). We have observed the same tendency in sperm viability. Mice from the HFD and $\mathrm{HFD_t}$ groups presented one-fifth less viable spermatozoa than mice from the control group (CTRL: $48 \pm 2\,\%$; HFD: $38 \pm 2\,\%$; $\mathrm{HFD_t}$: $35 \pm 2\,\%$) (Figure 3.2c). Concerning sperm morphology, smears from mice of the CTRL group displayed 6 % more normal spermatozoa ($40 \pm 2\,\%$) than mice fed with a HFD ($34 \pm 2\,\%$), while those mice had 6 % more ($44 \pm 2\,\%$) spermatozoa with tail defects than mice fed with $\mathrm{HFD_t}$ ($39 \pm 1\,\%$) (Figure 3.2d). In addition, the animals from the $\mathrm{HFD_t}$ group revealed the highest percentage of spermatozoa with head defects ($25 \pm 1\,\%$). Nevertheless, no changes were observed on any of the accessed reproductive parameters (reproductive success ratio, litter size and male ratio) (data not shown).

Diet switch in mice did not restore energy metabolism homeostasis in the testis

Overall, metabolome analysis revealed a unique metabolic signature for each group in mice testes. Regarding the content of metabolites related to energy producing metabolic pathways, it revealed evident differences between CTRL and groups fed with HFD (Figure 3.3). The AMP content in testes of mice from HFD and $\mathrm{HFD_t}$ groups was increased four-fold and three-fold respectively, when compared to the levels detected in the testis of CTRL mice (CTRL: $4.65 \pm 0.85 \times 10^{-4}$ a.u.; HFD: $1.96 \pm 0.18 \times 10^{-3}$ a.u.; $\mathrm{HFD_t}$: $1.38 \pm 0.36 \times 10^{-3}$ a.u.) (Figure 3.3a). Conversely, adenosine levels decreased by 30 % and 20 % in testes of mice from HFD and $\mathrm{HFD_t}$ (CTRL: $2.32 \pm 0.19 \times 10^{-3}$ a.u.; HFD: $1.55 \pm 0.10 \times 10^{-3}$ a.u.; $\mathrm{HFD_t}$: $1.84 \pm 0.27 \times 10^{-3}$ a.u.) (Figure 3.3b). Notably, the levels of lactate were not affected by diet regime (Figure 3.3c), whereas acetate levels were 50 % higher in the testis of mice from the CTRL group ($2.34 \pm 0.07 \times 10^{-3}$ a.u.) than in those from mice of the HFD ($1.56 \pm 0.08 \times 10^{-3}$ a.u.) and $\mathrm{HFD_t}$ ($1.49 \pm 0.06 \times 10^{-3}$ a.u.) groups (Figure 3.3d). Regarding succinate, the content in testes of mice from the $\mathrm{HFD_t}$ ($7.95 \pm 1.92 \times 10^{-3}$ a.u.) was more

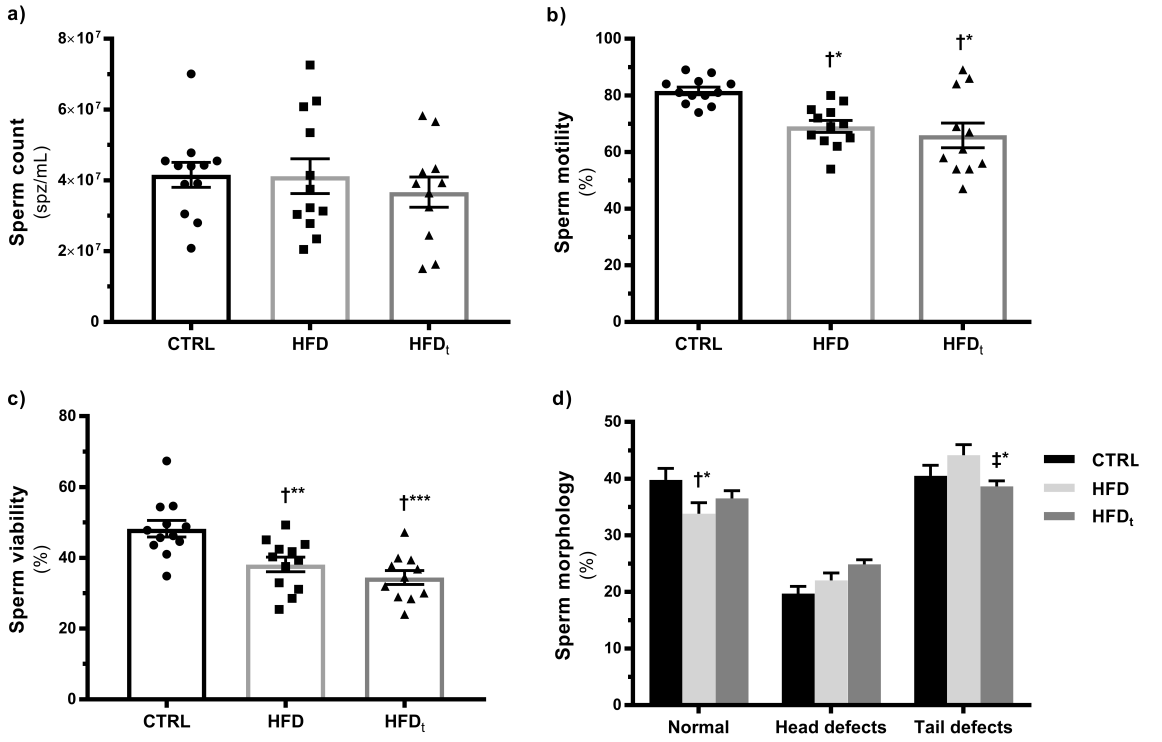


Figure 3.2: Characterisation of mice sperm parameters. Samples were directly obtained from mice epididymis. Results are expressed as the mean ± SEM (n = 12 for each group). Mice sperm concentration (a) and sperm motility (b) were assessed immediately after sample collection. Sperm viability (c) was evaluated in eosin-negrosin dyed smear, while sperm morphology (d) was classified in smears dyed with Diff-Kwik. Sperm morphology was grouped in three categories: normal, head defects (decapitated, pin head, flattened head), and tail defects (bent neck, coiled tail). Samples with more than 30 % decapitated sperm were not considered. The means for sperm counts, motility and viability were tested by one-way ANOVA with Tukey's multicomparison test. The means for sperm morphology classes were tested by mixed factorial ANOVA with Tukey's multicomparison test. Statistical significance was considered when $P < 0.05$. Significance marks stand as it follows: † vs CTRL; ‡ vs HFD; § vs HFD_t. *$P < 0.05$; **$P < 0.005$; *** $P < 0.0005$; ****$P < 0.0001$.

than two-fold higher than that in testes of mice from the CTRL group ($3.33 \pm 0.46 \times 10^{-3}$ a.u.), and more than three-fold the value obtained in testes of mice from the HFD group ($2.44 \pm 0.46 \times 10^{-3}$ a.u.) (Figure 3.3e). Creatine remained unchanged in testes of mice from the different experimental groups (Figure 3.3f).

Mice testicular content of amino acids remained altered even after diet switch

Testicular levels of six amino acids were analysed: three intermediaries of the alanine shuttle (alanine, glutamate and glutamine) and three branched-chain amino acids (BCAAs) (valine, leucine and isoleucine). No differences were found in the testicular content of alanine (Figure 3.4a). Glutamate was increased in testis of mice from the HFD_t group (Figure 3.4b), while testicular glutamine levels were higher in mice from the HFD and HFD_t groups (Figure 3.4c). Testes from mice fed with HFD_t were 10 % richer in glutamate ($1.61 \pm 0.03 \times 10^{-2}$ a.u.) than

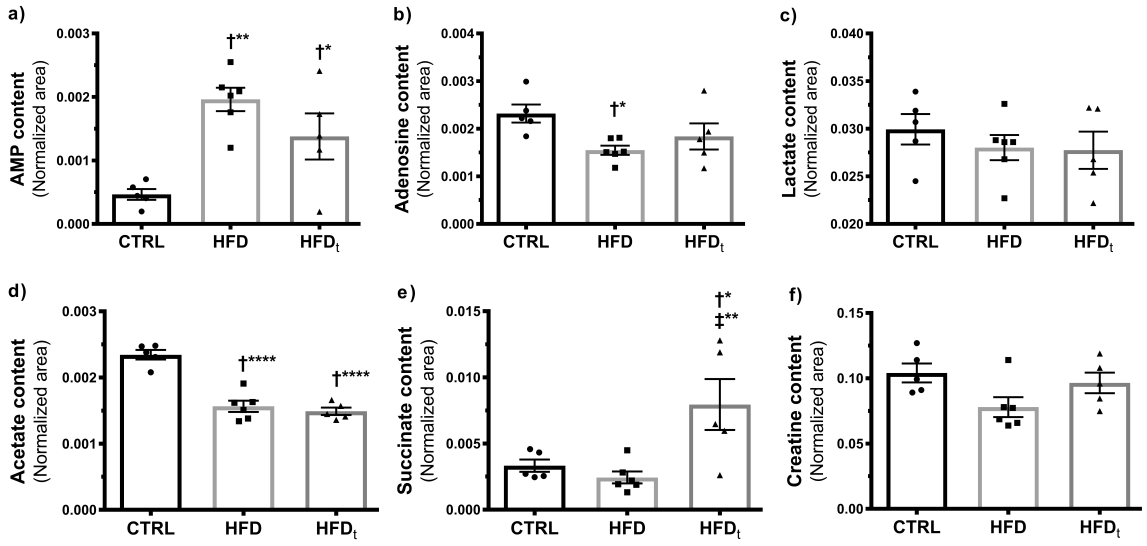


Figure 3.3: ^{1}H-NMR quantification of testicular metabolites related to energy-obtaining pathways. Absolute quantification of (a) AMP, (b) adenosine, (c) lactate, (d) acetate, (e) succinate and (f) creatine, metabolites involved metabolic processes related to energy obtaining, using ^{1}H-NMR. Results are expressed as mean ± SEM (n = 6 for each condition). Results were tested by one-way ANOVA with Tukey's multicomparison and were considered significant when $P < 0.05$. † vs CTRL; ‡ vs HFD; § vs HFD_t. *$P < 0.05$; **$P < 0.005$; *** $P < 0.0005$; ****$P < 0.0001$.

testes of mice from other groups (CTRL: $1.47 \pm 0.04 \times 10^{-2}$ a.u.; HFD: $1.49 \pm 0.04 \times 10^{-2}$ a.u.). The testicular content of glutamine in mice from HFD and HFD_t groups was respectively 15 ($5.57 \pm 0.21 \times 10^{-3}$ a.u.) and 20 % ($5.79 \pm 0.11 \times 10^{-3}$ a.u.) greater than those from the CTRL group ($4.82 \pm 0.24 \times 10^{-3}$ a.u.). Concerning BCAAs, valine (Figure 3.4d), leucine (Figure 3.4e) and isoleucine (Figure 3.4f), we have only observed alterations in the testicular content of leucine. Its levels were 19 % lower in testes of mice fed with HFD ($1.54 \pm 0.11 \times 10^{-3}$ a.u.) and 8 % lower in testes of mice fed with HFD_t ($1.75 \pm 0.07 \times 10^{-3}$ a.u.) than in testes of mice from CTRL group ($1.91 \pm 0.05 \times 10^{-3}$ a.u.).

Diet switch in mice was not able to restore the oxidative profile in testis

Changes were also observed in the testicular levels of metabolites related to antioxidative defence, in a diet-specific manner (Figure 3.5). Our results showed that betaine increased approximately by 20 % in testis of mice fed with HFD ($9.51 \pm 0.30 \times 10^{-2}$ a.u.), comparing to the levels detected in the testis of CTRL mice ($7.88 \pm 0.46 \times 10^{-2}$ a.u.) and mice fed with HFD_t ($8.20 \pm 0.31 \times 10^{-2}$ a.u.) (Supplementary Table 3.5a). No differences were found in testicular content of taurine (Supplementary Table 3.5b). Glutathione, the key cellular antioxidative component in mammals, was 60 % more abundant in testes from both mice fed with HFD ($3.68 \pm 0.28 \times 10^{-3}$ a.u.) and HFD_t ($3.65 \pm 0.24 \times 10^{-3}$ a.u.), comparing to CTRL mice ($2.27 \pm 0.46 \times 10^{-3}$ a.u.), suggesting increased oxidative stress (Supplementary

Figure 3.5c). However, unlike betaine, testicular glutathione levels were not restored by diet switch.

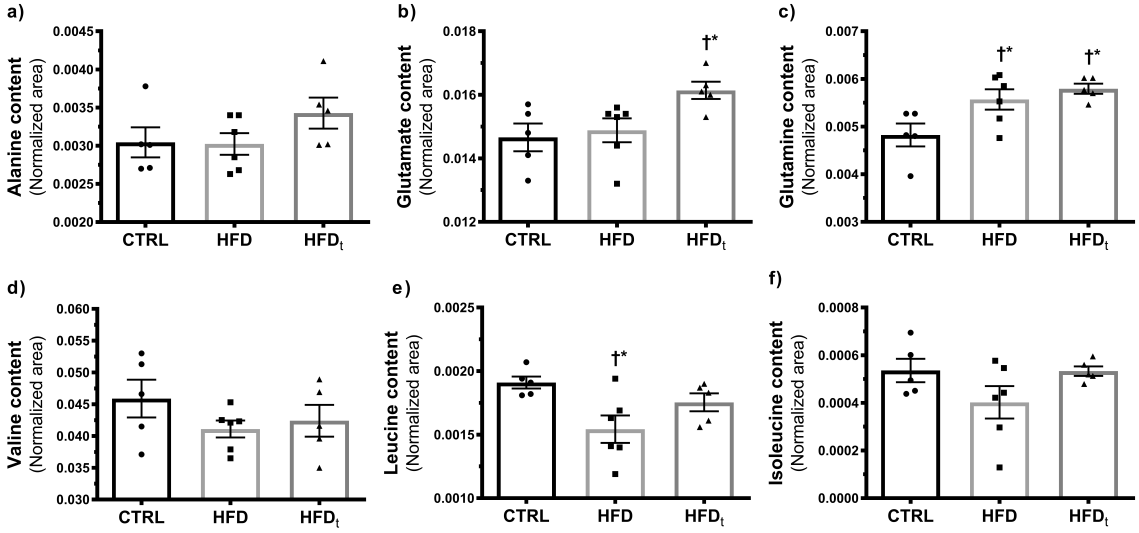


Figure 3.4: ^{1}H-NMR quantification of testicular amino acids (AAs). Absolute quantification of testicular content of the amino acids (a) alanine, (b) glutamate, (c) glutamine, (d) valine, (e) leucine and (f) isoleucine, using ^{1}H-NMR. Results are expressed as mean ± SEM (n = 6 for each condition). Results were tested by one-way ANOVA with Tukey's multicomparison, and were considered significant when $P < 0.05$. † vs CTRL; ‡ vs HFD; § vs HFD$_t$. *$P < 0.05$; **$P < 0.005$; *** $P < 0.0005$; ****$P < 0.0001$.

Testicular metabolic profile is associated with glucose (in)tolerance and sperm quality

Individual values for biometric data, glucose homeostasis, sperm parameters and testicular metabolites were mutually correlated (Supplementary Tables 3.S3, 3.S4, 3.S5, 3.S6, 3.S7, 3.S8, 3.S9 and 3.S10). A strong correlation was found between total body weight and fat mass ($r = 0.8209$, $P < 0.0001$), and individual fat depots; epididymal fat ($r = 0.8793$, $P < 0.0001$), perirenal fat ($r = 0.8959$, $P < 0.0001$) and retroperitoneal fat ($r = 0.7695$, $P < 0.0001$, Supplementary Table 3.S3). Strong correlation was observed between total body and liver weight ($r = 0.7015$, $P < 0.0001$). WAT weight and fat mass establish positive correlations with Area Under the Curve (AUC) in ipGTT and ipGTT (Supplementary Table 3.S4). WAT weight correlates with sperm defects and notably, retroperitoneal fat was correlated with pin head spermatozoa (a type of head defect) ($r = 0.4338$, $P < 0.05$) (Supplementary Table 3.S5). Insulin resistance is a risk factor towards abnormal spermatozoa ($r = -0.5029$, $P < 0.005$) (Supplementary Table 3.S6). Overall, WAT was associated with a decrease in leucine, isoleucine and succinate, while brown adipose tissue (BAT) negatively correlates with isoleucine ($r = -0.117$, $P < 0.05$) (Supplementary Table 3.S7). Total body weight showed a very strong positive correlation with taurine ($r = -0.8194$, $P < 0.0005$), whereas taurine was negatively correlated to testis weight. Glucose intolerance is correlated with betaine ($r = 0.5180$, $P < 0.05$), conversely to

succinate ($r = -0.5059$; $P < 0.05$) and creatine ($r = -0.7285$, $P < 0.005$) (Supplementary Table 3.S8). A correlation matrix illustrates the relation between testicular metabolites and sperm parameters (Figure 3.5a). Amino acids were negatively correlated with sperm counts (sperm concentration) and sperm viability; for example, glutamine is positively correlated to pin head defects ($r = 0.5771$, $P < 0.05$). Acetate was found to be a protective factor for sperm quality (Supplementary Table 3.S9).

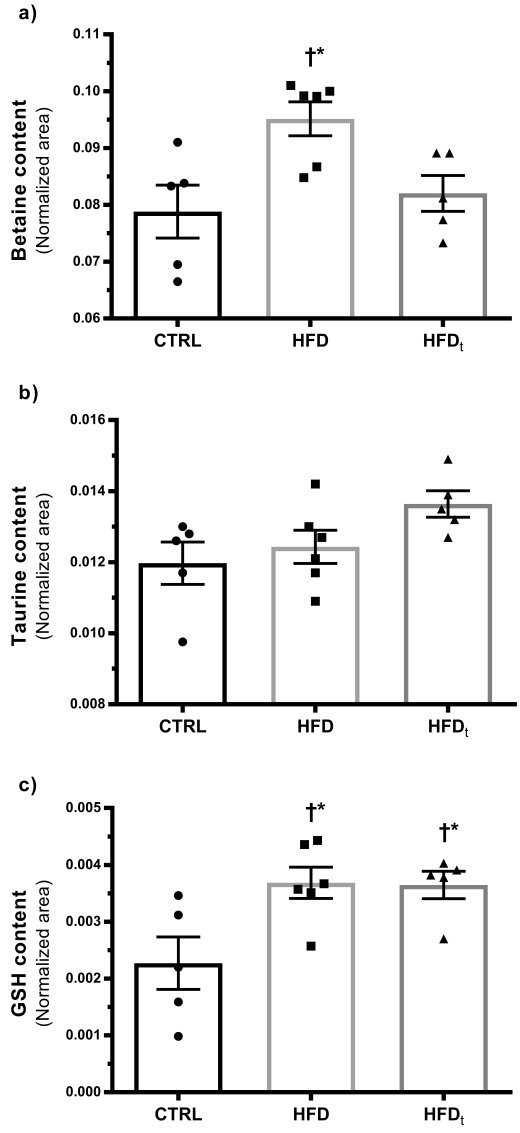


Figure 3.5: ^{1}H-NMR quantification of testicular antioxidants. Absolute quantification of (a) betaine, (b) taurine and (c) glutathione, metabolites involved in the antioxidative action, using ^{1}H-NMR. Results are expressed as mean ± SEM (n = 6 for each condition). Results were tested by one-way ANOVA with Tukey's multicomparison and were considered significant when $P < 0.05$. † vs CTRL; ‡ vs HFD; § vs HFD_t. *$P < 0.05$; **$P < 0.005$; *** $P < 0.0005$; ****$P < 0.0001$.

Male reproductive dysfunction in mice is caused by metabolic interference of HFD

After analysing all the individual correlations between biometric data, glucose homeostasis, sperm parameters and testicular metabolome of our experimental model, we explored how they associate with the differences observed in the first stage of our study (Figure 3.6a). For mice fed with HFD, four metabolic pathways were found impacted in testes (Figure

3.6b). Pyruvate (PI = 0.15, $P = 1.10 \times 10^{-3}$, False Discovery Rate (FDR) = 3.60×10^{-2}) and glutamate metabolism (PI = 0.14, $P = 1.46 \times 10^{-3}$, FDR = 3.60×10^{-2}) were the most significantly affected pathways in testes. Ethanol degradation (PI = 0.49, $P = 3.48 \times 10^{-3}$, FDR = 6.89×10^{-2}) and ammonia recycling (PI = 0.22, $P = 9.87 \times 10^{-3}$, FDR = 0.14) were also affected. The same metabolic pathways were affected in mice fed with HFD_t (Figure 3.6c). Glutamate metabolism was the most affected metabolic pathway (PI = 0.45, $P = 2.60 \times 10^{-6}$, FDR = 2.57×10^{-4}), three-fold more impacted than in HFD group. Ammonia recycling (PI = 0.31, $P = 5.75 \times 10^{-4}$, FDR = 1.14×10^{-2}) was 40 % more impacted in testes of mice fed with HFD_t. Pyruvate metabolism and ethanol degradation were equally impacted in testes of mice from HFD and HFD_t. Additionally, HFD_t mice suffered impact in testicular purine metabolism (PI = 0.12, $P = 5.25 \times 10^{-4}$, FDR = 1.14×10^{-2}), urea cycle (PI = 0.19, $P = 4.55 \times 10^{-4}$, FDR = 1.14×10^{-2}), cysteine metabolism (PI = 0.29, $P = 9.93 \times 10^{-3}$, FDR = 6.15×10^{-2}) and glutathione metabolism (PI = 0.41, $P = 9.10 \times 10^{-4}$, FDR = 6.01×10^{-2}).

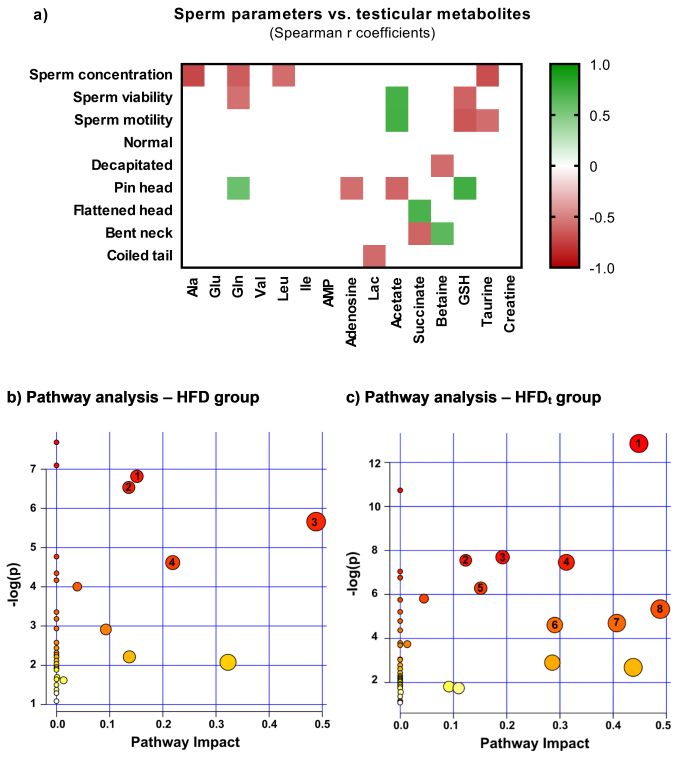


Figure 3.6: Metabolomics integration of NMR data. Summary of main metabolomic correlations drawn from our experimental model in testis. (a) Simplified correlation matrix of sperm parameters vs testicular metabolites. The scale represents Spearman r coefficient values, when $P < 0.05$ (n = 16, Exact P value). (b) MetaboAnalyst 4.0 Pathway Analysis Tool was used to identify the most affected metabolic pathways in HFD mice, using the list of metabolites which presented differences from CTRL group. Hence, input metabolites were: leucine, betaine, AMP, acetate, glutamine and glutathione. 1 – pyruvate metabolism; 2 – glutamate metabolism; 3 – ethanol degradation; 4 – ammonia recycling. (c) The same methodology as used to profile the most relevant metabolic changes in HFD_t group. Input metabolites were adenosine, succinate, glutamate, AMP, acetate, glutamine and glutathione. 1 – glutamate metabolism; 2 – purine metabolism; 3 – urea cycle; 4 – ammonia recycling; 5 – pyruvate metabolism; 6 – cysteine metabolism; 7 – glutathione metabolism; 8 – ethanol degradation.

3.4 Discussion

The obesity pandemic and the decline of sperm quality share a timeline with a common denominator: HFD (Skakkebaek et al., 2016; WHO, 2000). Yet, there is a gap on mechanistic studies on the subject. Clinical studies often have confounding factors. Thus, animal models can be a reliable choice to study the molecular mechanisms of HFD-induced male infertility. Herein, we used NMR-based metabolomics to identify testicular metabolic fingerprints induced by HFD and how weight loss caused by dietary switch from HFD to normal diet affects sperm quality, in a rodent model (*Mus musculus*). A number of metabolomics-based studies were already used to unveil the molecular mechanisms associated with male fertility (dys)function (Deepinder et al., 2007; Minai-Tehrani et al., 2016). Recently, we described ageing-associated testicular changes using NMR-based metabolomics in a rodent model (Jarak et al., 2018). In the present study, we detected distinct testicular metabolic signatures in animals fed by HFD, even if temporarily. Additionally, changes observed in testicular metabolome were correlated with general metabolic status and parameters of sperm quality.

Our group has extensively addressed the changes induced by HFD and related diseases in the male reproductive system. We have described mechanisms by which HFD induce a pre-diabetic state and cause functional changes in the testis (Rato et al., 2013). In addition, we demonstrated how energy homeostasis triggers specific metabolic shifts in SCs (Jesus et al., 2016; Martins et al., 2019; Martins et al., 2015; Martins et al., 2016). We further discussed how excess accumulation of lipids due to obesogenic substances impairs sperm maturation (Rato et al., 2017). Besides, deleterious effects associated with pathological states in energy homeostasis impair endocrine function and metabolic balance in testis, which can cause abnormal sperm parameters (Alves et al., 2015; Rato, Alves, Dias et al., 2015; Rato et al., 2014), suggesting functional changes in testicular cells beyond the somatic SCs. In the present work, we add a new level of evidence, based on Multivariate Analysis and Metabolomics, to show that the metabolic fingerprints induced by HFD in testes may not be fully reverted by dietary switch. Poorer sperm quality was found in mice fed with a HFD. Interestingly, even though mice from the HFD_t group were fed with a balanced diet for the majority of their lives, some metabolic changes and defects in sperm quality caused by HFD and/or previous obesity were not reversed by dietary change/weight loss. The period that mice from the HFD_t were fed with a HFD (60 days) overlaps with childhood, puberty and the early years of adulthood, a critical age for male reproductive maturation (Dutta & Sengupta, 2016). The first two spermatogenic cycles in mice occur during this period (Whittingham &

Wood, 1983), stressing the importance of testicular environment in this phase for successful reproductive outcomes later in life (Woodruff et al., 2008). This fact is even more interesting considering that diet switch and weight loss were effective in recovering mice weight to normal level and in preventing the onset of metabolic syndrome. In *Mus musculus*, a spermatogenic cycle takes approximately 9 days, while spermatogenesis takes 40 days (Costa et al., 2018). Therefore, in mice from the HFD_t group, the time between diet reversion and killing allows the completion of 11 spermatogenic cycles, which are enough to correctly assess the impact of the new diet in sperm parameters. Nevertheless, it is interesting that sperm parameters did not lead to poorer reproductive outcomes. No differences were found in pregnancy success rates, litter size or male ratio, though litter size was smaller than officially reported in this strain (Murray et al., 2010).

Our data show that WAT is the main source for body weight differences observed between groups. Besides, the differences detected in liver weight underline the role of this organ in mediating the effects induced by HFD. We found a very strong positive correlation between WAT and liver weight, particularly in HFD mice. This correlation has been widely described and reflects steatosis caused by diet (Fraulob et al., 2010; Gao et al., 2015; Lai et al., 2015; Samuel et al., 2004), a condition that may progress to non-alcoholic fatty liver disease (NAFLD) and ultimately to cirrhosis. In addition, WAT accumulation and liver enlargement (steatosis) are predictive factors for metabolic dysfunction, towards metabolic disease (Dixon et al., 2001; Kotronen & Yki-Jarvinen, 2008). We found poorer performance of mice fed with HFD during ipGTT and ipGTT, which reflect metabolic syndrome onset. In fact, WAT deposits are extremely correlated with poorer performance in ipGTT and ipGTT and can be regarded as the link between HFD and the onset of metabolic disease (Rato et al., 2013).

Testicular metabolome and evaluated sperm parameters were distinct in HFD and HFD_t groups, although globally negatively affected by HFD. In both groups, testicular metabolome was enriched in AMP, glutamine and glutathione, whereas acetate content was strikingly reduced. In addition, nucleic acid metabolism was significantly affected by HFD. AMP participates in energy production homeostasis, which is indispensable in highly metabolically active tissue. AMP is involved in the regulation of lipolysis and lipogenesis in response to insulin; thus, it is a marker for whole-body metabolic homeostasis (Hajer et al., 2008). The increase in AMP content was correlated with the weight of BAT, WAT and liver, but not total body weight. Also, AMP is involved in spermatogenesis through cAMP activated signalling pathways AMP-activated protein kinase (AMPK). AMPK signalling is an important regulator of the nutritional support of spermatogenesis by regulating SC metabolism

(Galardo et al., 2007), thus highlighting the relevance of these signalling pathways and how they respond to fat in the testicular tissue. However, the metabolic hallmarks observed, as glucose intolerance, are mostly correlated with epididymal fat, which humans lack (Wrońska & Kmieć, 2012). Thus, caution should be taken when discussing these effects in humans. Yet, the model was able to correlate retroperitoneal fat weight with the prevalence of head and tail defects in sperm of mice (X. Chen et al., 2013; McPherson & Lane, 2015), which was already reported in humans (Martini et al., 2010; Rato et al., 2017).

Concerning testicular metabolome, we found that glutamine and acetate were correlated with sperm quality. Glutamine is an important energetic substrate for the somatic Sertoli cells (P. F. Oliveira et al., 2015). Hence, testicular accumulation of glutamine may indicate SC metabolic dysfunction. On the other hand, acetate, an energy substrate obtained from lipolysis (Martins et al., 2015), was reduced in both groups of mice fed with HFD and HFD_t. We may hypothesise that acetate is being redirected for membrane remodelling, possibly due to increased cell turnover in testes (Alves et al., 2012). This assumption is supported by the changes found in antioxidant intermediaries and in purine metabolism. On the other hand, acetate excretion by SC may be inhibited by the effect of leptin (Martins et al., 2015). Glutathione content was upregulated in testes of mice from both HFD groups, while betaine content was only increased in those fed with HFD. Diet switch failed to attenuate oxidative stress as evidenced by the increased levels of glutathione. High levels of glutathione in testes of HFD-fed mice were associated with higher prevalence of sperm pin head defects, lower sperm viability and decreased sperm motility.

The consumption of HFD is associated with local inflammatory process in mice testes (X. Chen et al., 2013), which leads to glutathione overexpression. Recalling data obtained on testicular acetate content, this increase may be related to a local membrane remodelling process, associated with germ cell turnover by SCs (Martins et al., 2015). Although we should be cautious about this claim, as no apoptotic markers in testes were assessed, this response to HFD has been previously reported (Isidori et al., 1999; Mu et al., 2017). Yet, it is possible to assume that diet, rather than fat deposition, is the major contributor to changes in sperm viability, and glutathione and acetate testicular content. All those metrics were changed in HFD groups, but did not significantly correlated with fat mass or fat deposits. Testes of mice fed with HFD were further enriched in betaine, an intermediary of antioxidant pathways that is also associated with glucose intolerance and insulin resistance (T. J. Wang et al., 2011). Indeed, we have also found a strong correlation between betaine and the AUC of ipGTT in HFD-fed mice. Testicular betaine content was also strongly correlated with weight gain of

WAT pads, which may link the presence of this metabolite in testes to a direct effect of HFD. Decreased levels of testicular leucine is another hallmark of HFD. We found that leucine content in testes was negatively correlated with WAT weight. According to the metabolite correlation matrix (Supplementary Table 3.S10), leucine is negatively correlated with AMP, which may indicate an AMPK-related signalling cascade repressing leucine excretion or leucine consumption in testes of mice fed with HFD. Although increased serum concentration of leucine has been correlated with the onset of T2D in humans (T. J. Wang et al., 2011), we have observed an opposite effect in our study: mice fed with HFD, which displayed glucose intolerance and insulin resistance, had lower leucine levels in testes. This tissue-specific difference should not be overlooked and shows a functional specificity of the testis concerning amino acids metabolism, which should be further explored.

The most distinct metabolic feature in testes of mice fed with HFD_t was the enrichment in adenosine, glutamate and succinate. Overall these three metabolites are associated with WAT lipid mobilisation, weight loss, decreased percentage of sperm with bent neck, increased percentage of sperm pin head defects and improved glucose tolerance. Succinate has been correlated with sperm defects and glucose intolerance. A strong correlation was also observed with perirenal and epididymal fat lipid mobilisation. Previous studies reported changes in gene expression of epididymal WAT in response to diet restriction, namely in energy and lipid metabolism, with particular inhibition of genes related to mitochondrial pathways (Duivenvoorde et al., 2011). The authors reported the same phenotypic changes we observed in mice fed with HFD_t, such as weight loss and metabolic normalisation. The accumulation of succinate in testes of mice fed with a HFD_t also suggests increased TCA activity and mitochondrial involvement in the irreversible deleterious effects caused by a diet rich in fats. Although lipophagy is primarily described in adipocytes and hepatocytes, with pivotal implications in lipid homeostasis (Christian et al., 2013; K. Liu & Czaja, 2012), we should not overlook the possibility that a similar process occurs in testes, namely in SCs. Lipophagy allows the release of lipids from lipid droplets, which can then be recruited for metabolic functions (K. Liu & Czaja, 2012). This process is another energy-triggered phenomenon and HFD, obesity and hyperinsulinaemia inhibit it, prompting lipid accumulation (Christian et al., 2013; K. Liu & Czaja, 2012). Nevertheless, the process is triggered by cytoplasmatic concentrations of FFAs and cholesterol, rather than extracellular availability (K. Liu & Czaja, 2012). In fact, SCs accumulate lipid droplets as a result of phagocytosis of apoptotic germ cells (H. Wang et al., 2006).

Interestingly, we found that pyruvate metabolism, glutamate metabolism, ethanol degrad-

ation and ammonia recycling in testes were irreversibly affected by HFD, as the differences were verified even after dietary switch and weight loss. Furthermore, testes of mice fed with HFD presented alterations in purine metabolism, urea cycle, cysteine metabolism and glutathione. Those alterations, associated with antioxidant defences and metabolic reprograming, are linked to testicular metabolic adaptations to diet switch, posterior to sexual maturation.

Overall, our observations have a translational echo in a recent meta-analysis by Lee et al. (2019), which concluded that there are no improvements in sperm quality after metabolic bariatric surgery, despite the improvements on overall metabolic and endocrine function. In another review, P. F. Oliveira, Sousa et al. (2017) postulates that dietary interventions, such as bariatric surgery, exercise plans or caloric restriction may not present satisfactory results to male fertility due to collateral damages induced by the sudden weight loss and prospective accumulation of endogenous toxicants. Our study has, however, some limitations. We have only used polar testicular fractions to quantify metabolites, and there are dissimilarities between animal models and humans. Furthermore, our experimental model does not allow to differentiate between HFD and weight gain/obesity, because the switch from HFD to normal diet comprises dietary switch plus weight loss. Despite several sperm parameters have been affected by the consumption of HFD, we are cautious to draw conclusions regarding the reproductive success of these mice, as our protocol does not provide an extensive assessment of fertility outcomes. Nevertheless, this is the first study showing that excess of weight/obesity until early adulthood can cause irreversible damage to testicular function and sperm quality even when diet reversion restored normal global metabolic status. The biomarkers reported in this study are potential clinical targets for prevention and treatment of obesity-related infertility in males.

3.5 Author contributions

Pedro F. Oliveira, Marco G. Alves and Rachel L. Batterham contributed to study design, analysis and interpretation of data. Luís Rato, Ivana Jarak and **Luís Crisóstomo** performed experimental work. **Luís Crisóstomo** edited the images and tables, performed the statistics and contributed to analysis and interpretation of data. Branca M. Silva and João F. Raposo critically reviewed the manuscript and suggested modifications. All the authors contributed to manuscript writing/editing and approved the final version.

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3.7 Supplementary Material

Table 3.S1: Resonance assignment in the ^{1}H-NMR spectra of polar testicular extracts (s: singlet, d: doublet, dd: doublet of doublets, t: triplet, m: multiplet).

	Compound[a]	**δ^{1}H ppm (multiplicity, assigment)**
1	acetate	1.92 (s, αCH$_3$)
2	adenine	8.18 (s, C8H ring), 8.22 (s, C2H ring)
3	adenosine	3.83 (m, ribose), 3.91 (m, ribose), 4.28 (m, ribose), 4.44 (m, ribose), 4.79 (m, ribose), 6.10 (d, C1'H ribose), 8.23 (s, C8H ring), 8.35 (s, C2H ring)
4	alanine	1.48 (d, βCH$_3$), 3.78 (t, αCH)
5	aspartate	2.66 (dd, βCH), 2.81 (dd, 'CH), 3.86 (m, αCH)
6	betaine	3.26 (s, αCH$_3$), 3.90 (s, αCH$_2$)
7	choline	3.20 (s, CH$_3$), 3.51 (m, NCH$_2$), 4.06 (m, OCH$_2$)
8	citrate	2.49 (d, α,γCH), 2.69 (d, α',γ'CH)
9	creatine	3.03 (s, CH$_3$), 3.93 (s, CH$_2$)
10	dimethylglycine	2.91 (s, αCH), 3.72 (s, CH$_3$)
11	ethanolamine	3.14 (m, NCH$_2$), 3.82 (m, CH$_2$)
12	formate	8.46 (s, CH)
13	glutamate	2.07 (m, βCH), 2.14 (m, β'CH), 2.35 (m, γCH$_2$), 3.76 (m, αCH)
14	glutamine	2.13 (m, βCH$_2$), 2.45 (m, γCH$_2$), 3.75 (m, αCH)
15	glutathione	2.16 (m, βCH$_2$ Glu), 2.56 (m, γCH$_2$ Glu), 2.95 (m, βCH$_2$ Cys), 3.79 (m, αCH Gly), 4.59 (q, αCH$_2$ Cys)
16	glycerol	3.56 (dd, C1H$_2$), 3.65 (dd, C3H$_2$), 3.79 (m, C2H)
17	GPC	3.23 (s, CH$_3$), 3.68 (m, NCH$_2$), 3.88 (s, αCH$_2$), 4.33 (m, OCH$_2$)
18	glycine	3.55 (s, CH$_2$)
19	3OHBut	1.20 (d, γCH$_3$, 2.24 (dd, αCH), 4.15 (m, βCH))
20	hypoxanthine	8.19 (s, C2H), 8.21 (s, C8H)
21	IMP/AMP	3.75 (m, ribose), 4.03 (m, ribose), 4.36 (m, ribose), 4.50 (m, ribose), 6.14 (d, C1'H ribose), 8.26 (s, C8H ring), 8.59 (s, C2H ring)
22	isoleucine	0.94 (t, δCH$_3$), 1.01 (d, β'CH$_3$), 1.26 (m, γCH), 1.49 (m, γ'CH), 1.98 (m, βCH), 3.67 (m, αCH)
23	lactate	1.33 (d, βCH$_3$), 4.11 (q, αCH)
24	leucine	0.96 (t, δCH$_3$), 1.73 (m, βδCH$_2$), 3.73 (m, αCH)
25	lysine	1.72 (m, δCH$_2$), 1.92 (m, βCH$_2$), 3.01 (m, εCH$_2$), 3.76 (m, αCH)
26	*myo*-inositol	3.26 (t, C5H), 3.52 (dd, C1H, C3H), 3.62 (m, C4H, C6H), 4.06 (t, C2H)
27	PChol	3.22 (s, CH$_3$), 3.91 (m, NCH$_2$), 4.17 (m, OCH$_2$)
28	PEtAm	3.21 (m, NCH$_2$), 3.97 (m, OCH$_2$)
29	*scyllo*-inositol	3.35 (s, CH)
30	succinate	2.40 (s, CH$_2$)
31	taurine	3.26 (t, NCH$_2$), 3.43 (t, SCH$_2$)
32	tyrosine	6.87 (d, C3H, C5H ring), 7.18 (d, C2H, C6H ring)
33	uridine	4.33 (m, C2'H ribose), 5.89 (d, C1'H ribose), 5.91 (d, C5H ring), 7.87 (d, C6H ring)
34	valine	0.99 (d, γCH$_3$), 1.04 (d, γ'CH$_3$), 2.26 (m, βCH), 3.62 (m, αCH)

[a]all metabolites are putatively annotated (level 2 of identification according to Chemical Analysis Working Group (CAWG) Metabolomics Standards Initiative recommendations).

Table 3.S2: Mice organ and tissue weight. Organs and tissue were collected at sacrifice, 200 days after weaning. Results were tested by one-way ANOVA with Tukey's Multicomparison test. Statistical significance was considered when $p < 0.05$. Results are expressed as mean (g) ± SEM. † –vs CTRL; ‡ –vs HFD; § – vs HFDt. * $p < 0.05$; ** $p < 0.005$; *** < 0.0005; **** $p < 0.0001$.

	L. testis	R. testis	R. epididymis	Liver	Seminal vesicles	Epididymal fat	Perirenal fat	Retroperitoneal fat	BAT
CTRL	0.11 ± 0.00	0.11 ± 0.00	0.06 ± 0.00	1.15 ± 0.02	0.25 ± 0.02	0.73 ± 0.07	0.29 ± 0.04	0.32 ± 0.04	0.14 ± 0.01
HFD	0.12 ± 0.00	0.11 ± 0.00	0.06 ± 0.00	1.38 ± 0.09 †*	0.31 ± 0.01	2.21 ± 0.23 †****	1.56 ± 0.11 †****	2.52 ± 0.21 †****	0.18 ± 0.01
HFD_t	0.11 ± 0.00	0.11 ± 0.00	0.06 ± 0.00	1.08 ± 0.13 ‡***	0.27 ± 0.03	0.63 ± 0.09 ‡****	0.23 ± 0.04 ‡****	0.35 ± 0.06 ‡****	0.16 ± 0.01

Table 3.S3: Correlation factors between testicular mice total body weight and collected tissues. GSI and Fat mass were also considered in this correlation as they are result from weight measurements of gonads and adipose tissue, respectively. The correlation was obtained using the Spearman r with Approximate p-value (n = 35). * $p < 0.05$; ** $p < 0.005$; *** < 0.0005; **** $p < 0.0001$.

	R testis	L testis	R epididymis	Seminal Vesicles	Liver	GSI	BAT	Epididymal fat	Perirenal fat	Retroperiton. fat	Fat mass
r value	0.4021 *	0.0703	0.0241	0.4732 **	0.7015 ****	-0.7964 ****	0.4294 *	0.8793 ****	0.8959 ****	0.7695 ****	0.8209 ****
P (two-tailed)	0.0166	0.6882	0.8907	0.0041	< 0.0001	< 0.0001	0.0100	< 0.0001	< 0.0001	< 0.0001	< 0.0001

Table 3.S4: Correlation factors between biometric data and Area Under Curve (AUC) of ipGTT and ipITT. The correlation was obtained using the Spearman r with Approximate p-value (n = 35). * $p < 0.05$; ** $p < 0.005$; *** < 0.0005; **** $p < 0.0001$.

		Fasting glycaemia	R testis	L testis	R epididymis	Seminal Vesicles	Liver	GSI	BAT	Epididymal fat	Perirenal fat	Retroperiton. fat	Fat mass
AUC ipGTT	r value	0.4700 **	0.1386	-0.1492	0.2386	0.4069 *	0.3553 *	-0.6416 ****	0.2173	0.6457 ****	0.549 ***	0.5722 ***	0.5666 ***
	P (two-tailed)	0.0044	0.4271	0.3922	0.1675	0.0153	0.0362	< 0.0001	0.2099	< 0.0001	0.0006	0,0003	0.0004
AUC ipITT	r value	0.5399 ****	0.1747	-0.1257	0.2035	0.3245	0.2863	-0.5203 **	0.2764	0.5308 **	0.5156 **	0.5637 ***	0.5511 ***
	P (two-tailed)	0.0008	0.3154	0.4717	0.2409	0.0572	0.0955	0.0014	0.1080	0.0010	0.0015	0.0004	0.0006

Table 3.S5: Correlation factors between sperm parameters and biometric data. The correlation was obtained using the Spearman r with Approximate p-value (n = 35). * $p < 0.05$; ** $p < 0.005$; *** < 0.0005; **** $p < 0.0001$.

	R testis	L testis	R epididymis	Seminal Vesicles	Liver	GSI	BAT	Epididymal fat	Perirenal fat	Retroperiton. fat	Fat mass
Sperm concentration	0.2869	**0.4242** *	-0.2082	0.0058	0.0473	0.1696	-0.0371	0.1406	0.1546	0.1489	0.1927
Sperm viability	0.0527	-0.0785	-0.1004	-0.292	-0.0454	-0.0431	-0.1669	0.0149	-0.0423	-0.1168	-0.044
Sperm motility	0.2542	0.1162	**-0.3837** *	-0.0629	-0.0007	0.1647	**-0.3798** *	-0.0599	-0.0192	-0.0501	-0.0792
Normal	-0.0756	0.3157	-0.2495	-0.0425	**-0.4008** *	0.2987	-0.3354	-0.1499	-0.2671	-0.2648	-0.3013
Decapitated	0.1163	0.0694	0.3448	-0.1378	0.0852	0.1648	-0.0472	-0.1843	-0.1563	-0.2634	-0.1809
Pin head	-0.2398	-0.1684	0.0754	0.1480	-0.0029	-0.2028	**0.4357** *	0.1462	0.2265	**0.4338** *	0.3563
Flattened head	-0.2222	-0.0497	-0.2111	0.0246	-0.1914	0.1953	0.0086	-0.142	-0.0804	0.1130	0.0113
Bent neck	-0.1451	-0.1358	0.1985	0.0544	0.2566	**-0.5358** **	0.1645	**0.4836** *	**0.3720** *	**0.3955** *	**0.4323** *
Coiled tail	0.1455	-0.1922	0.0120	0.074	0.2426	-0.0664	0.0666	0.0309	0.1410	0.0370	0.0937

Table 3.S6: Correlation factors between Area Under Curve (AUC) of ipGTT and ipITT and sperm parameters. The correlation was obtained using the Spearman r with Approximate p-value (n = 35). * $p < 0.05$; ** $p < 0.005$; *** < 0.0005; **** $p < 0.0001$.

		Sperm concentration	Sperm viability	Sperm motility	Normal	Decapitated	Pin head	Flattened head	Bent neck	Coiled tail
AUC ipGTT	r value	-0.2017	-0.1793	-0.0756	-0.2365	-0.0883	0.1434	-0.1002	**0.5211** **	-0.0701
	P (two-tailed)	0.2452	0.3028	0.6659	0.2082	0.6425	0.4496	0.5985	0.0031	0.7129
AUC ipITT	r value	-0.1771	-0.1862	0.03712	**-0.5029** **	0.2450	0.1711	0.0098	**0.4045** *	-0.0427
	P (two-tailed)	0.3088	0.2843	0.8323	0.0046	0.1919	0.3659	0.9590	0.0266	0.8226

Table 3.S7: Correlation factors between biometric data and Testicular metabolites. The correlation was obtained using the Spearman r with Exact p-value (n = 15). * $p < 0.05$; ** $p < 0.005$; *** < 0.0005; **** $p < 0.0001$.

	Ala	Glu	Gln	Val	Leu	Ile	AMP	Adenosine	Lac	Acetate	Succinate	Creatine	GSH	Betaine	Taurine
R testis	-0.4709	-0.3567	-0.3514	-0.482	-0.3175	-0.4149	0.299	-0.2532	-0.359	0.2807	0.0092	-0.0214	-0.3783	0.3419	**-0.8194** ***
L testis	**-0.7021** **	-0.3553	-0.2882	**-0.6478** *	-0.395	-0.2742	0.3284	-0.2712	-0.4737	0.2501	-0.0060	-0.1206	-0.1778	**0.5035** *	**-0.7096** **
R epididymis	-0.0117	0.0273	-0.0399	-0.0563	0.1953	-0.2039	0.0485	0.0893	0.0039	-0.0680	-0.2447	-0.2769	-0.2738	0.0146	-0.2442
Seminal Vesicles	-0.3503	-0.2265	0.3146	**-0.5192** *	-0.3129	-0.1254	0.441	-0.3112	-0.4542	-0.1947	-0.0501	-0.2974	0.3378	0.476	-0.1345
Liver	0.0944	-0.1152	0.2041	-0.0044	-0.2754	-0.2046	**0.5725** *	-0.3591	-0.0892	0.1177	-0.2193	-0.2445	0.1148	0.4124	-0.1615
GSI	-0.0414	0.2581	-0.2657	-0.1945	0.2249	0.3508	-0.3847	0.2314	-0.1587	0.2918	**0.6175** *	0.4196	-0.2845	-0.3968	-0.0879
BAT	0.0885	0.1141	0.1835	-0.1706	-0.3706	**-0.5117** *	**0.5177** *	-0.4189	-0.1686	-0.1631	0.2783	-0.1041	0.3561	0.4222	-0.0262
Epididymal fat	-0.4598	-0.3867	0.1487	-0.2794	**-0.6313** *	-0.4971	**0.5735** *	-0.4559	-0.2798	-0.2324	**-0.5529** *	**-0.5416** *	0.1941	**0.7432** **	-0.2078
Perirenal fat	-0.403	-0.3806	-0.0022	-0.2548	**-0.5969** *	**-0.5700** *	**0.5287** *	-0.4138	-0.2367	-0.1105	**-0.5287** *	-0.4024	0.2239	**0.6809** **	-0.2952
Retroperiton. fat	-0.339	-0.1415	0.0973	-0.2329	**-0.6214** *	-0.4273	**0.7092** **	**-0.6054** *	-0.1241	-0.1395	-0.3531	-0.4499	0.2582	**0.8575** ****	-0.2729
Fat mass	-0.423	-0.3041	0.0206	-0.2735	**-0.7388** **	**-0.5588** *	**0.6412** *	**-0.5353** *	-0.2253	-0.2235	-0.4176	-0.4268	0.2588	**0.727** **	-0.2889
Body weight	-0.2552	**-0.5547** *	0.1186	-0.0662	-0.3166	**-0.5077** *	0.299	-0.2532	-0.359	0.2807	0.0092	-0.0214	-0.3783	0.3419	**-0.8194** ***

Table 3.S8: Correlation factors between testicular metabolite content and Area Under Curve (AUC) of ipGTT and ipITT. The correlation was obtained using the Spearman r with Approximate p-value (n = 35). * $p < 0.05$; ** $p < 0.005$; *** < 0.0005; **** $p < 0.0001$.

		Ala	Glu	Gln	Val	Leu	Ile	AMP	Adenosine	Lac	Acetate	Succinate	Creatine	GSH	Betaine	Taurine
AUC ipGTT	r value	-0.1489	0.07085	0.3093	0.02353	-0.262	-0.3294	0.3853	-0.3676	0.1325	-0.4471	**-0.5059** *	**-0.7285** **	-0.0382	**0.5180** *	-0.0486
	P (two-tailed)	0.5797	0.7937	0.2421	0.9345	0.3244	0.2127	0.1414	0.1618	0.6224	0.0844	0.0479	0.0019	0.8910	0.0418	0.8584
AUC ipITT	r value	0.0000	-0.1403	0.3589	-0.0898	-0.0309	-0.4312	0.3473	-0.3974	-0.0737	-0.3297	-0.1810	-0.3586	-0.1781	0.2121	-0.1372
	P (two-tailed)	>0.9999	0.6015	0.1712	0.7400	0.9099	0.0965	0.1866	0.1278	0.7848	0.2112	0.4995	0.1713	0.5066	0.4271	0.6092

Table 3.S9: Correlation matrix of Sperm parameters vs. Testicular metabolites. The correlation was obtained using the Spearman r with Exact p-value (n = 15). * $p < 0.05$; ** $p < 0.005$; *** < 0.0005; **** $p < 0.0001$.

	Ala	Glu	Gln	Val	Leu	Ile	AMP	Adenosine	Lac	Acetate	Succinate	Creatine	GSH	Betaine	Taurine	Creatine
Sperm concentration	**-0.7222** **	-0.1963	**-0.6421** *	-0.4059	**-0.574** *	-0.2706	0.3206	-0.3529	-0.3166	0.2147	-0.0353	-0.0486	-0.1294	0.3238	**-0.6942** **	-0.0486
Sperm viability	-0.2255	-0.4089	**-0.5626** *	0.2471	0.2104	-0.1029	-0.3559	0.4118	0.3314	**0.7294** **	-0.3118	0.1148	**-0.6118** *	0.0765	-0.4407	0.1148
Sperm motility	-0.2965	-0.3959	-0.4783	0.1251	0.3446	-0.2237	-0.3032	0.3385	0.2417	**0.7314** **	-0.3797	-0.0604	**-0.6637** *	0.1311	**-0.5752** *	-0.0604
Normal	-0.3951	-0.0232	0.0727	0.2090	0.1630	0.2046	-0.2134	0.3102	0.3517	0.2420	-0.2068	-0.1145	-0.1386	0.4736	0.0541	-0.1145
Decapitated	0.0132	-0.0840	-0.2750	0.1077	0.0682	0.2000	-0.2132	0.1648	0.0529	0.1165	0.1253	0.2552	-0.2747	**-0.5765** *	-0.0794	0.2552
Pin head	0.1678	0.2279	**0.5771** *	-0.3410	-0.2985	-0.1320	0.462	**-0.5699** *	-0.3407	**-0.6073** *	0.1672	-0.3051	**0.7459** **	0.3106	0.4801	-0.3051
Flattened head	0.0684	0.3050	0.3366	-0.0593	0.0000	0.1473	0.1165	-0.3011	-0.0815	-0.2527	**0.7055** *	0.3432	0.2571	-0.0330	0.3594	0.3432
Bent neck	-0.1004	0.0597	0.1200	0.2860	-0.3910	-0.4334	0.495	-0.3190	0.3308	-0.2486	**-0.6095** *	-0.5220	0.2112	**0.6421** *	-0.1634	-0.5220
Coiled tail	0.2029	-0.1238	-0.1980	-0.4681	0.0704	-0.2308	-0.0681	0.0374	-0.5859 *	0.0462	0.1341	0.0946	-0.0857	-0.2860	-0.1720	0.0946

Table 3.S10: Correlation factors between identified metabolites in the polar fraction of mice testicular extracts. The correlation was obtained using the Spearman r with Exact p-value (n = 15). * $p < 0.05$; ** $p < 0.005$; *** < 0.0005; **** $p < 0.0001$.

	Ala	Glu	Gln	Val	Leu	Ile	AMP	Adenosine	Lac	Acetate	Succinate	Betaine	GSH	Taurine	Creatine
Ala		**0.5296** *	0.4657	**0.5129** *	0.455	0.2476	-0.1489	0.1518	0.3454	-0.0221	0.3346	-0.385	0.1459	**0.6492** *	0.1792
Glu	**0.5296** *		0.2018	0.2937	0.0251	0.1683	0.1284	-0.248	0.3082	-0.2007	0.4812	0.0406	0.1077	0.4142	-0.1315
Gln	0.4657	0.2018		0.0471	0.0774	-0.0854	0.3152	-0.2975	-0.0501	-0.486	0.0781	0.185	0.4683	**0.693** **	-0.3522
Val	**0.5129** *	0.2937	0.0471		**0.518** *	0.2382	-0.4265	0.4765	**0.9087** ****	0.2000	-0.0941	-0.1383	-0.2529	0.4068	0.2620
Leu	0.455	0.0251	0.0774	**0.518** *		0.3267	**-0.6578** *	**0.6976** **	0.4296	0.4386	0.0559	-0.4345	-0.4680	0.2080	0.3166
Ile	0.2476	0.1683	-0.0854	0.2382	0.3267		-0.3118	0.3529	0.1458	0.1265	0.1029	-0.4739	0.0500	0.3110	0.2929
AMP	-0.1489	0.1284	0.3152	-0.4265	**-0.6578** *	-0.3118		**-0.9412** ****	-0.4153	**-0.5206** *	0.1059	**0.5710** *	0.3765	-0.1916	-0.4871
Adenosine	0.1518	-0.248	-0.2975	0.4765	**0.6976** **	0.3529	**-0.9412** ****		0.4433	**0.5647** *	-0.2176	**-0.5239** *	-0.3765	0.1444	0.4798
Lac	0.3454	0.3082	-0.0501	**0.9087** ****	0.4296	0.1458	-0.4153	0.4433		0.2018	-0.1267	0.0015	-0.2194	0.2627	0.2004
Acetate	-0.0221	-0.2007	-0.4860	0.2000	0.4386	0.1265	**-0.5206** *	**0.5647** *	0.2018		-0.1088	-0.1089	**-0.6441** *	-0.3125	0.2517
Succinate	0.3346	0.4812	0.0781	-0.0941	0.0559	0.1029	0.1059	-0.2176	-0.1267	-0.1088		-0.2826	0.1176	0.0987	0.4665
Betaine	-0.385	0.0406	0.1850	-0.1383	-0.4345	-0.4739	**0.571** *	**-0.5239** *	0.0015	-0.1089	-0.2826		0.0132	-0.2176	**-0.5523** *
GSH	0.1459	0.1077	0.4683	-0.2529	-0.468	0.05	0.3765	-0.3765	-0.2194	**-0.6441** *	0.1176	0.0132		0.4908	-0.0353
Taurine	**0.6492** *	0.4142	**0.6930** **	0.4068	0.208	0.311	-0.1916	0.1444	0.2627	-0.3125	0.0987	-0.2176	0.4908		-0.0354
Creatine	0.1792	-0.1315	-0.3522	0.262	0.3166	0.2929	-0.4871	0.4798	0.2004	0.2517	0.4665	**-0.5523** *	-0.0353	-0.0354	

Chapter 4

Diet during early life defines testicular lipid content and sperm quality in adulthood

This chapter was reproduced from the published work:

Crisóstomo, **L.**, Videira, R. A., Jarak, I., Starčević, K., Mašek, T., Rato, L., Raposo, J. F., Batterham, R. L., Oliveira, P. F., & Alves, M. G. (2020). Diet during early life defines testicular lipid content and sperm quality in adulthood. *American Journal of Physiology - Endocrinology and Metabolism*, *319*(6), E1061–E1073. https://doi.org/10.1152/ajpendo.00235.2020

Abstract

Childhood obesity is a serious concern associated with ill health later in life. Emerging data suggest that obesity has long-term adverse effects upon male sexual and reproductive health, but few studies have addressed this issue. We hypothesized that exposure to high-fat diet during early life alters testicular lipid content and metabolism, leading to permanent damage to sperm parameters. After weaning (day 21 after birth), 36 male mice were randomly divided into three groups and fed with a different diet regimen for 200 days: a standard chow diet (CTRL), a HFD (carbohydrate: 35.7 %, protein: 20.5 %, and fat: 30.0 %), and a high-fat diet for 60 days, then replaced by standard chow (HFD_t). Biometric and metabolic data were monitored. Animals were then euthanized, and tissues were collected. Epididymal sperm parameters and endocrine parameters were evaluated. Testicular metabolites were extracted and characterized by ^{1}H-NMR and GC-MS. Testicular mitochondrial and antioxidant activity were evaluated. Our results show that mice fed with a high-fat diet, even if only until early adulthood, had lower sperm viability and motility, and higher incidence of head and tail defects. Although diet reversion with weight loss during adulthood prevents the progression of metabolic syndrome, testicular content in fatty acids is irreversibly affected. Excessive fat intake promoted an overaccumulation of proinflammatory n-6 polyunsaturated fatty acids in the testis, which is strongly correlated with negative effects upon sperm quality. Therefore, the adoption of high-fat diets during early life correlates with irreversible changes in testicular lipid content and metabolism, which are related to permanent damage to sperm quality later in life.

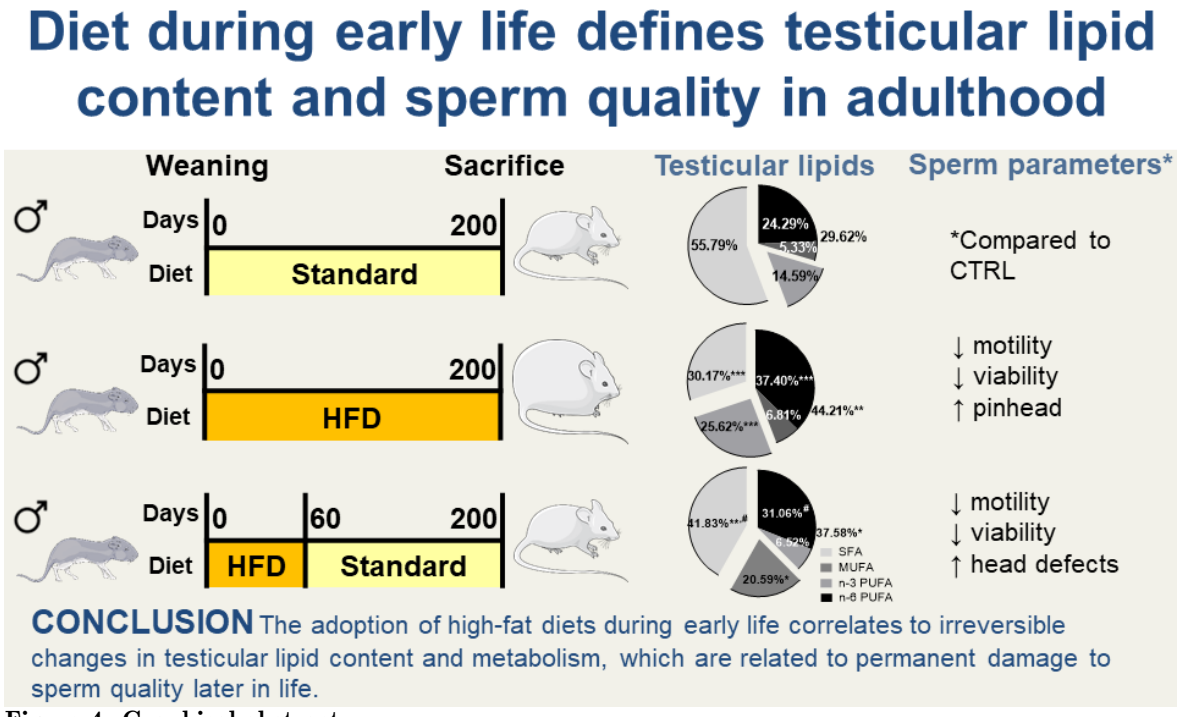


Figure 4: Graphical abstract.

4.1 Introduction

The fast and global increase of overweight/obesity in recent decades led the World Health Organization to declare it the "Epidemics of the XXI Century" (WHO, 2000). Excessive fat deposition has been associated with the onset of non-communicable diseases, particularly T2D (Hruby et al., 2016). Consumption of energy-dense, high-fat diet (HFD), and low physical activity are two major drivers of the obesity epidemic (Hruby et al., 2016). In developed countries, consumption of energy-dense foods occurs at an ever-younger age (Muecke et al., 1992). Childhood obesity rates continue to soar worldwide, with obesity-associated comorbidities occurring at a younger age (Llewellyn et al., 2016; WHO, 2014). Concurrently, concerns on the impact of excessive adiposity and T2D on sperm quality and fertility outcomes have been raised (Eisenberg et al., 2016a; Mulholland et al., 2011; Palmer, Bakos, Fullston, et al., 2012). Although there are not many studies demonstrating the impact of fat-rich diets in sperm quality, men attending fertility centers are often advised to lose weight and adopt a balanced diet. Unlike the other lifestyle interventions such as physical activity (Stanford et al., 2018), the effectiveness of dietary intervention in recovering normal sperm quality or its positive effects on testicular metabolism has never been demonstrated (Stokes et al., 2015).

Normal male reproductive function requires a well-orchestrated balance of endocrine and metabolic factors to secure proper energy flow towards meiosis and cellular remodeling of differentiating germ cells (Rato, Alves, Socorro, Duarte, et al., 2012). In this regard, testicular lipid dynamics are crucial, not only as energy substrates, but also as structural elements of the future sperm cells. Moreover, mitochondrial function and antioxidant defenses are crucial for providing energy for spermatogenesis and sperm motility, while avoiding oxidative damage which can critically compromise cell functions. Previously we have shown that HFD during early life causes irreversible changes in testicular metabolism, even after reversion, and those changes are correlated to defects in sperm motility, viability and morphology (**Crisóstomo** et al., 2019). The relevance of fatty acids in testicular physiology has been reported, specially the repercussions for spermatogenesis (Hale et al., 2019; Koeberle et al., 2012; Masaki et al., 2017; Roqueta-Rivera et al., 2010). We hypothesize that the adoption of HFDs affects testicular lipid dynamics resulting in poorer sperm quality. We further address whether an HFD early in life can irreversibly affect sperm quality later in life, due to testicular lipid dysmetabolism, even after diet intervention in early adulthood. We have performed our study in a rodent model. Biometry, glucose homeostasis, endocrine function, testicular antioxidant system, testicular bioenergetics and sperm parameters were assessed. We have further performed a multivariate analysis supported by targeted metabolomics and lipidomics, based on semi-

quantitative GC-MS and ^{1}H-NMR, to determine changes in testicular lipid metabolism.

4.2 Materials and Methods

Animal model

Animal model. *Mus musculus* C57BL6/J male mice (n = 36) were randomly divided into three groups after weaning (21 days–23 days): control (CTRL) (n = 12), HFD (n = 12), and transient HFD (HFD_t) (n = 12). All mice were generated from normoponderal males and females and were subjected to the same random *in utero* stimuli, although generated from different litters. Briefly, CTRL group mice were fed with a standard chow (no. F4031, BioServ: carbohydrate: 61.6 %, protein: 20.5 %, and fat: 7.2 %–16.3 $\%_{Kcal}$). The HFD group mice received an HFD (no. F3282, BioServ: carbohydrate: 35.7 % protein: 20.5 %, fat: 36.0 % – 59.0 $\%_{Kcal}$) for 200 days after weaning. HFD_t group mice were fed with an HFD (no. F3282; BioServ, Flemington, NJ) for 60 days and then switched to standard chow (no. F4031, BioServ). At 120 days postweaning, mice were randomly assigned to a normoponderal female, in mating pairs, 6 h/day, for seven consecutive days, for breeding. During this period, female breeders were maintained on the same standard diet. Mating pairs had no access to water or food during mating (6 h). Reproductive success rate, litter size, and litter male-to-female ratio were assessed. Animals 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 until euthanization. The animal model is compliant with the ARRIVE guidelines and was licensed by the Portuguese Veterinarian and Food Department (0421/000/000/2016).

Endocrine and metabolic function

Fasting glycemia was measured before euthanization (200 days postweaning), after overnight fast (8 h), using a glucometer (One Touch Ultra Lifescan-Johnson, Milpitas, CA) 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. Serum insulin was quantified using a rat/mouse insulin ELISA assay (EZRMI-13K; Millipore). Glucose homeostasis was evaluated according to the HOMA2 scores (Levy et al., 1998), using the HOMA2 calculator (University of Oxford, UK). Disposition Index (DI) was calculated as suggested by Caumo et al. (2006). Similarly, FSH, LH, 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).

Evaluation of epididymal sperm parameters

Epididymides were isolated and placed in prewarmed (37 °C) HBSS at pH 7.4, and then minced with a scalpel blade. Then the suspension was incubated for 5 min at (37 °C). Sperm parameters were evaluated as previously described (**Crisóstomo** et al., 2019; Rato et al., 2013). 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) in a diluted 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 (Kvist et al., 2002), 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) (Kvist et al., 2002).

NMR spectroscopy

A combined extraction of polar and nonpolar metabolites from testicular tissue was performed as previously described (Alves et al., 2011; **Crisóstomo** et al., 2019). Testes were washed with saline and decapsulated before metabolite extraction to minimize contamination by blood metabolites. The aqueous phase containing polar water-soluble metabolites was lyophilized and analyzed by ^{1}H-NMR spectroscopy, as described by Jarak et al. (2018). Lipid metabolites were identified by comparing recorded spectra with reference spectra and the Human Metabolome Database (Wishart et al., 2007), and according to Metabolomics Standards Initiative guidelines for metabolite identification (Sumner et al., 2007). Identification levels are indicated in Supplemental Table 4.S1 (all Supplemental material is available at https://doi.org/10.6084/m9.figshare.12302171 and in the *Supplementary Material* section). ^{1}H spectra were processed using previously described methods (Jarak et al., 2018). Obtained peak areas were normalized by the total spectral area and analyzed by univariate analysis. Areas were obtained using AMIX software (Bruker BioSpin, Rheinstetten, Germany) and expressed as arbitrary units defined by the software.

GC-MS analysis

Fatty acid methyl esters of total lipids were obtained by base-catalyzed transmethylation (2 M KOH in methanol) in the presence of nonadecanoic fatty acid (C19:0), used as the internal standard. The obtained hexane fatty acid 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 length, SGE, Austin, TX). 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 up to 170 °C, 4 °C min up to 220 °C and 40 °C min 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 m/z and event time: 0.3 s. All of the experimental measurements were repeated three times, and the average values were reported. Fatty acids were identified by retention time and fragmentation profile and were quantified by the internal standard procedure. Results were expressed as a percentage of total fatty acids.

Lipid peroxidation, activity of antioxidant enzymes, and activity of mitochondrial enzyme complexes

A single testis from each animal was homogenized in 2 mL of ice-cold extraction buffer (160 mM sucrose, 10 mM Tris · HCl, at pH 7.4), supplemented with a protease inhibitor cocktail (#B14001, Bimake, Munich, Germany) (1:10 $\frac{p}{v}$) using a glass-Teflon Potter Elvejhem (Kimble, Millville, NJ). The resulting homogenate was split into two fractions: one was used for adenine nucleotide (ATP, ADP, and AMP) extraction and quantification by HPLC, as described previously (Monteiro-Cardoso et al., 2015); the other was fractionated by differential centrifugation to obtain a mitochondria-free cytosolic fraction and a mitochondria-enriched fraction, as described previously (Mendes et al., 2018; Monteiro-Cardoso et al., 2015). The final protein content of each fraction was determined by the bicinchoninic acid (BCA) method (P. K. Smith et al., 1985). The mitochondria-enriched fraction was used to assess the activity of the mitochondrial enzymes (complexes mitochondrial complex I (CI), mitochondrial complex II (CII), mitochondrial complex IV (CIV), and mitochondrial complex V (CV) and citrate synthase (CS)), while the mitochondria-free cytosolic fraction was used to evaluate the activity of the enzymes of the antioxidant defense system [catalase (CAT), copper-zinc superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione-disulfide reductase

(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) (del Río et al., 1977). SOD, GPx and GSR activities were evaluated in 96-well plates, at 37 °C, as described previously (Neves et al., 2019). The reaction was conducted in 930 L of 100 mM potassium phosphate buffer (100 mM KH_2PO_4, 5 mM EDTA, pH 7.4) with 50 µL of testicular cytosol extract, and initiated by adding 20 µL of H_2O_2 (2 mM). CAT activity was expressed in nanomoles O_2 per minute per milligram protein ($nmol_{O_2}\,min^{-1}\,mg^{-1}_{protein}$).

SOD activity was evaluated by mixing 50 µL testicular cytosolic fraction with 170 µL potassium phosphate buffer, supplemented with 2 mM nitro blue tetrazolium (NBT), and 0.05 U xanthine oxidase (no. X1875-10UN, Sigma-Aldrich/Merck KGaA, Darmstadt, Germany). After addition of 10 µL hypoxanthine (2 mM), absorbance at 560 nm was measured every 20 s for 5 min. Results were expressed as units per minute per milligram of protein ($U\,min^{-1}\,mg^{-1}_{protein}$), where U is the enzyme activity that inhibited the reduction of NBT to blue formazan by 50 %.

GSR activity was evaluated by the rate of NADPH oxidation associated with the reduction of GSSG. Assays were carried out in 170 µL potassium phosphate buffer, supplemented with 10 mM NADPH and 10 mM GSSG. After adding 30 µL of testicular cytosolic fraction, NADPH oxidation was recorded by the fluorescence decrease at 450 nm (excitation: 360 nm) every 20 s for 5 min. NADPH oxidation not associated with GSSG reduction was assessed in assays carried out in the absence of GSSG. GSR activity was determined by difference between the rate of NADPH oxidation in the presence and absence of GSSG and expressed in nanomoles per minute per milligram of protein ($nmol_{NADPH}\,min^{-1}\,mg^{-1}_{protein}$).

GPx activity was evaluated by the rate of NADPH oxidation associated with H_2O_2 reduction. Assays were performed in 170 µL potassium phosphate buffer, supplemented with 10 mM GSH and 1–3 U GSR (no. G3664-500UN, Sigma-Aldrich/Merck KGaA, Darmstadt, Germany), and 50 µL of testicular cytosol fraction. Reaction was initiated by the addition of 10 µL H_2O_2 (0.6 mM), and fluorescence at 450 nm (excitation: 360 nm) was measured every 20 s for 5 min. GPx activity was determined by the rate of NADPH consumption in nanomoles per minute per milligrams of protein ($nmol_{NADPH}\,min^{-1}\,mg^{-1}_{protein}$).

Testicular lipid peroxidation levels were evaluated by the production of TBARS assay. Shortly, 50 µL testicular cytosolic fraction were mixed with 600 µL of reaction solution [thiobarbituric acid (TBA) 0.38 %$\frac{m}{v}$, trichloroacetic acid (TCAA) 37 %$\frac{m}{v}$ and 2,6-ditertbutyl-4-methylphenol (BHT) 0.02 %$\frac{m}{v}$ (Mendes et al., 2018)] and incubated at 95 °C for 30 min. Malondialdehyde

(MDA) formation was measured by colorimetric methods ($\varepsilon = 156\,\mathrm{mM}^{-1}\,\mathrm{cm}^{-1}$), and results were expressed in nanomoles of MDA per milligram of protein ($\mathrm{nmol}_{\mathrm{MDA}}\,\mathrm{mg}^{-1}_{\mathrm{protein}}$).

Activities of the mitochondrial complexes I, II and IV, and CS were assessed, at 37 °C, in 96-well plate by adapting previously described protocols (Monteiro-Cardoso et al., 2015). CS activity was determined spectrophotometrically, by monitoring the reduction of DTNB. The reaction mixture consisted of 200 mM Tris · HCl buffer (pH 8.0), 0.02 $\%\frac{m}{v}$ Triton X-100, 10 mM DTNB, 1 mM oxaloacetate, and 20 µL of testicular mitochondrial fraction. The reaction was initiated by adding 0.37 mM acetyl-CoA, and the absorbance at 412 nm was recorded every 30 s for 10 min. CS activity was calculated by DTNB ($\varepsilon = 13.6\,\mathrm{mM}^{-1}\,\mathrm{cm}^{-1}$) reduction rate, determined in the linear range of the plot and expressed as nanomoles per minute per milligram of protein ($\mathrm{nmol\,min}^{-1}\,\mathrm{mg}^{-1}_{\mathrm{protein}}$).

Complex I (CI) activity was assessed following NADH oxidation. The reaction was performed in a potassium phosphate buffer (25 mM KH_2PO_4, 10 mM $MgCl_2$; pH 7.4) supplemented with 1 mM KCN, 162.5 µM decylubiquinone, 3.0 µM rotenone (or equal volume of buffer) and 20 µL of testicular mitochondrial fraction. The reaction was triggered by the addition of NADH (50 µM), and fluorescence at 450 nm (excitation: 366 nm) was measured every 20 s for 15 min. CI activity was determined by the difference between NADH oxidation rate in rotenone-inhibited wells and noninhibited wells. This value was expressed in nanomoles of NADH per minute per milligram ($\mathrm{nmol}_{\mathrm{NADH}}\,\mathrm{min}^{-1}\,\mathrm{mg}^{-1}_{\mathrm{protein}}$).

Complex II (CII) activity was assessed monitoring 2,6-dichlorophenolindo-phenol (DCPIP) reduction at 600 nm ($\varepsilon = 20.7\,\mathrm{mM}^{-1}\,\mathrm{cm}^{-1}$) in a potassium phosphate buffer, supplemented with 2 mM KCN, 6.5 µM rotenone, 6.5 µM antimycin A, 0.05 mM DCPIP, 0.1 mM decylubiquinone, and 20 µL of testicular mitochondrial fraction. Negative control wells were further supplemented with 0.5 mM oxaloacetate (CII inhibitor). The reaction was initiated by adding 20 mM succinate and absorbance measure every 30 s for 20 min. CII activity was expressed as the DCPIP reduction rate (corrected for spontaneous reduction) in nanomoles of DCPIP per minute per milligram protein ($\mathrm{nmol}_{\mathrm{DCPIP}}\,\mathrm{min}^{-1}\,\mathrm{mg}^{-1}_{\mathrm{protein}}$).

Complex IV (CIV) activity was evaluated by the cytochrome c (CytC) oxidation rate. CytC (50 mM) was fully reduced by the addition of small volumes of saturated sodium dithionite solution. CytC oxidation reaction was performed in extraction buffer, supplemented with 3 µM rotenone, 0.1 µM antimycin A (inhibitor of mitochondrial complex III (CIII)), 2 mM KCN (specific inhibitor of CIV), or equal volume of buffer, and 20 µL of testicular mitochondrial fraction. The reaction was initiated by adding 15 µM reduced CytC, and the absorbance at 550 nm was recorded every 15 s for 5 min. CIV activity was calculated by CytC ($\varepsilon =$

$29.5\,\mathrm{mM}^{-1}\,\mathrm{cm}^{-1}$) oxidation rate, corrected by the spontaneous oxidation rate obtained in the KCN-inhibited wells, and expressed as nanomoles of CytC per minute per milligram of protein ($\mathrm{nmol}_{\mathrm{CytC}}\,\mathrm{min}^{-1}\,\mathrm{mg}^{-1}_{\mathrm{protein}}$).

Maximal activity of the mitochondrial complex V was measured using a MitoCheck Complex V activity assay kit (Cayman Chemical, Ann Arbor, MI). All colorimetric and fluorometric readings described in this section were obtained using a Biotek Synergy H1 plate reader (Winooski, VT).

Determination of testicular adenosine nucleotides levels by HPLC

Testicular adenosine nucleotides levels were assessed in a Waters 600 HPLC system (Waters, Milford, MA) equipped with 2487 dual-λabsorbance detector. ATP, ADP, and AMP were separated on a reverse-phase chromatography column (Lichrospher RP-18 HPLC Column, 5 µm particle size, 25 cm × 4.6 mm [Length × Inner Diameter]), using a gradient mobile phase that consisted of phase A (100 mM KH_2PO_4 buffer with 1.2 $\%\frac{\mathrm{V}}{\mathrm{V}}$ methanol, at pH = 7.0) and phase B (100 mM KH_2PO_4 buffer with 10 $\%\frac{\mathrm{V}}{\mathrm{V}}$ methanol, at pH = 7.0) The elution program was the following: 100 % of A from 0 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 $1\,\mathrm{mL}\,\mathrm{min}^{-1}$, 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). 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 moles per gram wet tissue ($\mathrm{mol}\,\mathrm{g}^{-1}$).

Statistical and exploratory factor analysis

Different statistical methods were applied depending on the objective and the nature of the analyzed data. Kolmogorov-Smirnoff test was used to test data normality. Whenever normality was ensured, one-way ANOVA with the Tukey's HSD *post hoc* test was set as the default test. Whenever this assumption was violated, extreme values of more than three standard deviations from the mean were omitted. Otherwise, the nonparametric Kruskal-Wallis test was performed, using the Mann-Whitney U test as a *post hoc* test. The overall distribution of sperm defects between groups was further tested using the χ^2 test. The nonparametric Spearman correlation was used to correlate fertility and sperm parameters. The paramet-

ric Pearson correlation (r coefficients) was used to correlate the different sets of variables in the study (sperm parameters, endocrine and metabolic function, mitochondrial function, bioenergetics, and antioxidant system), after variables were successfully tested for normality using the Kolmogorov-Smirnoff test, and as part of exploratory factor analysis. The correlation strength was classified according to ranks (Taylor, 1990). Variables with significant ($P < 0.05$) linear correlation with sperm parameters were then considered for Multivariate Analysis based on principal components analysis (PCA). Although PCA is an unsupervised multivariate method, it was used as a sparse and supervised method considering the variable selection criteria based on linear (Pearson) correlation. Forced factor extraction was performed to extract two principal components (PCs), based on the interpretability criterion, and using Varimax with Keiser's normalization as rotation method. Regression factors were used to plot sample distribution in the two-dimensional Euclidean space. All methods were performed using IBM SPSS statistics v25 (Armonk, NY). Independently of the statistical method used, significance was considered whenever $P < 0.05$.

4.3 Results

Hormonal balance is not affected by high-fat diets, and glucose homeostasis is recovered after dietary correction

In our previous study (**Crisóstomo** et al., 2019), we found that dietary switch from a fat-rich diet to a standard chow was able to normalize biometric parameters (Supplemeantal Figure 4.1, A and B) and glucose homeostasis (Figure 4.1). Hereby, we further characterized the model by studying the endocrine function, including insulin and reproductive hormones such as FSH, LH, E_2, and testosterone (Table 4.1). As observed before, the severe impairment in all the parameters related to metabolic homeostasis (HOMA2), significantly improved after diet reversal (Figure 4.1). HFD mice had a β-cell function (HOMA2-%B) ≈3- and 2-fold higher than those of CTRL and HFD_t (Figure 4.1A). Influence on glucose sensitivity (HOMA2-%S) (Figure 4.1B) and insulin resistance (HOMA2-IR) (Figure 4.1C) was even more pronounced; a 10- and 5-fold increase was observed in HFD animals when compared with CTRL and HFD_t, respectively. Plasma insulin levels were found to be increased in HFD mice, and notably, diet correction restored plasma insulin levels (Table 4.1).

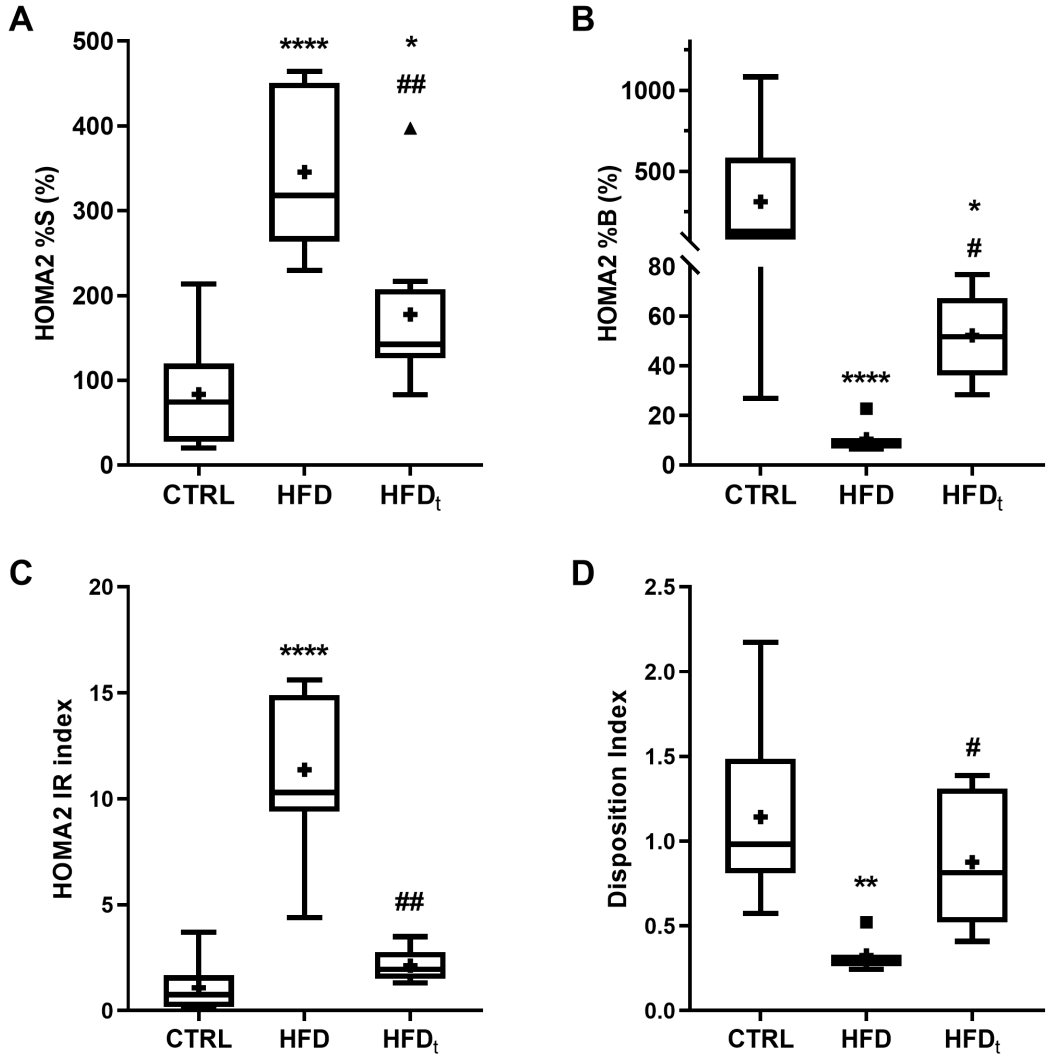


Figure 4.1: HOMA2 indexes and disposition index of mice fed standard chow (CTRL) or lifelong high-fat diet (HFD) or subjected to diet correction after 60 days (HFD$_t$). Fasting glycemia and insulin were used as inputs for the interactive HOMA2 calculator (University of Oxford, UK). Results are expressed as Tukey whisker boxes [median, 25th to 75th percentiles $\pm$ 1.5 IQR]. Extreme values are represented individually (■ denotes HFD, high-fat diet; ▲ denotes HFD$_t$, transient high-fat diet). Data were tested by one-way ANOVA with the Tukey HSD for group comparison. Significance was considered when $P < 0.05$. ✚ Group average. * vs. CTRL. # vs. HFD. *$P < 0.05$; **$P < 0.01$; ****$P < 0.0001$; #$P < 0.05$; ##$P < 0.01$.

Dietary correction in adulthood after HFD in early life does not restore the decreased sperm quality

We collected epididymal sperm immediately after euthanization. Sperm motility and concentration were promptly assessed, according to standard protocols. Sperm viability and morphology were evaluated after specific staining techniques and using optical microscopy. We have found no differences in sperm counts between groups (Figure 4.2). However, mice fed HFD, even if transiently, displayed reduced sperm motility (CTRL: 81.6 ± 4.6 %; HFD:

Table 4.1: Fasting glycemia and serum concentrations of insulin, FSH, LH, E_2 and testosterone in mice fed standard chow (CTRL), life-long high-fat diet (HFD), and those subjected to diet correction after 60 days (HFD_t). Fasting glycemia was evaluated using a glucometer (One Touch Ultra Lifescan-Johnson, Milpitas, CA, USA). Hormones were quantified by ELISA assay kits. Results are expressed as mean ± SD (units disclosed in the table). CTRL – standard chow; HFD – high-fat diet; HFD_t – transient high-fat diet. Data was tested by one-way ANOVA with Tukey's HSD for group comparison. Significance was considered when $p<0.05$. * vs. CTRL; # vs. HFD. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$.

Analyte	**CTRL** (n = 8)	**HFD** (n = 8)	**HFD_t** (n = 8)
Fasting glucose, mg dL^{-1}	94.50 ± 7.94	116.08 ± 15.26***	84.91 ± 9.91####
Insulin, nmol mL^{-1}	0.49 ± 0.68	3.56 ± 1.75****	0.67 ± 0.25####
FSH, nmol mL^{-1}	6.60 ± 2.83	4.25 ± 3.18	3.77 ± 2.67
LH, nmol mL^{-1}	435.99 ± 79.50	406.89 ± 61.62	470.30 ± 28.34
E_2, pmol mL^{-1}	348.89 ± 46.55	335.46 ± 114.04	413.87 ± 108.97
Testosterone, nmol mL^{-1}	15.88 ± 0.58	15.88 ± 0.48	15.23 ± 0.97

69.1 ± 7.4 %; HFD_t: 65.9 ± 14.5 %) and sperm viability (CTRL: 48.2 ± 8.1 %; HFD: 38.1 ± 7.2 %; HFD_t: 34.4 ± 6.6 %). Regarding sperm morphology (Table 4.2), we used three different classification systems: descriptive (six defects), categorical (three categories) and binomial (normal/abnormal). On the basis of the χ^2 test, we found that sperm morphology distribution is different between groups. Notably, recurring to ANOVA and Tukey's HSD test, we have found differences in the prevalence of pinhead, flattened head, bent neck, and overall head defects, especially relative to HFD_t. Despite the observed changes in sperm parameters, diet did not influence reproductive outcomes (Supplementary Table 4.S2). No significant correlations were found between sperm and fertility parameters (data not shown).

Table 4.2: Sperm morphology distribution, according to different classifications, in mice fed standard chow (CTRL), lifelong high-fat diet (HFD), and high-fat diet with diet correction after 60 days (HFD_t). Results are expressed as mean (% of total sperm cells) ± standard deviation. Sperm count distributions across groups were tested using the χ^2 test. Differences between groups for each morphological classification were tested by one-way ANOVA with Tukey's HSD. For both methods, significance was considered when $P<0.05$. #$P<0.05$ vs. HFD. *$P<0.05$, vs. CTRL; **$P<0.01$; ***$P<0.001$; ****$P<0.0001$.

Sperm morphology	**CTRL** (n = 9)	**HFD** (n = 12)	**HFD_t** (n = 9)	**χ^2 test**
Normal, %	39.79 ± 6.01	33.82 ± 6.74	36.51 ± 4.08	106.83****
Decapitated, %	12.10 ± 5.27	11.19 ± 3.61****	12.18 ± 3.11	
Pinhead, %	3.70 ± 1.30	6.20 ± 1.26**	6.68 ± 1.94**	
Flatenned head, %	3.92 ± 0.76	4.62 ± 1.20	6.02 ± 0.86***,#	
Bent neck, %	6.49 ± 0.97	9.38 ± 3.64	5.30 ± 1.97##	
Coiled tail, %	34.00 ± 5.73	34.77 ± 7.06	33.32 ± 2.46	
Normal, %	39.79 ± 6.01	33.82 ± 6.74	36.51 ± 4.08	48.57****
Head defects, %	19.72 ± 3.82	22.03 ± 4.52	24.88 ± 2.45*	
Tail defects, %	40.49 ± 5.58	44.15 ± 6.41	38.61 ± 3.00	
Normal, %	39.79 ± 6.01	33.82 ± 6.74	36.51 ± 4.08	26.16****
Abnormal, %	60.21 ± 6.01	66.18 ± 6.74	63.49 ± 4.08	

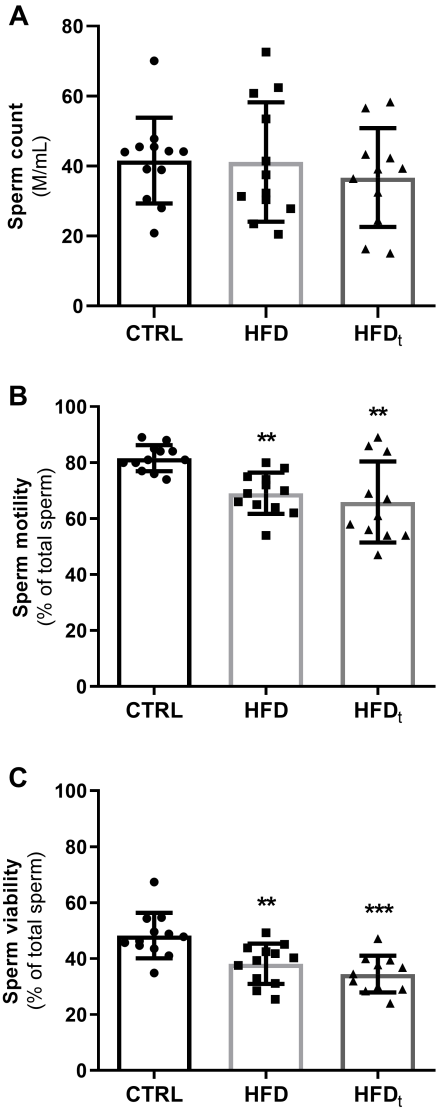


Figure 4.2: Epididymal sperm parameters of mice fed standard chow (CTRL) or lifelong high-fat diet (HFD) or subjected to diet correction after 60 days (HFD$_t$). Results are expressed as means ± SD. Sperm count is expressed as million spermatozoa per milliliter (M/mL), while other parameters are expressed as the percentage of total sperm cells. Individual values are represented (● denotes CTRL, standard chow; ■ denotes an HFD, high-fat diet; ▲ denotes HFD$_t$, transient high-fat diet). Data were tested by one-way ANOVA with the Tukey's HSD test for group comparison. Significance was considered when $P < 0.05$. **$P < 0.01$; ***$P < 0.001$.

Testicular mitochondrial function and bioenergetics are not affected by high-fat diets

Mitochondria were isolated from a whole testis to evaluate relevant parameters related to its activity and physiology. Particularly, relative protein expression of mitochondrial Oxidative Phosphorylation (OXPHOS) complexes was assessed by Western blot analysis, and the activities of CI, CII, CIV, and CS were evaluated by colorimetric/fluorometric assays (Supplementary Table 4.S3). Testicular bioenergetics were evaluated on the basis of testicular content of adenine nucleotides (ATP, ADP, and AMP), quantified by HPLC (Supplementary Table 4.S4). These nucleotides were extracted from the same testis used to isolate the mitochondria-rich fraction. There were no differences between any of the experimental groups.

A high-fat diet promotes a decrease in the activity of antioxidant defenses in testis

Lipid peroxidation and activity of enzymes of the antioxidant defense system were evaluated in mitochondria-free cytosolic fraction obtained from a whole-testis homogenate. The dietary regime did not influence testicular levels of lipid peroxidation products (Figure 4.3A). Additionally, activities of the cytosolic GPx and SOD were similar in the testes of the mice from the different groups (Figure 4.3B and C). Conversely, the mitochondria-free cytosolic fraction obtained from the testes of HFD group mice had reduced enzymatic activity of CAT (CTRL: $747.66 \pm 141.46\,\mathrm{nmol_{O_2}\,min^{-1}\,mg^{-1}_{protein}}$; HFD: $552.41 \pm 91.51\,\mathrm{nmol_{O_2}\,min^{-1}\,mg^{-1}_{protein}}$; $\mathrm{HFD_t}$: $670.02 \pm 107.21\,\mathrm{nmol_{O_2}\,min^{-1}\,mg^{-1}_{protein}}$) and GSR (CTRL: $133.14 \pm 11.31\,\mathrm{nmol_{NADPH}\,min^{-1}\,mg^{-1}_{protein}}$; HFD: $114.00 \pm 11.53\,\mathrm{nmol_{NADPH}\,min^{-1}\,mg^{-1}_{protein}}$; $\mathrm{HFD_t}$: $120.44 \pm 9.14\,\mathrm{nmol_{NADPH}\,min^{-1}\,mg^{-1}_{protein}}$).

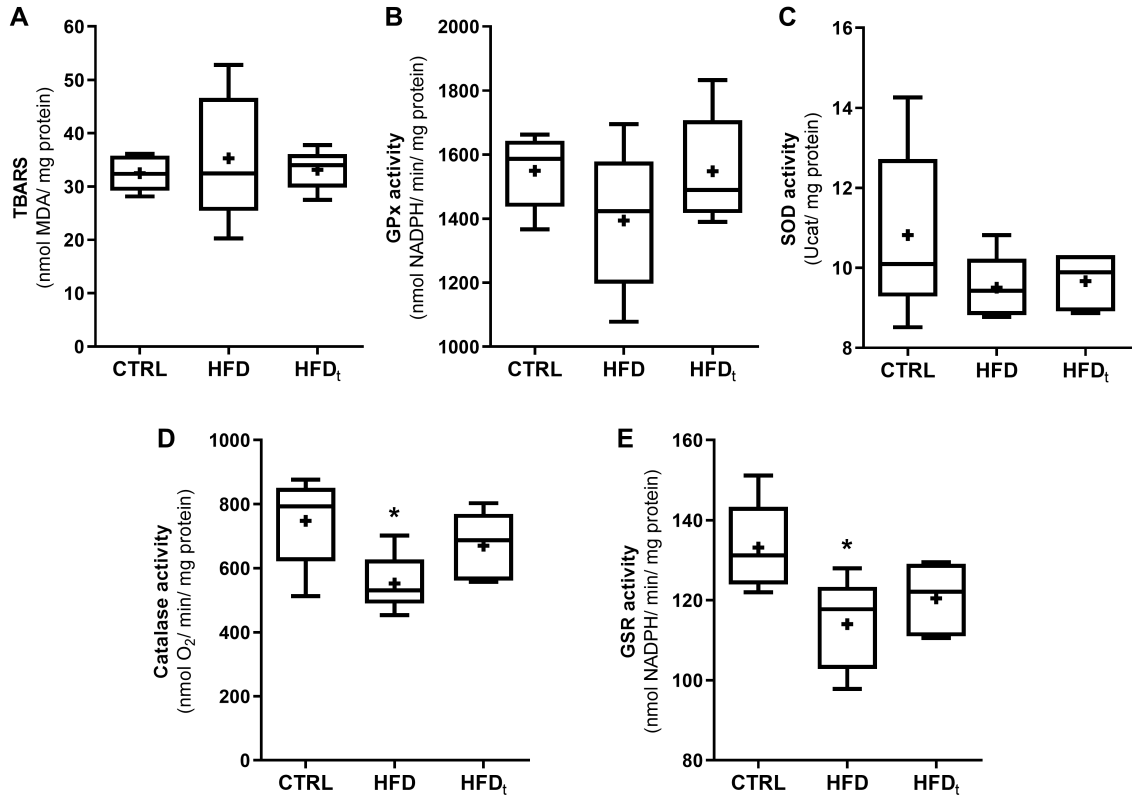


Figure 4.3: Lipid peroxidation (thiobarbituric acid reactive species (TBARS) assay) and activity of the four main enzymes of the antioxidative defense metabolism: superoxide dismutase (SOD), glutathione-disulfide reductase (GSR), glutathione peroxidase (GPx) and catalase (CAT) in the testis of mice standard chow (CTRL) or lifelong high-fat diet (HFD) or subjected to diet correction after 60 days ($\mathrm{HFD_t}$). Two outliers were excluded from the analysis resulting in $n = 5$ for every group. Results are expressed as Tukey whisker boxes [median, 25th to 75th percentiles $\pm$ 1.5 IQR. Data were tested by one-way ANOVA with the Tukey highly significant difference test for group comparisons. Significance was considered when $P < 0.05$. ✚ Group average. * vs. CTRL. *$P < 0.05$.

Testicular lipid metabolism is irreversibly affected by an HFD during early life

Metabolites were obtained from a whole testis using a protocol based on chloroform-methanol-water extraction, resulting in a polar and apolar fractions. In the polar fraction, analyzed by ^{1}H-NMR, various phospholipid-related metabolites and lipid precursors were detected (Table 4.3). HFD had a marked influence on the composition of polar lipids, as observed in the levels of phospholipid head groups like myoinositol, choline, or glycerol, or choline-related GPC. Reversal from HFD to normal diet tended to normalize metabolite levels.

Table 4.3: Polar lipid metabolites detected by ^{1}H-NMR in testicular extracts of mice fed standard chow (CTRL), lifelong high-fat diet (HFD), and those subjected to diet correction after 60 days (HFD_t). Results are expressed as mean (arbitrary units) ± SD. CTRL – standard chow; HFD – high-fat diet; HFD_t – transient high-fat diet. Results were tested by one-way ANOVA with Tukey's HSD, except where stated otherwise. Significance was considered when $P < 0.05$. [a]Kruskal-Wallis test, unadjusted P value. *$P < 0.05$, vs. CTRL; $^{\#}P < 0.05$ vs. HFD; **$P < 0.01$; ****$P < 0.0001$; $^{\#\#}P < 0.01$. Abbreviations: 3OHBut - 3-hydroxybutyrate; EtAm - ethanolamine; GPC - glycerophosphocholine; PChol - phosphocholine; PEtAm - phosphoethanolamine

	Metabolite	**CTRL** (n = 6)	**HFD** (n = 6)	**HFD_t** (n = 5)
Lipid Intermediaries	Glycerol	$(7.50 \pm 0.85) \times 10^{-3}$	$(9.26 \pm 0.26) \times 10^{-3}$**	$(7.82 \pm 0.71) \times 10^{-3}$##
	EtAm[a]	$(1.75 \pm 0.22) \times 10^{-3}$	$(1.74 \pm 0.14) \times 10^{-3}$	$(1.48 \pm 0.13) \times 10^{-3}$*,#
	PEtAm	$(2.01 \pm 0.18) \times 10^{-2}$	$(2.01 \pm 0.06) \times 10^{-2}$	$(1.99 \pm 0.09) \times 10^{-2}$
	myo-Inositol	$(4.94 \pm 0.48) \times 10^{-3}$	$(5.54 \pm 0.27) \times 10^{-3}$*	$(4.58 \pm 0.32) \times 10^{-3}$##
	3OHBut	$(6.68 \pm 1.08) \times 10^{-2}$	$(5.70 \pm 0.34) \times 10^{-2}$	$(5.97 \pm 0.64) \times 10^{-2}$
Choline Metabolism	Choline	$(1.32 \pm 0.15) \times 10^{-2}$	$(0.98 \pm 0.13) \times 10^{-2}$**	$(1.12 \pm 0.13) \times 10^{-2}$
	PChol	$(2.01 \pm 0.18) \times 10^{-2}$	$(2.01 \pm 0.06) \times 10^{-2}$	$(1.99 \pm 0.09) \times 10^{-2}$
	GPC	$(3.00 \pm 0.34) \times 10^{-3}$	$(4.26 \pm 0.37) \times 10^{-3}$****	$(3.81 \pm 0.27) \times 10^{-3}$*

The apolar fraction of the testicular extract was characterized by GC-MS after transmethylation. The identified fatty acids (FAs) were quantified, and their relative abundance is displayed in Supplemental Table 4.S5. Dietary intervention promoted a meta-state between CTRL and HFD conditions for most of the identified FAs (*e.g.*, palmitic acid and stearic acid). The only exception was vaccenic acid (C18:1n-7), which is twice more abundant in testes of HFD_t mice than in CTRL. After grouping FAs by degree of unsaturation and the position of the double bonds (Supplemental Table 4.S5 and Figure 4.4), we observed that the most abundant FA family in the testis of CTRL and HFD_t groups are the SFAs (55.79 % and 41.83 %, respectively), while PUFAs are the most abundant in the testis of mice from the HFD group (44.21 %). HFD and HFD_t mice also had increased testicular relative abundance of MUFAs (26 % and 21 %, respectively), with increased accumulation of oleic acid (C18:1n-9). Moreover, Supplemental Table 4.S5 also shows indirect measures for the anti-inflammatory/proinflammatory potential via lipid mediators (C22:6n-3/C20:4n-6 ratio), the combined activity of Δ5- and Δ6-desaturases (D5D and D6D) (C20:4n-6/C18:2n-6 ratio), and the activity of Δ4-desaturases (D4D) (C22:5n-6/C20:4n-6 ratio).

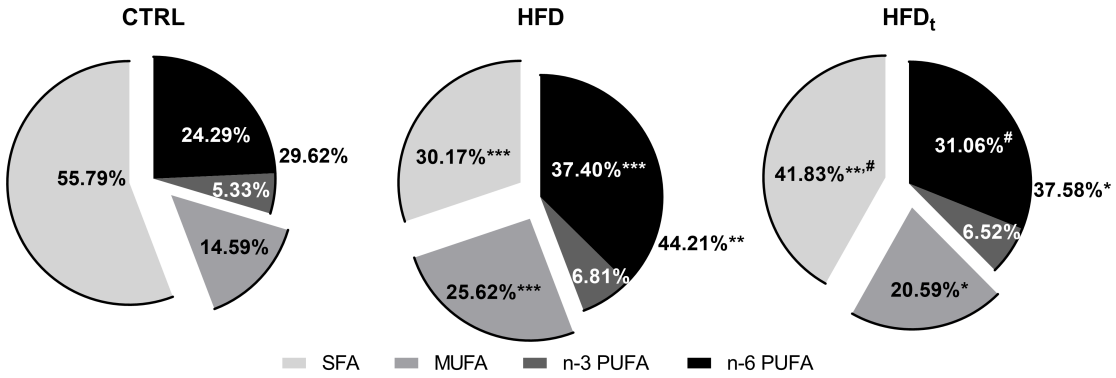


Figure 4.4: Relative abundance of lipids in the apolar fraction of testicular extracts of mice fed standard chow (CTRL) or lifelong high-fat diet (HFD) or subjected to diet correction after 60 days (HFD$_t$), grouped by saturation. CTRL, standard chow; HFD, high-fat diet; HFD$_t$, transient high-fat diet. The results were tested by one-way ANOVA with the Tukey's HSD test for *post hoc* group comparisons. Significance was considered when $P < 0.05$. * versus CTRL; # vs. HFD. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; #$P < 0.05$. MUFAs, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids; SFA, saturated fatty acids.

Male reproductive dysfunction caused by HFD is correlated to lipid dysmetabolism in testes

Our main aim was to evaluate whether a dietary intervention in early adulthood can prevent damage to male reproductive health. However, as in previous studies (**Crisóstomo** et al., 2019; Jarak et al., 2018), groups were not considered when testing for correlations. Assuming that all mice are equivalent before being fed by a specific diet regimen, our test hypothesis is that some pairs of variables vary proportionally in response of HFD. Those pairs reflect the most relevant (discriminant) variables; therefore, the variables that must be included in the multivariate model. In a correlation matrix (Figure 4.5A), we tested variables of mitochondrial function, bioenergetics, endocrine function, and antioxidant defenses against sperm parameters. We found a significant negative correlation between CI activity and the prevalence of pin head sperm defect ($r = -0.577$; $P = 0.024$). Regarding bioenergetics, the increase in testicular content in ATP ($r = 0.517$; $P = 0.034$) and ATP/ADP ratio ($r = 0.592$; $P = 0.012$) was correlated with elevated sperm counts. AMP ($r = -0.638$; $P = 0.011$) and AMP/ATP ratio ($r = -0.703$; $P = 0.003$) were inversely correlated to sperm head defects, particularly decapitated sperm ($r = -0.627$; $P = 0.012$ and $r = -0.689$; $P = 0.005$). Conversely, testicular energy charge was positively correlated to both parameters ($r = 0.733$; $P = 0.002$ and $r = 0.633$; $P = 0.011$). Concerning endocrine function, most correlations were found in glucose homeostasis. Elevated serum levels of insulin ($r = -0.425$; $P = 0.043$), HOMA2-%B ($r = -0.523$; $P = 0.010$) and HOMA2-IR ($r = -0.438$; $P = 0.037$) were correlated with lower sperm viability. HOMA2-%S behaved contrarily to the former ($r = 0.424$; $P = 0.044$). FSH showed a

protective effect against sperm head defects ($r = -0.464$; $P = 0.039$), namely pin ($r = -0.505$; $P = 0.023$) or flattened head ($r = -0.673$; $P = 0.001$) defects. Concerning antioxidant defenses, only SOD activity presented a significant inverse correlation to sperm concentration ($r = -0.527$; $P = 0.030$). Significantly correlated variables were selected for PCA, to evaluate their predictive power (*i.e.*, if samples were clustered according to their diet using these variables as predictors). These correlations promote a significant clustering of samples, according to their experimental group, and there is no overlap between groups, considering two PCs (Figure 4.5B).

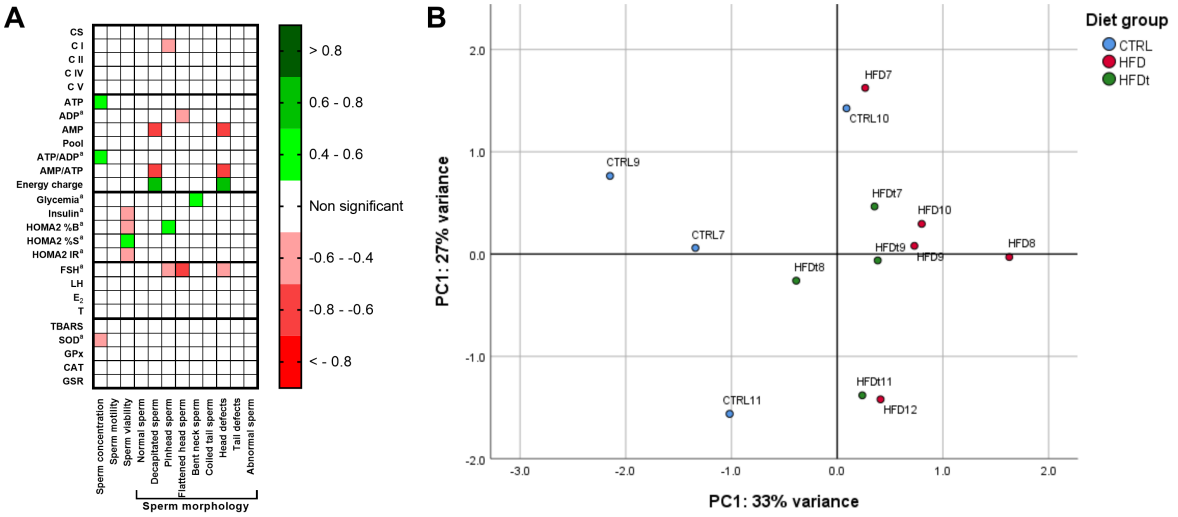


Figure 4.5: Correlations between sperm parameters to endocrine, antioxidant, mitochondrial, and bioenergetic parameters. A: individual scores for the previously assessed parameters were correlated using the parametric Pearson correlation ($n = 24$). Values are represented as Pearson r coefficients, according to the color scale, when $P < 0.05$. B: sample distribution according to two principal components extracted by principal components analysis, and on the basis of correlations between sperm parameters to endocrine, antioxidant, mitochondrial, and bioenergetic parameters ($n = 10$). [a]After base-10 logarithmic transformation. CS, citrate synthase; C I, complex I activity; C II, complex II activity; C IV, complex IV activity; C V, complex V activity; Pool, total adenosine nucleotide pool; glycemia, fasting glycemia; FSH, serum follicle-stimulating hormone; LH, serum leutinizing hormone; E2,serum 17β-estradiol; T, serum testosterone; TBARS, thiobarbituric acid-reactive species assay (lipid peroxidation); SOD, superoxide dismutase activity; GPx, glutathione peroxidase activity; CAT, catalase activity; GSR, glutathione-disulfide reductase activity.

In the second correlation matrix (Figure 4.6A), sperm parameters were correlated against testicular lipid metabolite levels. Testicular content in SFAs and PUFAs had a mirror effect over the prevalence of bent neck and coiled tail sperm defects, while SFAs were negatively correlated with bent sperm neck defects ($r = -0.668$; $P = 0.035$) and positively correlated with coiled sperm tail defects ($r = 0.649$; $P = 0.042$). MUFAs had the opposite relation to bent sperm neck ($r = 0.744$; $P = 0.014$) and to coiled sperm tail ($r = -0.663$; $P = 0.037$). Globally, lipid precursors correlated positively with sperm viability. Testicular glycerol ($r = 0.612$; $P = 0.020$) and myoinositol ($r = 0.592$; $P = 0.026$), however, displayed a positive correlation with the prevalence of bent neck sperm. Choline is positively correlated to normal mor-

phology of sperm (r = 0.609; P = 0.021), while negatively correlated to tail defects (r = 0.614; P = 0.019). The two-dimensional projection of the PCs extracted by PCA (Figure 4.6B) reveals a clear separation of the samples according to their group.

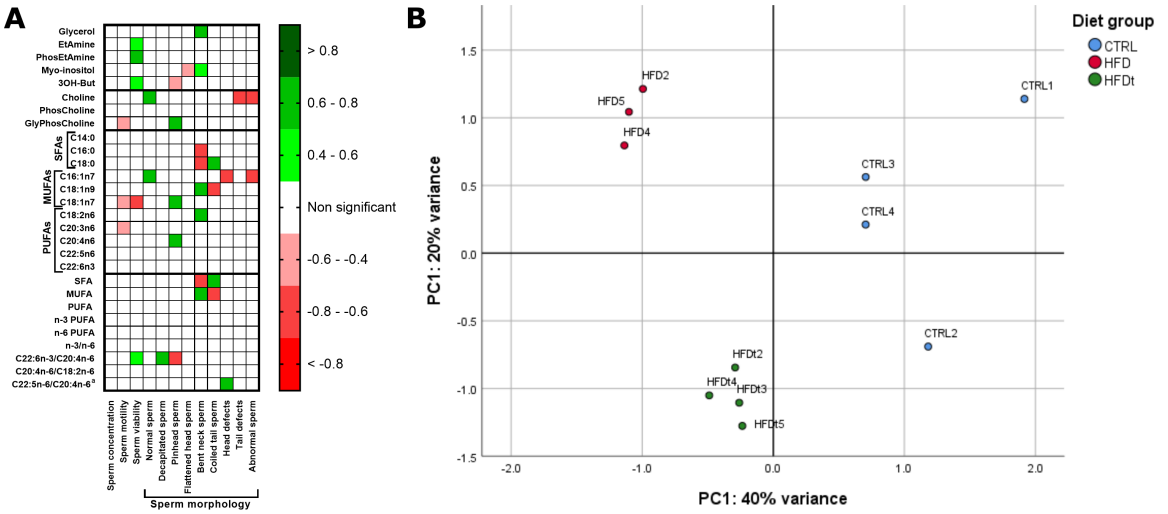


Figure 4.6: Correlation of sperm parameters to testicular lipids and lipid precursors. A: individual scores for the previously assessed parameters were correlated using the parametric Pearson correlation (n = 14). Values are represented as Pearson r coefficients, according to the color scale, when P < 0.05. B: sample distribution according to two PCs extracted by PCA, and based on correlations between sperm parameters to testicular lipids and lipid precursors (n = 11). [a]Spearman rho coefficients, as the normality assumption was violated for this variable. EtAmine, ethanolamine; PhosEtAmine, phosphoethanolamine; 3OH-But, 3 -hydroxybutyrate; PhosCholine, phosphocholine; GlyPhosCholine, glycerophosphocholine; SFAs, saturated fatty acids; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

4.4 Discussion

Overweight and obesity have reached pandemic proportions worldwide. Simultaneously, the prevalence of childhood obesity is increasing, leading to the onset of obesity-associated comorbidities, such as T2D, at younger ages. Reproductive function depends on metabolic homeostasis. Several reports link the prevalence of metabolic disease with lower sperm quality (Eisenberg et al., 2016a; D. Guo et al., 2017a; Palmer, Bakos, Fullston, et al., 2012). In this study, we used a rodent model to investigate whether a dietary intervention to replace an HFD with a balanced diet in early adulthood, is effective in reversing or preventing the negative effects of childhood obesity in fat accumulation, metabolic homeostasis, and sperm quality. Particularly, we evaluated whether diet intervention is effective in normalizing testicular metabolic and lipid balance, and sperm quality later in life. Further, we integrated all data of metabolomics and lipidomics to elucidate the importance of lipids in mechanisms involved in the onset and/or recovery of the fertility phenotypes induced by HFD in early life.

We have previously demonstrated that this model induces phenotypic traits related to obesity and metabolic syndrome, based on biometric and physiological tests for glucose intolerance (ipGTT) and insulin resistance (ipGTT) (**Crisóstomo** et al., 2019). We have shown that HFD promoted fat deposition in white adipose tissue pads and liver, which were positively correlated with poorer performance in ipGTT and ipGTT. In this report, we calculated HOMA2 indexes based on fasting glycemia and fasting insulin levels (Levy et al., 1998). Although HOMA2 application to animal models is often criticized (T. M. Wallace et al., 2004), as it has been developed on the basis of a large human database, it has shown an acceptable goodness of fit for mouse and other mammals (Antunes et al., 2016; Avtanski et al., 2019), and has a high correspondence to ipGTT, ipGTT, and their respective AUCs (Allison et al., 1995). As its precursor, HOMA2 is based upon serum fasting glycemia and insulinemia, therefore providing a better physiological characterization of glucose homeostasis. The methodology for quantifying hormonal serum concentrations at euthanization was ELISA, for all selected hormones. Insulin was the only hormone measured that was found to be altered when comparing hormonal levels in the serum of mice from the different groups. HOMA2 showed that dietary intervention is not capable to completely reverse the effects of an HFD during early life in β-cell function and insulin sensitivity, despite total recovery of insulin resistance index. Despite these results not being novel (**Crisóstomo** et al., 2019), it is relevant to state the high degree of correspondence with our previous results, obtained using different methods for glucose homeostasis assessment.

Although testosterone aromatization into estradiol has been widely reported in patients with obesity, our results support recent research showing that this effect may be negligible (Bekaert et al., 2015; Rastrelli et al. [APA], 2018). Although another study suggests marked changes in sex hormone levels in HFD rodents (Borges et al., 2017), circulating levels of fertility-related hormones were not influenced by dietary regimes in this particular study (Table 4.1). Yet, endocrine function is strongly correlated with sperm quality, especially glucose homeostasis and FSH secretion (**Crisóstomo** et al., 2018; Rato, Alves, Socorro, Duarte, et al., 2012) (Figure 4.5A). In this context, SCs may be the mediator between both factors and sperm quality. SCs nurse differentiating germ cells during spermatogenesis, and their function is modulated by metabolic (Jesus et al., 2016; Rato et al., 2014) and endocrine (Martins et al., 2019; Martins et al., 2015; Martins et al., 2016; Rato, Alves, Duarte, et al., 2015) factors, with significant impact on mature spermatozoa (P. F. Oliveira, Sousa, et al., 2017). Fat deposition in the abdominal area, in humans, is correlated with poor sperm quality (Faure et al., 2014; A. O. Hammoud et al., 2008), likely due to increased testicular

temperature (Garolla et al., 2015; Hucik et al., 2019) and local inflammation (G.-L. Liu et al., 2013). In our previous work (**Crisóstomo** et al., 2019), we also reported reduced sperm quality, which was associated with fat deposition. Moreover, there is a decrease in the activity of antioxidant enzymes (CAT and GSR) in the testes of lifelong HFD-fed mice. Therefore, it is possible that extra lipid intake, notably in PUFAs, is the cornerstone in testicular antioxidant balance. This hypothesis is supported by the differences in lipid fractions between groups (Figure 4.4), the differences in the correlations of lipid fractions against sperm parameters (Figure 4.6A), and the sample separation achieved by the corresponding PCA (Figure 4.6B). Nonetheless, more studies will be needed to confirm this hypothesis, particularly by using a model in which HFD is replaced by a high-fat, low-PUFA diet. Moreover, we have not considered the potential contamination of testicular lipid profile by serum. Yet, testes were decapsulated before homogenization to hinder this issue. Besides, the interference of serum lipid profile is unlikely considering blood to testis volume (Kaliss & Pressman, 1950) and a previous study comparing testicular and serum lipid profiles (Napoli & McCormick, 1981).

HFD is the culprit for testicular lipid dysmetabolism. The proportion of SFAs is the lowest in testes of mice from the HFD group, and the highest in testis from mice of the CTRL group. Contrastingly, MUFA and PUFA fractions were more abundant in HFD than in the CTRL group. As lard, one of the main components of the diet formulations, is rich in oleic acid (18:1n9), it is not surprising that HFD and HFD_t testes are enriched in this fatty acid. Yet, the relative content of MUFAs compared with PUFAs, in both groups, suggests that oleic acid and other MUFAs are unsaturated into PUFAs. Besides, the detected n-3 and n-6 long-chain PUFAs can only be synthesized after their precursors, the linoleic and linolenic acid are obtained by diet, as they cannot be synthesized de novo in mammals (Miyazaki & Ntambi, 2008). According to Koeberle et al. (2012), mammals store large amounts of PUFAs in testes during puberty, by the action of the lysophosphatidic acid acyltransferase 3, resulting in a unique FA profile. For instance, docosapentaenoic acid (C22:5n-6, n-6 DPA) is more abundant in the testis than in other mammalian tissues (Bieri & Prival, 1965; Zanetti et al., 2007). Although several studies describe n-6 DPA-enrichment in various tissues due to HFD (Garg et al., 1988; Naoe et al., 2019; Ostermann et al., 2017), testicular content of DPA was unchanged in our model, but we observed testicular enrichment in other n-6 FAs (Supplemental Table 4.S5).

Regarding PUFAs, the n-3/n-6 ratio in testis supports an amelioration in testicular metabolism after ceasing HFD feeding at early adulthood. The D6D has been associated with the deleterious effects of HFD, particularly regarding glucose resistance (Hucik et al.,

2019). The dietary n-3/n-6 ratio intake, for humans, is ideally close to 1, and lower ratios (n-6 enrichment) are associated with health deterioration, including increased cardiovascular risk (Simopoulos, 2002). In human testis, a lower n-3/n-6 ratio has been reported in oligo and asthenozoospermia (Zalata et al., 1998). Herein, we found a much lower n-3/n-6 ratio (0.22) in testicular FA content, in the CTRL group. Yet, this ratio was significantly lower (0.18) in HFD-fed mice during their lifetime. Considering the critical role of docosahexaenoic acid (C22:6n-3, DHA) in spermatogenesis, the detrimental content of n-3 in testis may be associated with the observed phenotype, as it cannot balance the proinflammatory environment induced by HFD. Effectively, DHA supplementation was shown to improve testicular n-3/n-6 ratio, concomitantly improving antioxidant balance (Starčević et al., 2018). However, in our study, sperm parameters did not correlate to the testicular n-3/n-6 ratio.

Although the HFD formulation had similar relative amounts of SFAs, MUFAs, and PUFAs comparing to the standard chow, the C18:3n-3/C18:2n-6 molar ratio is an order of magnitude higher in HFD (according to the manufacturer information). Interestingly, testicular cells responded to this difference by accumulating even more n-6 PUFAs, without changing their n-3 PUFA content (Figure 4.4/Supplemental Table 4.S5). Moreover, n-6 PUFA changes are distinguished not only by an increase in the relative abundance of dietary available linoleic acid (C18:2n-6) but also by an increase of dihomo-γ-linolenic acid (C20:3n-6), and arachidonic acid (C20:4n-6; ARA), which are obtained from linoleic acid by an enzyme-catalyzed desaturation-elongation process. This may reflect an overall inhibition of the D4D and D5D induced by HFD, notably by the relative enrichment in the dietary n-3. D6D is considered the rate-limiting enzyme in the desaturation-elongation processes in mammalian cells, but according to the relative abundance of n-6 PUFAs to n-3 PUFAs and the C20:4n-6/C18:2n-6 ratio, HFD does not change its activity nor is it overwhelmed by the extra dietary n-3. Therefore, testicular metabolic pathways involving n-6 PUFA remodeling and metabolism exhibit high sensitivity to the excess of dietary fat (Figure 4.4).

The structural importance of phospholipids and its FA composition to cell membrane fluidity should not be overlooked (Lingwood & Simons, 2010). Testicular SFA content is negatively correlated with bent neck defects, while it is positively correlated with coiled tail defects. Interestingly, MUFAs present mirrored correlations. This observation may be linked to the different membrane composition of the midpiece (neck) and the tail, or even to different membrane lipid needs of germ cells at different spermatogenic stages (Wolf et al., 1986). However, because of the limited availability of testicular samples, it was not possible to estimate the detailed testicular content of neutral lipid and phospholipid classes, besides the

total lipid profile. In this context, it is noteworthy that FAs from the same lipid saturation family can result in opposite outcomes in testicular function. DHA is reduced in testes of acyl-CoA synthetase isoform 6 (ACSL6) knockout (KO) mice, leading to an enrichment in the ARA (Hale et al., 2019). This change fosters a proinflammatory environment, as ARA is an n-6 PUFA and precursor of proinflammatory eicosanoids, whereas DHA is an n-3 PUFA and precursor of resolvins. According to those authors, ACSL6-KO mice suffered from subfertility, with hypogonadism, oligozoospermia, reduced number of germ cells, and morphological abnormalities of the seminiferous epithelium. Our data show that HFD group presented overlapping phenotypes, such as ARA accumulation in testes and poorer sperm parameters, although without DHA depletion in testes, likely due to its dietary intake. Particularly, testicular ARA content is positively correlated to sperm pin head defects. However, Hale et al. (2019) further reported that DHA-rich and ARA-rich phospholipids are delocalized in testes of ACSL6-KO mice. So, the relative testicular content in those FAs is crucial to maintain a normal testicular ultrastructure and spermatogenesis, although some caution must be taken, as the characterization of FAs was not quantitative. Despite the different proportions in lipid fractions between diet regimens, it is not possible to state whether absolute testicular FA content is enriched after HFD.

HFD promotes a positive feedback toward a proinflammatory state in testis. Both n-3/n-6 and C22:6n-3/C20:4n-6 reflect a shift toward proinflammatory pathways in the testis of mice continuously fed by HFD (Supplemental Table 4.S5). Interestingly, the same group presented lower CAT and GSR activity and lower potential to prevent oxidative damage. The testicular enrichment in C18:1n-9, in HFD mice, may promote cell membrane fluidity in testicular cells and, consequently, their vulnerability to peroxidation and phospholipase A2. Ultimately, it leads to increased release of PUFAs, particularly ARA, a central precursor of pro-inflammatory pathways (leukotrienes, thromboxanes and prostaglandins). Overall, the present data indicate that HFD shifts the cellular redox environment toward a more prooxidant and pro-inflammatory state, enhancing testes susceptibility for oxidative stress and inflammatory processes development. Thus, the absence of changes in lipid peroxidation (TBARS) and in the activity of mitochondrial ETC activity complexes suggest that HFD by itself seems not to be enough stimulus to trigger oxidative stress. It was reported that HFD causes an increase in antioxidant metabolites in testis, notably GSH and betaine (**Crisóstomo** et al., 2019). Although we cannot rule out that the decreased activity of CAT and GSR can emerge from post-translational changes (*e.g.*, acetylation, phosphorylation and ubiquitination), it was reported that HFD has not only a negative impact (decrease) on the

expression of testicular antioxidant enzymes (Ghosh & Mukherjee, 2018; G.-L. Liu et al., 2013), but also promotes an increase in antioxidant metabolites in testes, notably GSH and betaine (**Crisóstomo** et al., 2019). Thus, the increased testicular GSH and betaine levels may be enough to keep the redox balance in testis despite the decreased enzymatic antioxidant protection.

In addition to this, lipolysis can be another contributor to the observed differences in lipid fractions, and a critical process to mediate membrane integrity and fluidity of all cells in testicular tissue. It is well known that dietary MUFAs promote lipolysis (O'Connor & Rudkowska, 2019). Changes in testicular glycerol, choline, myo-inositol and glycerophosphocholine in HFD (and partially in HFD_t) are likely due to lipolysis and indicate increased phospholipid turnover and/or degradation. Nevertheless, lipolysis raises another threat to seminiferous tubules stability. Glycerol accumulates in the testes of HFD and HFD_t mice. Glycerol destabilizes tight junctions and desmosomes between SCs, causing a leaky blood-testis barrier (**Crisóstomo**, Alves, et al., 2017). We also observed an accumulation of the organic osmolytes glycerophosphocholine and myoinositol in testes, after HFD. Previously, we reported an accumulation of betaine, glutamine, and glutamate in the same condition (**Crisóstomo** et al., 2019). Interestingly, this phenomenon has been previously reported in kidney medullar cells, as a response against hypertonic stress (Neuhofer & Beck, 2005). FA oxidation requires a hydration step; therefore, the extra dietary intake of FAs can induce hypertonic stress in testes, triggering this response in testicular cells, notably SCs. Our data also suggest a protective effect of choline against sperm tail defects. A recent study linked choline (and betaine) supplementation with lipolysis activation, due to elevated succinate concentration (Sivanesan et al., 2018) in plasma. Interestingly, we have obtained opposite results in testes, after dietary intervention (**Crisóstomo** et al., 2019). Yet, our data support a promotion of lipolysis in testes by HFD in early adult life. Again, SCs are likely involved in this outcome, as their metabolic activity has been described to be remodeled toward lipolysis as a response to a high-energy environment, as it is the case of obesity (Rato et al., 2014). In our model, we have observed that AMP and AMP/ATP ratio inversely correlated with sperm head defects suggesting that energy-consuming pathways can be beneficial to spermatozoa. Conversely, increased testicular energy charge promoted sperm head defects. Additionally, sperm motility was reduced in groups where our data suggest lipolysis overactivation (HFD and HFD_t groups), as supported by the changes observed in metabolites related to phospholipids. An exaggerated accumulation of lipid droplets in the testis leads to lower sperm motility, but a proper FA supply is needed for sperm capacitation in the epididymis (Masaki

et al., 2017). Thus, different metabolic pathways related to different lipid classes have different roles and importance not only in different testicular cells, but also in different parts of the spermatozoon (Takei et al., 2014).

In summary, our data demonstrate that an HFD during early life (akin to childhood and puberty) causes an excessive accumulation of unsaturated FAs is testes. A diet intervention, replacing HFD for a balanced diet was proven effective in protecting/preventing metabolic dysfunction. However, an HFD during early-life caused irreversible metabolic remodeling in testes, with long-term sperm defects. Dietary intervention in early adulthood promotes lipolysis in testes, particularly from unsaturated FAs, toward the CTRL state, but this process is apparently too slow to recover normal sperm parameters. Mechanistically, our data suggest that HFD promotes a proinflammatory state in testis, aggravated by a positive feedback system that favors the accumulation of n-6 PUFAs, precursors of inflammatory response signaling molecules. Our model did not allow us to verify whether testicular lipid composition and normal sperm quality could be achieved later in life, but we must also consider that sperm quality declines with age, even in rodents (Jarak et al., 2018). Epigenetic modifications are likely involved in the observed phenotypes after HFD, especially those which have not been reversed by diet switch. Indeed, the prepubertal period is critical for epigenetic remodeling of germ cells (E. E. Nilsson & Skinner, 2015; Schagdarsurengin & Steger, 2016), and we plan to investigate the influence of those mechanisms in our model in future work. Our findings highlight the importance of preventing childhood obesity, to avoid irreversible damage for the reproductive health of the fathers of tomorrow, with unpredicted effects on their progeny.

4.5 Author contributions

Marco G. Alves, João F. Raposo, Rachel L. Batterham, and Pedro F. Oliveira conceived and designed research; **Luís Crisóstomo**, Marco G. Alves, Romeu A. Videira, Ivana Jarak, Kristina Starčević, Tomislav Mašek, and Luís Rato performed experiments; **Luís Crisóstomo**, Marco G. Alves, Romeu A. Videira, and Pedro F. Oliveira analyzed data; **Luís Crisóstomo**, Marco G. Alves, Romeu A. Videira, Ivana Jarak, Kristina Starčević, Tomislav Mašek, Rachel L. Batterham, and Pedro F. Oliveira interpreted results of experiments; **Luís Crisóstomo** prepared figures; **Luís Crisóstomo**, Marco G. Alves, and Pedro F. Oliveira drafted manuscript; **Luís Crisóstomo**, Marco G. Alves, Romeu A. Videira, Ivana Jarak, Kristina Starčević, Tomislav Mašek, João F. Raposo, Rachel L. Batterham, and Pedro F. Oliveira edited and revised manuscript; **Luís Crisóstomo**, Marco G. Alves, Romeu A. Videira, Ivana Jarak, Kristina Starčević, Tomislav Mašek, Luís Rato, João F. Raposo, Rachel L. Batterham, and Pedro F.

Oliveira approved final version of manuscript.

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4.7 Supplementary Material

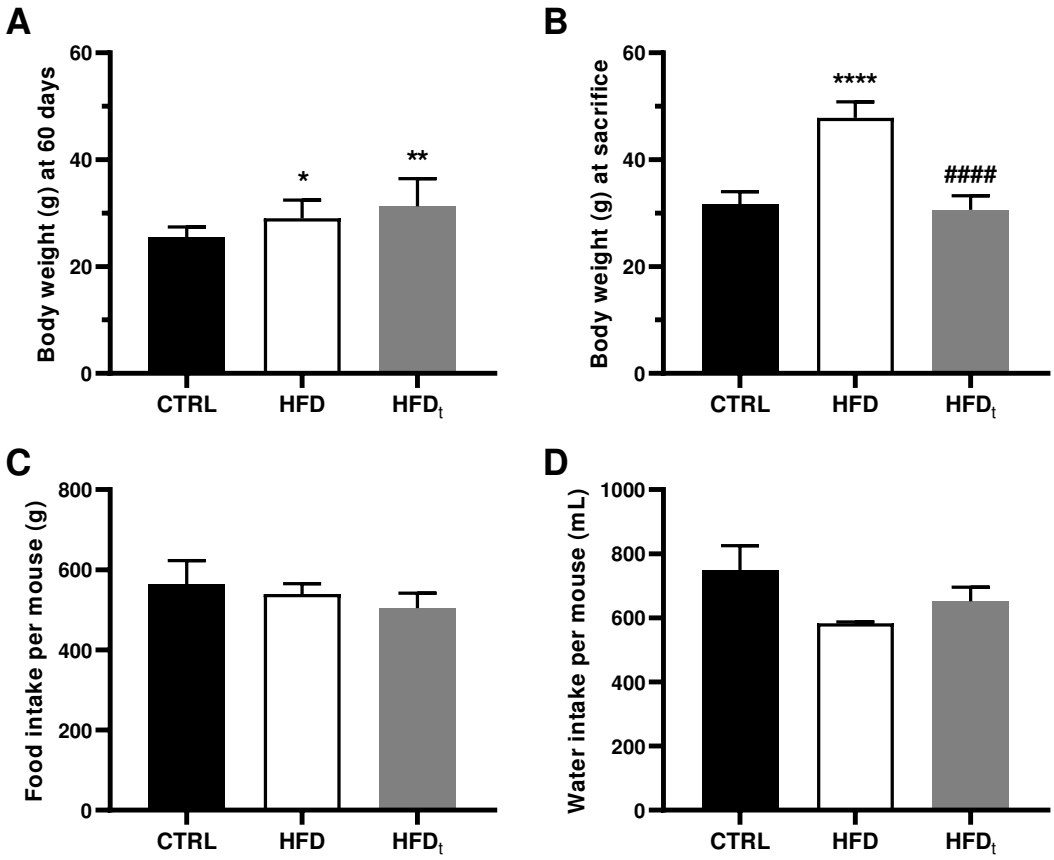


Figure 4.S1: Body weight and food/water intake monitoring throughout the experiment. A) Average body weight 60 days after weaning, when diet reversion was performed to HFD_t group. B) Average body weight at the end of the experiment, 200 days after weaning. C) Cumulative food intake per mouse. D) Cumulative water intake per mouse. Results are expressed as mean ± standard deviation. Data were tested using one-way ANOVA corrected by Tukey's HSD. Significance was considered when $p < 0.05$. * vs. CTRL; # vs. HFD. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Table 4.S1: ^{1}H-NMR resonance assignment of polar testicular lipid precursors (s: singlet, d: doublet, dd: doublet of doublets, t: triplet, m: multiplet).

	Compound[a]	**δ^{1}H ppm (multiplicity, assigment)**
1	choline	3.20 (s, CH_3), 3.51 (m, NCH_2), 4.06 (m, OCH_2)
2	ethanolamine	3.14 (m, NCH_2), 3.82 (m, CH_2)
3	glycerol	3.56 (dd, C1H_2), 3.65 (dd, C3H_2), 3.79 (m, C2H)
4	GPC	3.23 (s, CH_3), 3.68 (m, NCH_2), 3.88 (s, αCH_2), 4.33 (m, OCH_2)
5	3OHBut	1.20 (d, γCH_3, 2.24 (dd, αCH), 4.15 (m, βCH))
6	*myo*-inositol	3.26 (t, C5H), 3.52 (dd, C1H, C3H), 3.62 (m, C4H, C6H), 4.06 (t, C2H)
7	PChol	3.22 (s, CH_3), 3.91 (m, NCH_2), 4.17 (m, OCH_2)
8	PEtAm	3.21 (m, NCH_2), 3.97 (m, OCH_2)

[a]all metabolites are putatively annotated (level 2 of identification according to Chemical Analysis Working Group (CAWG) Metabolomics Standards Initiative recommendations).

Table 4.S2: Reproductive parameters. Results are expressed as the mean (%) ± standard error of the mean. Data tested using χ^2 test, based on the number of successful matings to the number of attempts (success rate), and on the number of male offspring to the litter size (male pups).

Parameter	**CTRL**	**HFD**	**HFD$_t$**	χ^2
Success rate (%)	48.83 ± 10.40	55.56 ± 11.70	47.06 ± 12.10	0.32
Litter size[a]	5.81 ± 1.97	4.93 ± 1.07	4.30 ± 1.42	–
Male pups (%)	53.33 ± 9.10	48.28 ± 9.30	47.62 ± 10.90	0.22

[a]Tested by one-way ANOVA, Tukey's HSD, and expressed as the mean (liveborn pups) ± SD.

Table 4.S3: Enzymatic activity of mitochondrial complexes in testes of mice fed standard chow (CTRL), life-long high-fat diet (HFD), and those subjected to diet correction after 60 days (HFD_t). Results are expressed as mean ± standard deviation. Experimental groups were compared by one-way ANOVA with Tukey's HSD. Significance was considered when $p < 0.05$. * vs. CTRL; # vs. HFD. * p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001.

	Parameter	CTRL (n=6)	HFD (n=6)	HFD_t (n=5)
Mitochondrial Complex activity	*Citrate Synthase*[a]	193.47 ± 50.11	175.42 ± 67.62	165.00 ± 18.78
	Complex I[b]	35.43 ± 10.59	30.65 ± 9.57	32.14 ± 6.85
	Complex II[c]	49.58 ± 8.47	40.23 ± 15.53	46.84 ± 9.47
	Complex IV[d]	84.22 ± 15.60	69.31 ± 22.38	64.98 ± 16.41
	Complex V[e]	1578.16 ± 399.68	1499.32 ± 468.87	1283.78 ± 299.98
	CI/CS ratio	0.19 ± 0.05	0.18 ± 0.04	0.19 ± 0.03
	CII/CS ratio	0.26 ± 0.04	0.23 ± 0.06	0.29 ± 0.07
	CIV/CS ratio	0.46 ± 0.14	0.42 ± 0.10	0.40 ± 0.12
	CV/CS ratio	9.03 ± 2.16	7.73 ± 0.63	7.77 ± 1.58

[a]Units: $nmol_{DTNB}\,min^{-1}\,mg^{-1}_{protein}$; [b]Units: $nmol_{NADH}\,min^{-1}\,mg^{-1}_{protein}$; [c]Units: $nmol_{DCPIP}\,min^{-1}\,mg^{-1}_{protein}$; [d]Units: $nmol_{CytC}\,min^{-1}\,mg^{-1}_{protein}$; [e]Units: $\mu mol_{NADH}\,min^{-1}\,mg^{-1}_{protein}$.

Table 4.S4: Testicular bioenergetics of mice fed standard chow (CTRL), life-long high-fat diet (HFD), and those subjected to diet correction after 60 days (HFD_t). Results are expressed as mean ± standard deviation. Experimental groups were compared by one-way ANOVA with Tukey's HSD. Significance was considered when $p < 0.05$. * vs. CTRL; # vs. HFD. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Bioenergetics	CTRL (n=6)	HFD (n=6)	HFD_t (n=5)
ATP ($nmol\,mg^{-1}$)	1355.05 ± 409.86	1242.37 ± 305.59	1381.14 ± 215.19
ADP ($nmol\,mg^{-1}$)	898.80 ± 339.79	510.97 ± 184.30	828.81 ± 718.97
AMP ($nmol\,mg^{-1}$)	595.85 ± 340.32	467.75 ± 294.22	478.63 ± 217.89
Adenine nucleotide pool	2849.70 ± 938.94	2221.09 ± 520.29	2688.57 ± 751.63
ATP/ADP	1.60 ± 0.63	2.80 ± 1.28	2.62 ± 1.54
AMP/ATP	0.41 ± 0.18	0.36 ± 0.16	0.34 ± 0.12
Energy charge	0.65 ± 0.05	0.68 ± 0.05	0.68 ± 0.04

Table 4.S5: Fatty acid composition of the apolar testicular extracts of mice fed standard chow (CTRL), life-long high-fat diet (HFD), and those subjected to diet correction after 60 days (HFD_t). FAs are presented as: Common name (Lipid numbers). Results are expressed as the mean (% of total lipids) ± standard deviation. Results were tested by one-way ANOVA with Tukey's HSD. Significance was considered when $p < 0.05$. * vs. CTRL; # vs. HFD. * p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001. Abbreviations: SFAs – Saturated Fatty Acids; MUFAs – Monounsaturated Fatty Acids; PUFAs – Polyunsaturated Fatty Acids.

Fatty acid	**CTRL (n=5)**	**HFD (n=3)**	**HFD_t (n=5)**
Myristic acid (C14:0)	0.92 ± 0.28	0.59 ± 0.13	0.91 ± 0.09
Palmitic acid (C16:0)	29.14 ± 3.31	17.04 ± 0.51***	22.37 ± 2.83**
Palmitoleic acid (C16:1n-7)	1.71 ± 0.76	1.25 ± 0.05	1.44 ± 1.17
Stearic acid (C18:0)	25.73 ± 3.50	12.54 ± 0.32***	18.56 ± 3.59*
Oleic acid (C18:1n-9)	11.08 ± 0.90	21.95 ± 4.16***	16.49 ± 1.68**,#
Vaccenic acid (C18:1n-7)	1.79 ± 0.41	2.42 ± 0.38	2.65 ± 0.55*
Linoleic acid (C18:2n-6)	5.01 ± 0.68	9.19 ± 0.89***	6.85 ± 1.30*,#
Dihomo-γ-linolenic acid (C20:3n-6)	0.75 ± 0.29	1.32 ± 0.22*	1.25 ± 0.29*
Arachidonic acid (C20:4n6)	9.25 ± 2.25	14.58 ± 2.00**	12.19 ± 0.78
Docosapentaenoic acid (C22:5n-6)	9.28 ± 2.92	12.31 ± 1.39	10.77 ± 0.81
Docosahexaenoic acid (C22:6n-3)	5.34 ± 0.93	6.81 ± 1.00	6.52 ± 0.84
SFAs	55.79 ± 6.38	30.17 ± 0.45***	41.83 ± 6.14**,#
MUFAs	14.59 ± 0.94	25.62 ± 4.04***	20.59 ± 3.22*
PUFAs	29.62 ± 5.56	44.21 ± 3.61**	37.58 ± 2.97*
n-3 PUFAs	5.34 ± 0.93	6.81 ± 1.00	6.52 ± 0.84
n-6 PUFAs	24.29 ± 4.65	37.40 ± 2.63***	31.06 ± 2.14#
n-3/n-6	0.22 ± 0.01	0.18 ± 0.01**	0.21 ± 0.01#
C22:6n-3/C20:4n-6	0.59 ± 0.07	0.47 ± 0.01**	0.53 ± 0.04
C20:4n-6/C18:2n-6	1.92 ± 0.70	1.61 ± 0.22	1.83 ± 0.36
C22:5n-6/C20:4n-6	1.00 ± 0.14	0.85 ± 0.03	0.88 ± 0.01

Chapter 5

Inheritable testicular metabolic memory of high-fat diet causes transgenerational sperm defects in mice

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Abstract

The consumption of energy-dense diets has contributed to an increase in the prevalence of obesity and its comorbidities worldwide. The adoption of unhealthy feeding habits often occurs at early age, prompting the early onset of metabolic disease with unknown consequences for reproductive function later in life. Recently, evidence has emerged regarding the intergenerational and transgenerational effects of high-fat diets (HFD) on sperm parameters and testicular metabolism. Hereby, we study the impact of high-fat feeding male mice (F_0) on the testicular metabolome and function of their sons (F_1) and grandsons (F_2). Testicular content of metabolites related to insulin resistance, cell membrane remodeling, nutritional support and antioxidative stress (leucine, acetate, glycine, glutamine, inosine) were altered in sons and grandsons of mice fed with HFD, comparing to descendants of chow-fed mice. Sperm counts were lower in the grandsons of mice fed with HFD, even if transient. Sperm quality was correlated to testicular metabolite content in all generations. Principal Component Analysis of sperm parameters and testicular metabolites revealed an HFD-related phenotype, especially in the diet-challenged generation and their grandsons. Ancestral HFD, even if transient, causes transgenerational "inherited metabolic memory" in the testicular tissue, characterized by changes in testicular metabolome and function.

5.1 Introduction

Modern societies promote a fast-paced daily life that often leads to poor lifestyle choices. Notably, readily available and palatable fast food contributes to a HFD that has led to a marked increase in the prevalence of overweight and obesity worldwide (WHO, 2014). Obesity-related comorbidities have increased proportionally, including non-communicable diseases, such as T2D (WHO, 2016a). Of interest, male infertility has been also correlated to this increase in obesity prevalence. Several studies report a coincident temporal trend in sperm quality decline (Geoffroy-Siraudin et al., 2012; Levine et al., 2017). Others report how male obesity is associated with infertility (Campbell et al., 2015; Nguyen et al., 2007) and lower success of ART (Palmer, Bakos, Fullston, et al., 2012; Umul et al., 2015). Recently, evidence has emerged concerning the intergenerational and transgenerational effects of male obesity on the health of their progeny (Bygren et al., 2001; Fullston et al., 2013; Rando & Simmons, 2015), particularly on sexual health (Craig et al., 2017; Pavlinková et al., 2017). HFD cause acute changes in human sperm (Nätt et al., 2019), and permanent changes in murine testicular metabolite content (**Crisóstomo** et al., 2019). The age of onset of obesity and its comorbidities are occurring at an ever younger age (IDF, 2017; WHO, 2016a). Therefore, it is of utmost relevance to understand the metabolic factors transmitted via paternal lineage

to the progeny.

Rodent models have been widely applied in transgenerational studies involving ancestral exposure to HFD (Baxter & Drake, 2019; Bošković & Rando, 2018), including studies restricted to paternal inheritance. Although the male gamete can only carry a minimal amount of sncRNAs besides the DNA content (Bošković & Rando, 2018), these studies show metabolic phenotypic changes inherited by direct progeny (sons) and subsequent descendants (grandsons) (Q. Chen et al., 2016; Cropley et al., 2016; Grandjean et al., 2016; Pavlinková et al., 2017). Moreover, seminal fluid composition potentially affects early phases of embryo development (Sharma & Rando, 2017). Epigenetic mechanisms respond to metabolic inputs, such as metabolite availability at cellular level, which is affected by diet (Sharma & Rando, 2017). "Metabolic memory" has been coined with metabolic adaptations in somatic cells, in the context of glycemic control and T2D complications (Ihnat et al., 2007). Previous studies demonstrate that both somatic and germline cells adapt in response to metabolic, environmental cues, particularly HFD, and these adaptations are potentially inherited by the offspring via the paternal lineage (Q. Chen et al., 2016; Cropley et al., 2016; Grandjean et al., 2016; Pavlinková et al., 2017). Therefore, we advocate that "metabolic memory" could be adapted to the context of transgenerational epigenetic inheritance. This "inherited metabolic memory" refers to the inheritance of acquired adaptations to environmental variables in ancestry, such as diet, by progeny which has not been exposed to the same stimuli, for several generations. In this study we adopted a multivariate analysis of untargeted ^{1}H-NMR metabolomics, to assess the effects of lifelong or temporary paternal HFD upon testicular metabolome and sperm parameters of the direct offspring (sons) and second-generation offspring (grandsons) in mice. Biometric and glucose homeostasis data was also recorded individually across generations. Based on these data, we demonstrate "inherited metabolic memory" caused by ancestral exposure to HFD in the paternal lineage.

5.2 Material and Methods

Animal Model

This study was performed in three generations of *Mus musculus* C57BL6/J mice (Supplementary Figure 5.S1). The first generation (Generation F_0) was reared as previously described (**Crisóstomo** et al., 2019; **Crisóstomo** et al., 2020). Mice were 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 libitum*. Af-

ter weaning (21–23 days), F_0 mice (n = 36) were randomly divided in three groups: CTRL (n = 12), HFD (n = 12) and HFD_t (n = 12). Mice from the CTRL group were fed with a standard chow. Mice from the HFD group received HFD (#F3282, BioServ, USA - Carbohydrate: 35.7 %, Protein: 20.5 %, Fat: 36.0 % [59.0 $\%_{Kcal}$]). The mice from HFD_t group were fed with a HFD for 60 days (#F3282, BioServ, New Jersey, USA) and then switched to standard chow (#F4031, BioServ, New Jersey, USA). F_0 mice were mated starting at 120 days days of age with normoponderal, chow-fed, randomly selected females of same age to generate the F_1 generation. Mating lasted for 8 days days and involved placing a male and a female in the same cage for 6 h each day, without water or food supply. After weaning, F_1 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 the generation F_2 were fed with standard chow after weaning. Food and water were supplied without restrictions. All mice in all generations were kept in 12 h dark:light cycles, 4 mice per cage. Mice from all generations were euthanized 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 is compliant with the ARRIVE guidelines, was internally reviewed by the Organization for Animal Welfare (ORBEA) and approved and licensed by the Portuguese Veterinarian and Food Department (DGAV) with the registration number 0421/000/000/2016. All animal experiments were performed according to the "Guide for the Care and Use of Laboratory Animals" published by The US National Institutes of Health (NIH Publication No. 85–23, revised 1996) and European rules for the care and handling of laboratory animals (Directive 86/609/EEC).

Glucose homeostasis assessment

Glucose tolerance and insulin resistance were assessed experimentally using the ipGTT and ipGTT protocols (McGuinness et al., 2009), at 196 days and 198 days post-weaning age. ipGTT was performed after overnight fast, whereas ipGTT was performed in the morning after a 4 h fast. Fasting glycemia was measured before sacrifice (200 days post-weaning) 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). Glucose homeostasis was evaluated according to the HOMA2 scores (Levy et al., 1998), using the HOMA2 calculator (University of Oxford, United Kingdom).

Evaluation of epididymal sperm parameters

Epididymides were isolated immediately after sacrifice (200 days post-weaning age) and placed in pre-warmed (37 °C) 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 (**Crisóstomo** et al., 2019; Rato et al., 2013). 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 (Kvist et al., 2002), 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) (Kvist et al., 2002).

Evaluation of fertility outcomes

After the mating of animals from F_0 and F_1 generations, we assessed the number of pregnant females to calculate the fertility rate of mice in different groups. Matings were performed until a sufficient number of pups to continue the experiment was achieved (12 pups per group in each generation). The total number of matings performed in each group and generation is available in the Supplementary Table 5.S1. The litter size and the male/female ratio was assessed after weaning off the pups, not to stress the female progenitor. Only completed pregnancies resulting in at least one surviving pup were considered successful.

NMR spectroscopy

A combined extraction of polar and nonpolar metabolites from testicular tissue was performed as previously described (Alves et al., 2011). The aqueous phase containing polar water-soluble metabolites was lyophilized and analyzed by ^{1}H-NMR spectroscopy, following a semi-quantitative methodology as described previously (Jarak et al., 2018). Metabolites were identified by comparing recorded spectra with reference spectra and the HMDB (Wishart et al., 2007) and according to MSI guidelines for metabolite identification (Sumner et al., 2007). Identification levels are indicated in the Supplementary Table 5.S2. ^{1}H spectra were processed as described previously and well-resolved metabolite multiplets were integrated (Jarak et al., 2018). Obtained areas were normalized to total spectral area, and then represented as the $\log_2$ Fold Change (FC) to the CTRL group of the corresponding generation. Univariate and multivariate statistical analysis was conducted based on these values (Supplementary Table 5.S3).

Statistical analysis

Data normality and homoscedasticity were tested using Kolmogorov-Smirnoff test with Lilliefors's correction and Levene's test. Parametric statistics were adopted if one of the above assumptions was met. Pairwise comparisons were tested using univariate ANOVA corrected by Tukey's HSD. Testicular metabolites were further corrected for multiple hypothesis testing, controlling the FDR, using Benjamini–Hochberg method (Benjamini & Hochberg, 1995). Two-way ANOVA with Šidak correction was used to test simple main effects of diet across generations F_1 and F_2. Repeated-measures ANOVA with Šidak correction was used to compare groups in time-course assays (body weight curves, ipGTT and ipGTT). Tabular data was tested for independence by χ^2 test, and column proportions were tested using z-test with Bonferroni's correction. Correlations between variables coding for sperm parameters and testicular metabolites were calculated using Pearson's method. Correlation strength was classified according to ranks (Taylor, 1990), and adjusted p-values for FDR were calculated using Benjamini–Hochberg method. PCA based on testicular metabolites and sperm parameters was performed to assess sample clustering according to diet and generation. Sample adequacy was assessed by the Kaiser–Meyer–Olkin Test and by the Bartlett's Test of Sphericity. Forced factor extraction was performed to extract two principal components, using Varimax with Keiser's Normalization as Rotation Method. These methods were performed using IBM SPSS Statistics v26 (Armonk, NY, USA). Euclidean distances between samples were obtained from the rotated PCA solution. Groups were compared according

to distances in the Euclidean space using PERMANOVA (M. J. Anderson, 2017). Pairwise comparisons were corrected by controlling the FDR using the Benjamini–Hochberg method. These methods were performed on R 4.0.1 (R Core Team, 2020), running the R-packages vegan 2.5-6 (Oksanen et al., 2020) and RVAideMemoire, 0.9-77 (Hervé, 2020). The significance level for pairwise corrections (Bonferroni, Tukey's HSD and Šidak) was set to $p < 0.05$. The significance level for FDR-controlling methods was considered when adjusted $p < 0.1$.

5.3 Results

HFD increases weight after weaning but does not promote weight gain across generations

The experimental design of this study is detailed in "Materials and methods" section and in Supplementary Figure 5.S1. HFD mice sons (Generation F_1) were significantly heavier than sons of CTRL mice (19 ± 2 g vs 15 ± 4 g). In generation F_2, no differences were found between groups (Figure 5.1a). Body weight curves for each group were obtained for F_0 Generation (Figure 5.1b), F_1 Generation (Figure 5.1c) and F_2 Generation (Figure 5.1d). As previously reported (**Crisóstomo** et al., 2019), in F_0 Generation both HFD and HFD_t mice gain more weight than CTRL from early age. After diet reversion (DR), 60 days after weaning, HFD_t mice start losing weight, and thereafter HFD mice became heavier than both CTRL and HFD_t mice. In F_1 Generation no differences were found between groups at any period, except at weaning (as previously mentioned). Similarly, no differences were found between the grandsons of the different groups (F_2 Generation). The weight of various organs, fat mass and GSI were also assessed (Supplemental table 5.S4). In this dataset, more differences were found in F_1 generation than in F_2. HFD mice generated F_1 mice with smaller left testis than CTRL (0.11 ± 0.02 g vs 0.12 ± 0.01 g). Comparing to mice from CTRL, mice from the HFD_t generated F_1 mice with smaller testes (Left: 0.10 ± 0.02 g vs 0.13 ± 0.01 g; Right: 0.10 ± 0.01 g vs 0.12 ± 0.01 g), less epididymal (0.55 ± 0.14 g vs 0.76 ± 0.20 g) and perirenal (0.21 ± 0.07 g vs 0.29 ± 0.10 g) fat, lower fat mass (4.19 ± 0.96 % vs 5.32 ± 1.01 %) and GSI (0.74 ± 0.09 % vs 0.85 ± 0.10 %).

The adoption of HFD does not affect glucose homeostasis in progeny

The adoption of lifelong HFD induced a pre-diabetic state in HFD mice (F_0 Generation) characterized by increased insulin resistance (10.40 ± 4.64 a.u., Figure 5.2a) and fasting glycemia (123 ± 16 mg dL^{-1}, Figure 5.2b). Interestingly, an increase in average fasting glycemia was

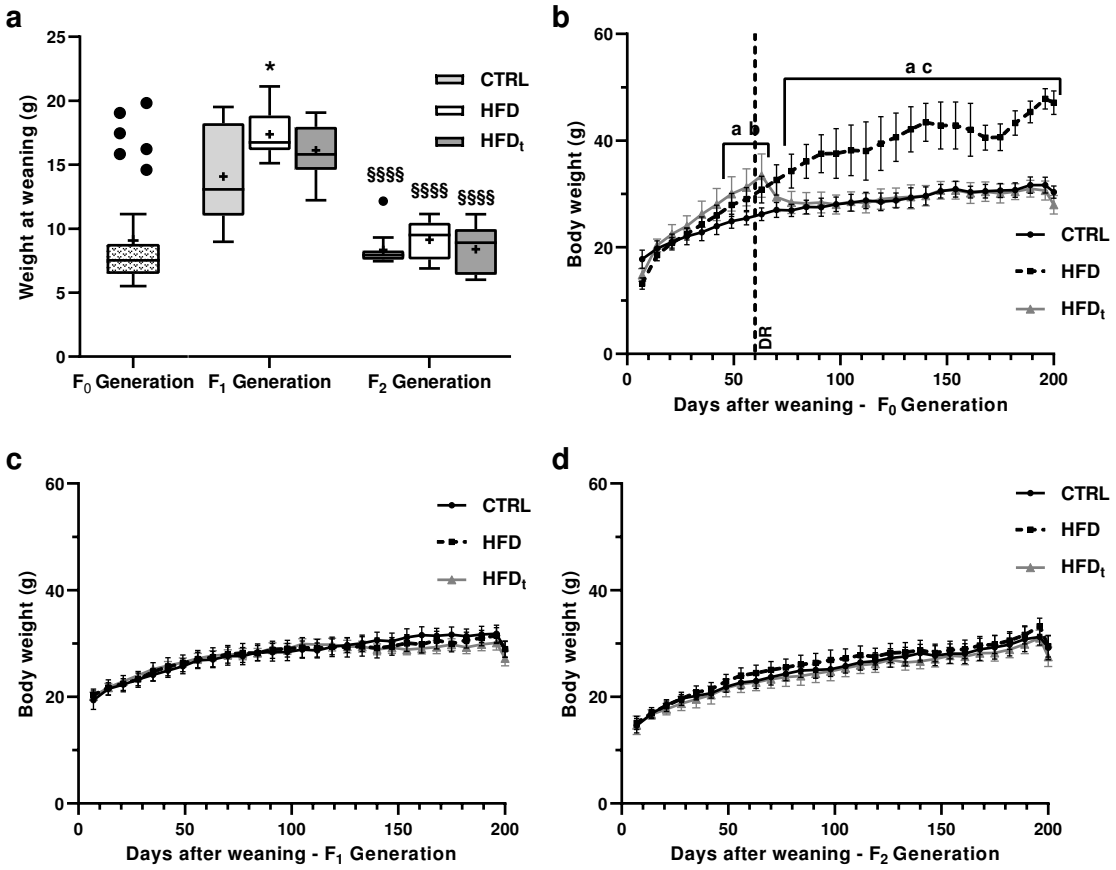


Figure 5.1: The evolution of body mass from weaning to sacrifice, across generations. (a) Body weight at weaning (g), expressed as Tukey's whisker boxes (median, 25th to 75th percentiles ± 1.5 interquartile range). Mice of F_0 Generation were weighted and then randomly assigned to an experimental group (n = 12 per group in each generation). Data was tested by one-way ANOVA with Tukey's HSD for group comparison within the same generation. Two-way ANOVA with Šidak correction was used to compare each diet group in Generation F_1 against its F_2 counterparts. Significance was considered when $p < 0.05$. * vs. CTRL; $^{\#}$ vs. HFD; § vs. Generation F_1. Multiple significance signs represent different significance levels: *p <0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001; ● Extreme values; **+** Group mean. Body weight curves for (b) F_0 Generation, (c) F_1 Generation and (d) F_2 Generation were obtained by monitoring the mice weight weekly (12 mice per group in each generation). Results are expressed as the mean body weight (g), with whiskers representing the 95 % Confidence Interval. The main and simple effects between groups were tested by Repeated Measures ANOVA corrected for family-wise comparisons using the Šidak method, and by Greenhouse–Geisser correction for sphericity. Graphs were obtained using the software GraphPad Prism 8 (San Diego, CA, USA). Significance was considered when p <0.05. a — CTRL vs. HFD; b — CTRL vs. HFD$_t$; c — HFD vs. HFD$_t$. *DR* diet reversion.

observed in groups CTRL and HFD_t in generation F_2, compared to their progenitors (97 ± 10 mg dL^{-1} and 105 ± 27 mg dL^{-1} respectively) (Figure 5.2b). This difference is not observed in fasting insulinemia (Figure 5.2c). No changes in this parameter were found in F_1 and F_2 Generations. Regarding the ipGTT and the ipGTT (Supplemental table 5.S5), the adoption of a lifelong HFD led to glucose intolerance and insulin resistance, but diet reversion prevented this affect. Interestingly, during ipGTT, the offspring of HFD, generation F_1, reached higher serum glycemia levels after 90 min and 120 min than the offspring of CTRL and HFD_t.

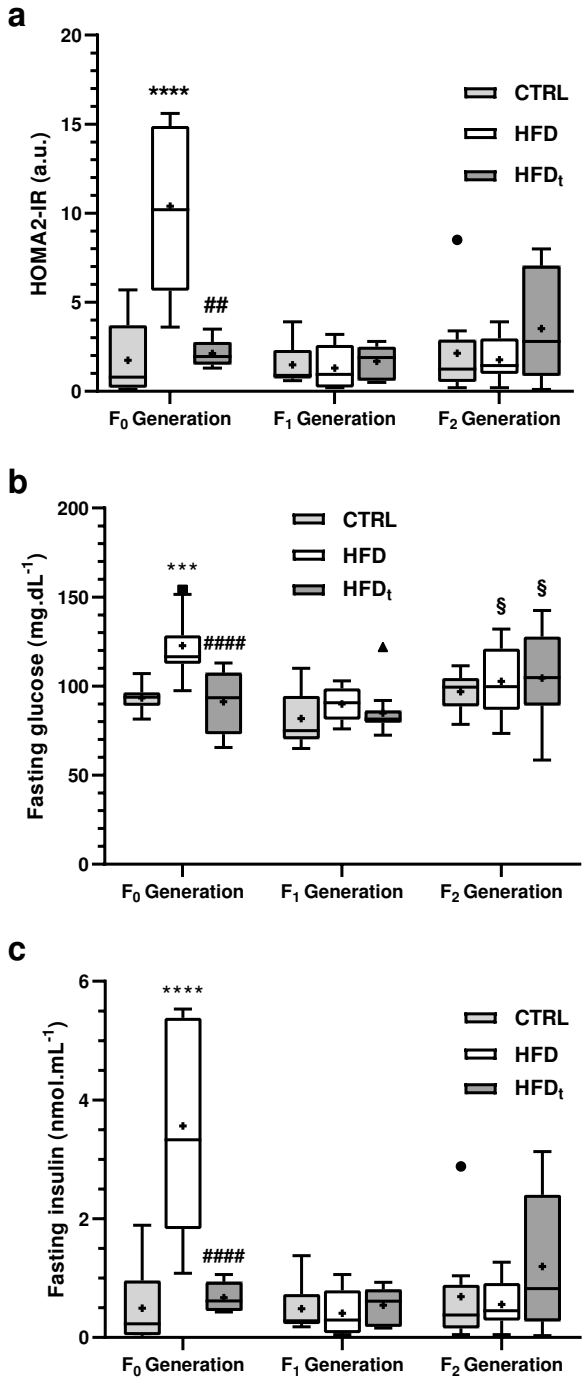


Figure 5.2: Glucose homeostasis across all the generations (n = 8 per group in each generation). (a) HOMA2-IR index; (b) fasting glycaemia; (c) serum insulin. Results are expressed as Tukey's whisker boxes (median, 25th to 75th percentiles ± 1.5 interquartile range). Extreme values are represented individually according to the experimental group (● CTRL—standard chow; ■ HFD—high-fat diet; ▲ HFD_t—transient high-fat diet). The samples were obtained in the day of sacrifice, 200 days post-weaning. Data was tested by one-way ANOVA with Tukey's HSD for group comparison within the same generation. Two-way ANOVA with Šidak correction was used to compare each diet group in F_1 Generation against its F_2 counterparts. Graphs were obtained using the software GraphPad Prism 8 (San Diego, CA, USA). Significance was considered when $p < 0.05$. * vs. CTRL; # vs. HFD; § vs. F_1 Generation. Multiple significance signs represent different significance levels: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$. ✚ Group mean.

Paternal HFD during early life induces sperm defects that persist for two generations

Sperm counts, viability, motility and morphology were assessed across generations (Figure 5.3, Table 5.1). In F_1 generation, differences were only found in sperm morphology. The offspring of HFD_t mice had a greater proportion of normal sperm ($37 \pm 6\,\%$) and lower proportion of decapitated sperm ($9 \pm 2\,\%$) than those from HFD ($32 \pm 5\,\%$ and $11 \pm 6\,\%$) and CTRL ($33 \pm 5\,\%$ and $13 \pm 6\,\%$). The offspring of HFD mice also had a lower proportion of decapitated sperm ($11 \pm 6\,\%$) than CTRL ($13 \pm 6\,\%$), but higher proportions of pin head ($9 \pm 2\,\%$) and bent neck ($8 \pm 4\,\%$) defects. Interestingly, the F_2 generation from HFD and HFD_t mice had decreased sperm counts compared to CTRL ($36.42 \pm 8.62\,\mathrm{M\,L^{-1}}$ and $33.60 \pm 6.12\,\mathrm{M\,mL^{-1}}$ respectively). Moreover, although no changes in sperm viability were found between groups in F_2 generation, it is interesting to note that F_2 from HFD and HFD_t mice had lower values of sperm viability ($32 \pm 8\,\%$ and $36 \pm 6\,\%$ respectively) than their F_1 progenitors ($43 \pm 4\,\%$ and $32 \pm 8\,\%$). Regarding sperm morphology in generation F_2, those mice obtained from HFD mice had significantly lower proportion of normal sperm ($34 \pm 5\,\%$) than those F_2 obtained from the other groups. Mice of the F_1 generation had a significantly higher proportion of head defects (CTRL: $27 \pm 6\,\%$; HFD: $27 \pm 7\,\%$; HFD_t: $25 \pm 5\,\%$) than their offspring in F_2 generation (CTRL: $18 \pm 5\,\%$; HFD: $22 \pm 4\,\%$; HFD_t: $19 \pm 3\,\%$). The proportion of head defects in the HFD mice, generation F_2, explains the lower proportion of normal sperm in this group. Reproductive parameters (success rate, litter size and male ratio per litter) were assessed in Generations F_0 and F_1 (Supplemental Table 5.S1). However, no changes were found.

Changes in testicular metabolism caused by HFD during early life prevail for several generations

Testicular polar metabolites were extracted and analyzed by ^{1}H-NMR. Results are presented as the $\log_2$ FC to CTRL of the corresponding generation. The full list of metabolites identified by ^{1}H-NMR is available as Supplementary Table 5.S2. Testicular leucine levels (Figure 5.4a) were 20 % higher in the sons of mice fed HFD ($0.26 \pm 0.10\,\log_2$ FC) than in sons of CTRL mice (F_1 generation). The grandsons of mice fed HFD (F_2 generation) had lower levels of testicular leucine ($0.03 \pm 0.21\,\log_2$ FC) than their F_1 progenitors. Testicular glutamine levels (Figure 5.4b), in F_0 generation, were increased in the HFD_t group ($0.23 \pm 0.06\,\log_2$ FC). No changes were found in the sons of the diet-challenged mice (F_1 generation). However, the grandsons of HFD_t mice had decreased levels of testicular glutamine, comparing to grandsons of CTRL ($-0.18 \pm 0.07\,\log_2$ FC). HFD-fed mice, even transiently, decreased testicular acetate content

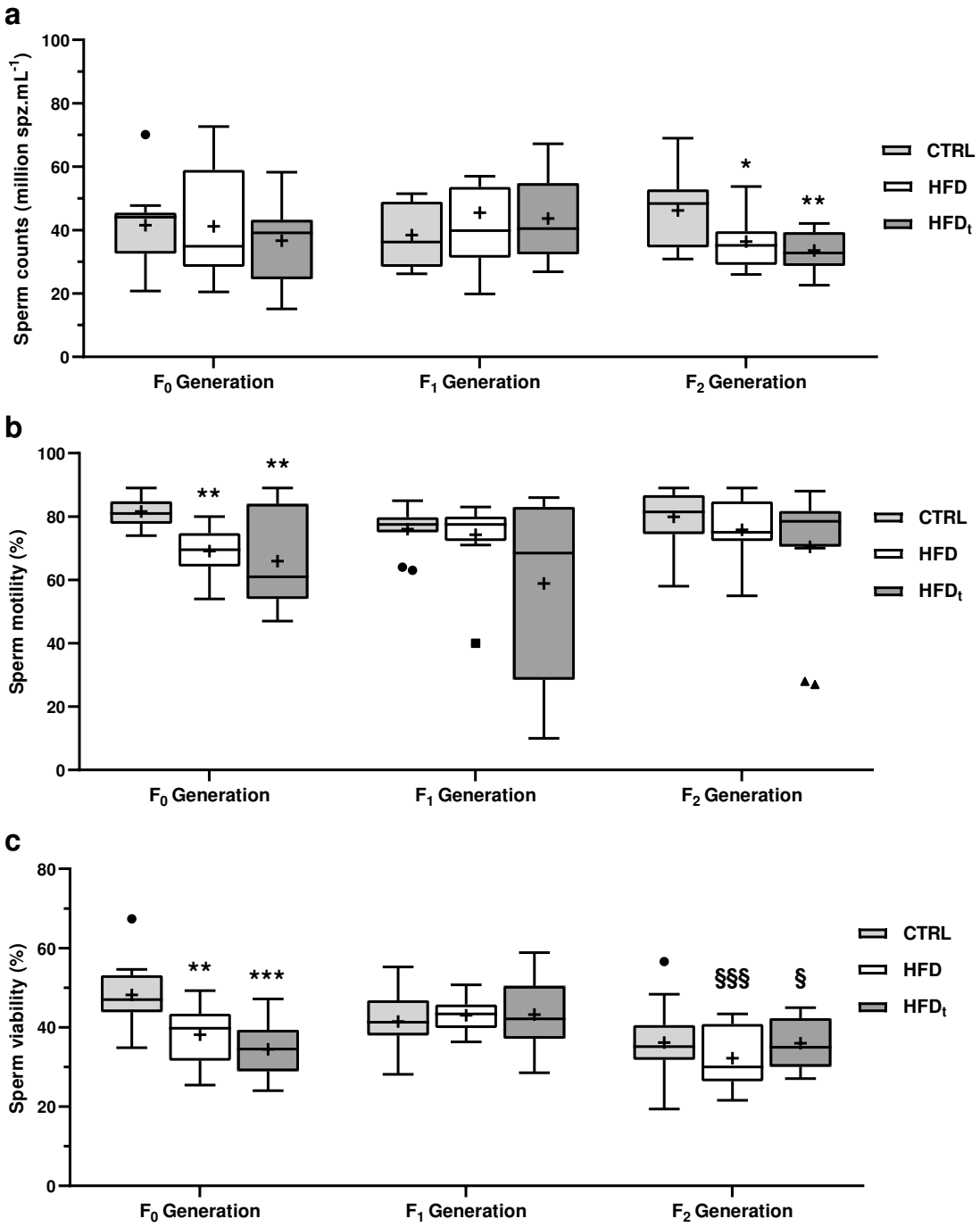


Figure 5.3: Sperm parameters across all the generations (n = 12 per group in each generation). (a) Sperm concentration; (b) sperm motility; (c) sperm viability. Results are expressed as Tukey's whisker boxes (median, 25th to 75th percentiles ± 1.5 interquartile range). Extreme values are represented individually according to the experimental group (● CTRL—standard chow; ■ HFD—high-fat diet; ▲ HFD_t—transient high-fat diet). Epididymal sperm was obtained from mice immediately after sacrifice, at 200 days days post-weaning. Data was tested by one-way ANOVA with Tukey's HSD for group comparison within the same generation. Two-way ANOVA with Šidak correction was used to compare each diet group in F_1 Generation against its F_2 counterparts. Graphs were obtained using the software GraphPad Prism 8 (San Diego, CA, USA). Significance was considered when $p < 0.05$. * vs. CTRL; # vs. HFD; § vs. F_1 Generation. Multiple significance signs represent different significance levels: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$. ✚ Group mean.

Table 5.1: Sperm morphology across generations (n = 12 per group in each generation). Data is presented as the mean (%) ± SD. Epididymal sperm was obtained from mice immediately after sacrifice, at 200 days post-weaning. The distribution of sperm defects across different experimental groups, in each generation, was tested by χ^2 test of independence. Comparisons within sperm morphology categories were obtained with the Z-test for column proportions with Bonferroni correction. Two-way ANOVA with Šidak correction was used to compare each diet group in F_1 Generation against its F_2 counterparts. Significance was considered when $p < 0.05$. * vs. CTRL; # vs. HFD. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

	Sperm morphology	CTRL	HFD	HFD_t	χ^2 test
F_0 Generation	Normal	40 ± 6	$34 \pm 7^{***}$	$37 \pm 4^{***}$	
	Decapitated	12 ± 5	11 ± 4	$12 \pm 3^{\#}$	
	Pin head	4 ± 1	$6 \pm 1^{***}$	$7 \pm 1^{*****\#\#}$	145.57****
	Flattened head	4 ± 1	5 ± 1	$6 \pm 1^{******\#\#\#}$	
	Bent neck	6 ± 1	$9 \pm 4^{***}$	$5 \pm 2^{\#\#\#}$	
	Coiled tail	34 ± 6	35 ± 7	$33 \pm 2^{*\#\#}$	
	Normal	40 ± 6	34 ± 7	$37 \pm 4^{***}$	
	Head defects	20 ± 4	22 ± 4	$25 \pm 2^{******\#\#\#}$	94.02****
	Tail defects	40 ± 6	$44 \pm 6^{**}$	$38 \pm 3^{\#\#\#}$	
	Normal	40 ± 6	$34 \pm 7^{***}$	$37 \pm 4^{***}$	
	Abnormal	60 ± 6	$67 \pm 7^{***}$	$63 \pm 4^{***}$	31.22****
F_1 Generation	Normal	33 ± 5	32 ± 5	$37 \pm 6^{*****\#\#\#}$	
	Decapitated	13 ± 6	$11 \pm 6^{*}$	$9 \pm 2^{***\#}$	
	Pin head	8 ± 2	9 ± 2	9 ± 2	62.96****
	Flattened head	7 ± 1	7 ± 2	7 ± 2	
	Bent neck	6 ± 2	$8 \pm 4^{**}$	7 ± 1	
	Coiled tail	33 ± 4	33 ± 5	31 ± 5	
	Normal	33 ± 5	32 ± 5	$37 \pm 6^{*****\#\#\#}$	
	Head defects	27 ± 6	27 ± 7	$25 \pm 5^{*\#}$	29.04****
	Tail defects	40 ± 4	41 ± 5	38 ± 5	
	Normal	33 ± 5	32 ± 5	$37 \pm 6^{*****\#\#\#}$	
	Abnormal	67 ± 5	68 ± 5	$63 \pm 6^{*****\#\#\#}$	28.02****
F_2 Generation	Normal	$40 \pm 8^{\S\S}$	$34 \pm 5^{***}$	$38 \pm 5^{\#\#}$	
	Decapitated	$6 \pm 3^{\S\S\S}$	8 ± 3	$6 \pm 3^{\#\ \S}$	
	Pin head	4 ± 1	$5 \pm 1^{\S\S\S\S}$	$6 \pm 2^{***\#\ \S}$	76.18****
	Flattened head	8 ± 2	$9 \pm 2^{*}$	$7 \pm 3^{\#\#\#}$	
	Bent neck	12 ± 4	10 ± 4	$13 \pm 4^{\#\#\ \S\S\S}$	
	Coiled tail	30 ± 6	$34 \pm 3^{**}$	$30 \pm 4^{\#\#}$	
	Normal	$40 \pm 8^{\S\S}$	$34 \pm 5^{***}$	$38 \pm 5^{\#\#}$	
	Head defects	$18 \pm 5^{\S\S\S}$	$22 \pm 4^{***\S}$	$19 \pm 3^{\#\ \S\S}$	31.62****
	Tail defects	42 ± 8	44 ± 4	$43 \pm 6^{\S}$	
	Normal	$40 \pm 8^{\S\S}$	$34 \pm 5^{***}$	$38 \pm 5^{\#\#}$	
	Abnormal	60 ± 8	$66 \pm 5^{***}$	$62 \pm 5^{***}$	26.86****

more than 1.5-fold, compared to CTRL, in generation F_0 (Figure 5.4c). The sons of both HFD and HFD_t (F_1 generation) overcompensated their progenitors' defect in testicular acetate, particularly the sons of HFD_t ($0.48 \pm 0.35 \log_2$ FC). This effect persisted in the grandsons of HFD-fed mice (F_2 generation), notably in the grandsons of HFD ($1.33 \pm 0.16 \log_2$ FC), which had more than 2.5-fold more testicular acetate than the grandsons of CTRL. Despite the mean testicular acetate content of the grandsons of HFD_t being 1.2-fold increased comparing to CTRL, this difference was not significant due to the large standard deviation of the results ($0.27 \pm 0.78 \log_2$ FC). Concerning inosine (Figure 5.4d), lifelong HFD promoted the decrease in testicular levels ($-0.68 \pm 0.22 \log_2$ FC) compared to CTRL ($0.00 \pm 0.31 \log_2$ FC) in generation F_0. Again, no differences were found for the testicular content of inosine in the sons of the diet-challenged mice (F_1 generation). Interestingly, the grandsons of HFD_t mice had lower inosine levels in testes ($0.68 \pm 0.40 \log_2$ FC) than the grandsons of CTRL ($0.00 \pm 0.15 \log_2$ FC). Detailed values are shown in Supplementary Table 5.S3.

Changes in testicular metabolism promoted by diet, even if transient, are correlated to sperm defects up to F_2

Sperm parameters and testicular metabolites were correlated using Pearson's method (Figure 5.5). Correlation matrixes were obtained for each generation in the study independently, and relevant correlations were displayed when $r < |0.4|$ and $p < 0.05$ (Figure 5.5a), or when $r < |0.4|$ and FDR p-adjusted < 0.1 (Figure 5.5b). Whenever a correlation between variables is mentioned, it abides to the former mathematical criteria. The number of significant correlations decreased across generations, (F_0: 31 correlations; F_1: 17 correlations; F_2: 9 correlations), especially when the p-value is adjusted for multiple testing (F_0: 11 correlations; F_1: 1 correlation; F_2: 0 correlations). Before FDR p-adjustment, acetate, glutamine and myo-inositol showed significant correlations with sperm parameters in all generations, whereas glutamine had the highest number of significant correlations with sperm parameters (9), most of them in generation F_0 (4). After FDR p-adjustment, acetate and succinate had the highest number of significant correlations with sperm parameters (2), all in F_0 generation.

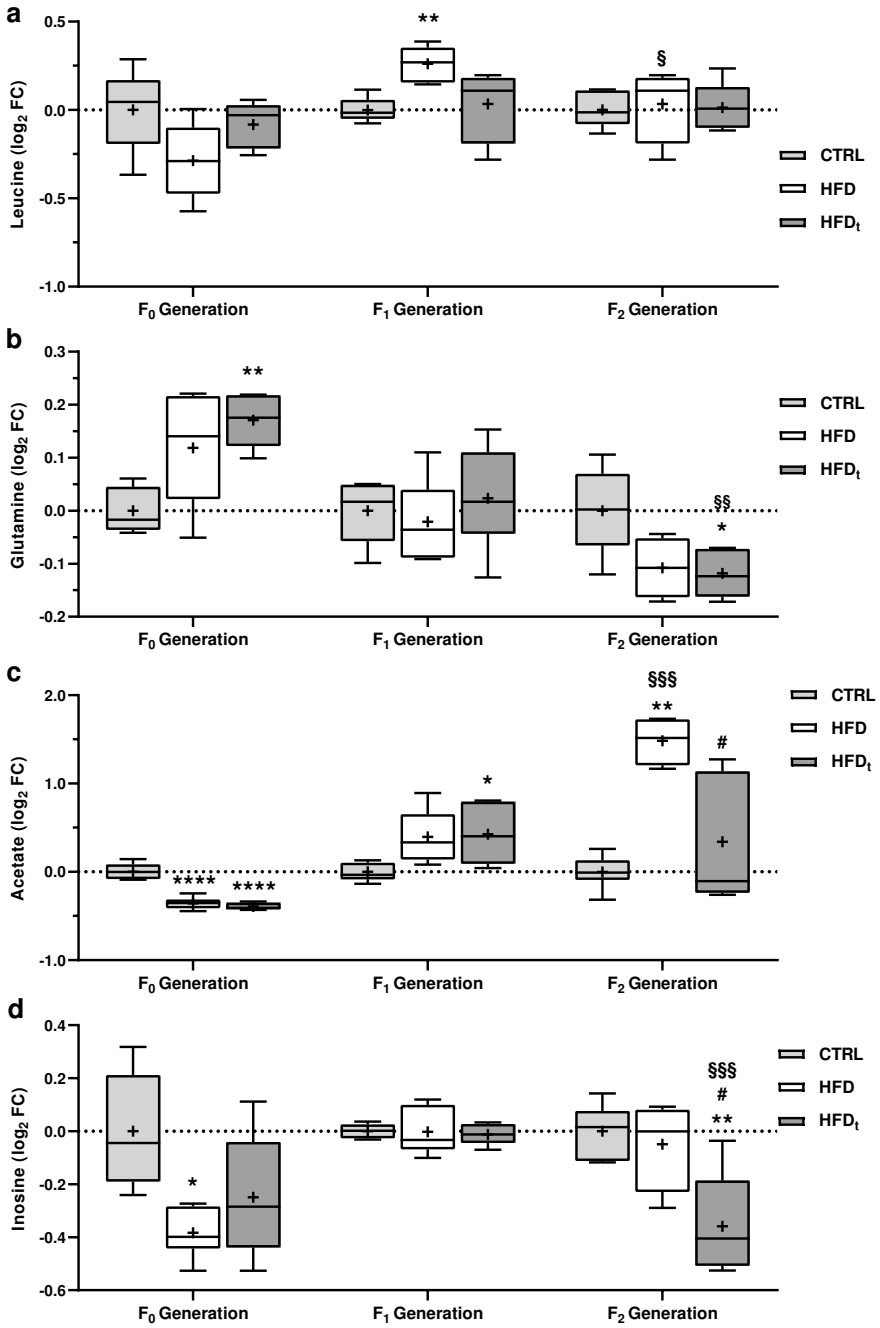


Figure 5.4: Testicular metabolites with variations across all generations (n = 6 per group in each generation). (a) Leucine; (b) Glutamine; (c) Acetate and (d) Inosine. Results are expressed as the $\log_2$ FC to the CTRL of the corresponding generation, as Tukey's whisker boxes (median, 25th to 75th percentiles ± 1.5 Interquartile range). Metabolites were extracted from the testes collected during sacrifice (200 days after mice weaning). Data was tested by univariate ANOVA with Tukey's HSD for pairwise comparisons. Two-way ANOVA with Šidak correction was used to compare each diet group in F_1 Generation against its F_2 counterparts. Graphs were obtained using the software GraphPad Prism 8 (San Diego, CA, USA). Pairwise significance was considered when $p < 0.05$. * vs. CTRL; # vs. HFD; § vs. F_1 Generation. Multiple significance signs represent different significance levels: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$. ✚ Group mean.

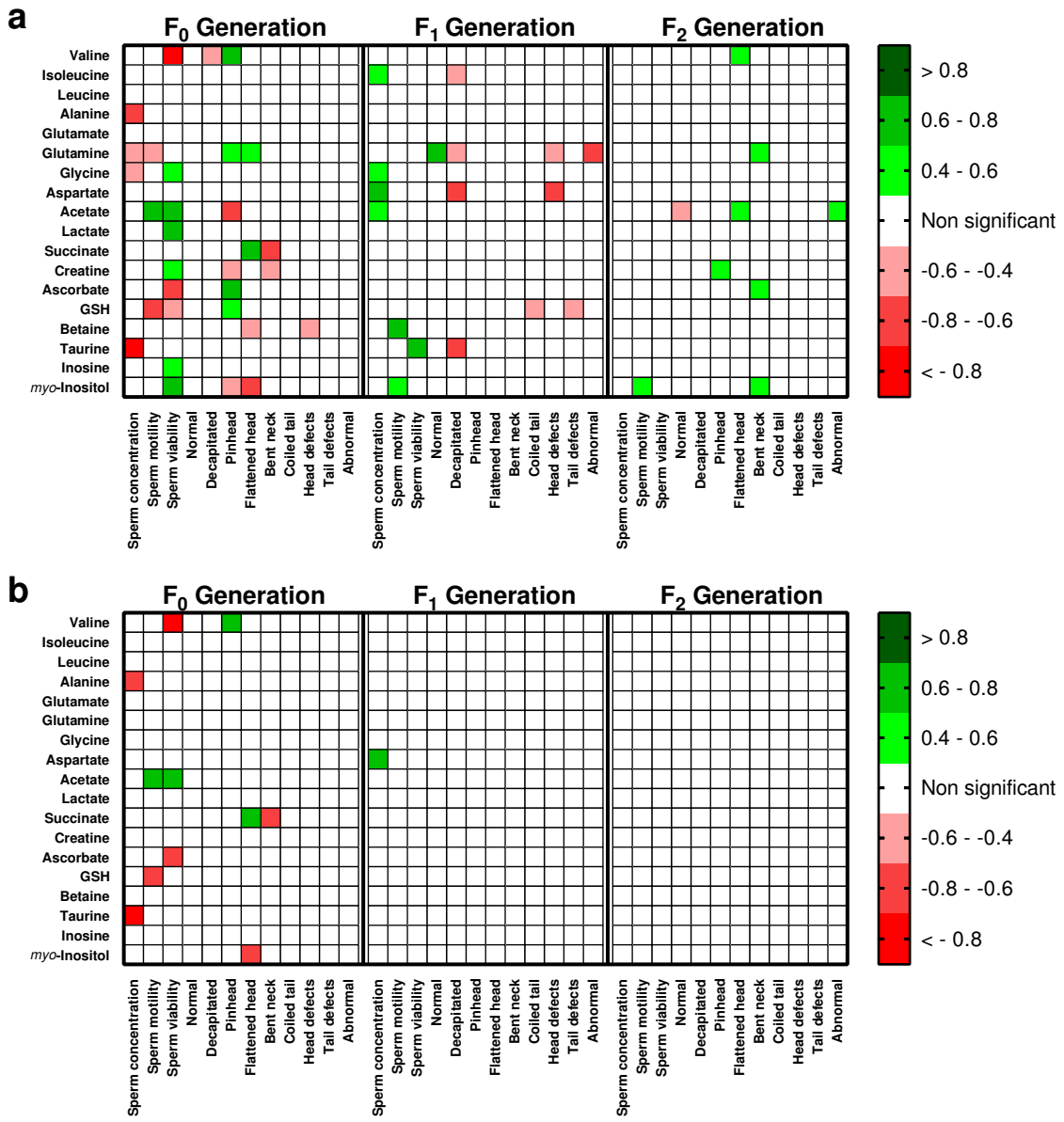


Figure 5.5: Correlations between sperm parameters and testicular metabolites, in each generation of the study (n = 6 per group in each generation). Correlation factors were obtained by Pearson's method using SPSS 26 (Armonk, NY, USA). (a) Strong ($|r| > 0.4$) and significant correlations considering unadjusted two-tailed $p < 0.05$. (b) Strong ($|r| > 0.4$) and significant correlations considering FDR-adjusted $p < 0.1$. Heatmaps were obtained using the software GraphPad Prism 8 (San Diego, CA, USA).

Exposure to HFD, even if transient, induces intergenerational and transgenerational effects in testicular metabolism and sperm parameters

A multivariate analysis including sperm parameters (counts, viability, motility and normal morphology) and all tested testicular metabolites was performed using PCA. Two principal components were extracted to represent each sample in a two-dimensional Euclidean space (Figure 5.6). A spatial dispersion graph was obtained for each generation individually, and as a transgenerational model, to evaluate clustering. PERMANOVA was then applied to detect overlapping of group clusters (M. J. Anderson, 2017) (Supplementary Table 5.S6). In generation F_0 clustering and well-separated groups are evident ($F = 17.56$, $p < 0.001$) (Figure 5.6a). In generation F_1, although there is overlap between groups, especially among the sons of HFD and HFD_t (p.adj = 0.430), the discrimination between groups was still significant ($F = 2.70$, p.adj = 0.036) (Figure 5.6b). The grandsons of diet-challenged mice (generation F_2) were better separated than their F_1 progenitors ($F = 6.45$, $p < 0.001$) (Figure 5.6c). Both the grandsons of HFD and the grandsons of HFD_t did not overlap with the grandsons of CTRL ($p = 0.046$ and $p = 0.006$, respectively). There is also a significant difference between HFD and HFD_t in F_2 Generation ($p = 0.089$). When analyzing all the groups at once (Figure 5.6d) there was a clear separation between the animals which have been HFD-challenged (HFD and HFD_t, generation F_0). Also, regardless of the generation, all CTRL samples are clustered near the origin. In this model, all the factors are significant predictors, although generation is the most relevant predictor (Generation: $F = 20.19$, $p < 0.001$; Diet: $F = 7.79$, $p < 0.001$; Generation*Diet: $F = 7.00$, $p < 0.001$). It was not possible to calculate multiple comparisons in this scenario.

5.4 Discussion

Metabolic diseases have reached pandemic proportions worldwide. Excess energy intake is considered the primary driver for the development of metabolic diseases. However, it is now clear that there are hereditable latent signals that trigger metabolic dysfunction. We used a multivariate analysis approach based on untargeted testicular metabolomics and analyzed sperm quality in fathers exposed to different diets and their offspring up to F_2. Our data show that there are paternally-mediated intergenerational and transgenerational effects on testicular metabolome and sperm quality caused by a HFD, even if transient (Table 5.2). Exposing fathers to HFD induces intergenerational and transgenerational effects. The former are defined as phenomena observed in offspring caused by direct exposure of the progenitor

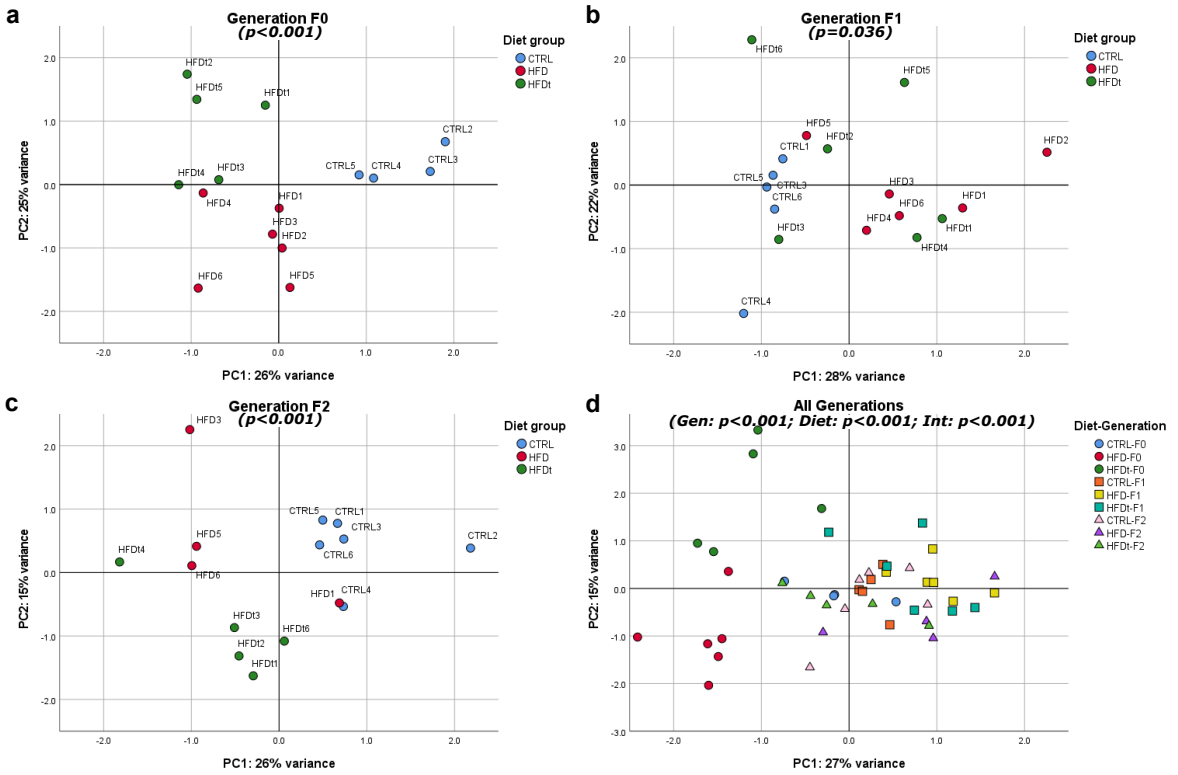


Figure 5.6: Sample dispersion in the two-dimensional space spanned by the two principal components extracted by PCA. Animals with at least one missing value in any of the variables considered (sperm parameters and testicular metabolites) were not included in the analysis. All the animals included in the model are represented in the graphs. Significance (p) values refer to overall permANOVA significance, obtained by the vegan R package. Significance was considered when adjusted $p < 0.1$. PC1 - principal component 1, PC2 - principal component 2, Gen - generation factor, Int - interaction (Generation*Diet) factor. PCA data visualization was obtained using the software SPSS 26 (Armonk, NY, USA) and edited using the software Inkscape 1.0 (Boston, MA, USA).

to an condition (*i.e.* environmental or contaminant) while the latter are caused by an ancestral exposure to which the previous generation has not been exposed (E. Nilsson et al., 2019; Perez & Lehner, 2019). Our study is based on paternal lineage and focuses on sons (intergenerational effects) and grandsons (transgenerational effects) of the diet challenged fathers.

Maternal metabolic dysfunction has been associated with a reprogramming that promotes diabetes or obesity in the offspring (Isganaitis et al., 2014; O'reilly & Reynolds, 2013) and the metabolic health of the father has been overlooked. However, an increasing number of studies show that the metabolic profile of the father at conception also influences the metabolic profile of the offspring. For instance, paternal obesity has an impact on the offspring metabolic health (Fullston et al., 2013; Fullston et al., 2015; Ng et al., 2014) as fathers with obesity are more likely to have children with obesity (L. Li et al., 2009). Interestingly, we have found limited evidence on this phenomenon. No differences were found in HOMA2-IR, fasting glucose and fasting insulinemia in the sons and grandsons of mice fed with HFD. Yet, the sons of HFD

mice had increased body weight at weaning than the sons of CTRL and HFD_t, whereas the sons of HFD_t had lower fat mass than the other groups, which elicits an effect of the father's diet in the body composition of the progeny. Also, the sons of HFD mice had increased serum glucose levels at 90 min and 120 min during insulin tolerance test than the sons of CTRL and HFD_t mice, reflecting an abnormal insulin response. These intergenerational effects of HFD in body adiposity and glucose homeostasis have been associated with altered DNA methylation patterns (Wu & Suzuki, 2006). Particularly, it has been shown that paternal exposure to HFD is associated with aberrant DNA methylation of adipogenic genes and genes related to increased diabetic risk (Masuyama et al., 2016; Ng et al., 2010), even in humans (Soubry et al., 2015). We have found that sons of mice fed with HFD_t had lower GSI than sons of CTRL and HFD mice, suggesting potential fertility problems. Infertility has been described as one of the most striking silent co-morbidities caused by metabolic diseases (Maresch et al., 2018). We have previously showed that consumption of HFD causes irreversible changes in testicular metabolism even after switching to normal diet, using the same experimental model used here for the progenitors (**Crisóstomo** et al., 2019). Sperm quality is the first line assessment of male fertility potential and a central indicator of testicular function. Our data show a transgenerational effect on sperm quality, particularly on sperm counts. The grandsons of individuals fed with HFD, even if transient, had a decreased sperm counts when compared with grandsons of individuals fed with a normal diet. A recent systematic review and meta-regression analysis showed that sperm counts are decreasing worldwide to dangerous levels (Levine et al., 2017). Concomitantly, metabolic diseases incidence and prevalence are also rising (Saeedi et al., 2019; The Global Health Observatory & World Health Organization [WHO], 2020a; 2020b; 2020c; Vardell, 2020). Thus, a relationship between these two factors has been proposed and discussed (P. F. Oliveira, Sousa, et al., 2017). Our data show that HFD, even if transient, has a transgenerational effect on sperm counts. We also detected inter- and transgenerational effects of diet on sperm morphology, particularly on head defects. Thus, our data suggest that unhealthy dietary habits induce inter- and transgenerational effects on sperm quality.

To further determine how diet-mediated inter- and transgenerational effects on sperm are mediated in the testis, we performed an exploratory analysis based on untargeted metabolomics from testicular extracts and multivariate analysis. Testicular metabolome reflected inter- and transgenerational effects of HFD consumption even when this occurred only until early adulthood. We found that the sons of mice fed with HFD, even temporarily, have higher levels of testicular glycine than sons of mice fed with standard chow. Glycine supplementation has

Table 5.2: Intergenerational and transgenerational effects of HFDs in the paternal lineage. The affected lineage is reported in brackets, comparing to the CTRL group of the corresponding generation.

Intergenerational effects	**Transgenerational effects**
⇓ Left testis weight (HFD/HFD_t)	⇓ Sperm counts (HFD/HFD_t)
⇓ Right testis weight (HFD/HFD_t)	⇓ Normal sperm (HFD)
⇓ Epididymal fat weight (HFD_t)	⇑ Head defects (HFD)
⇓ Perirenal fat weight (HFD_t)	⇑ testicular acetate (HFD)
⇓ Fat mass (HFD_t)	⇓ testicular glutamine (HFD_t)
⇓ GSI (HFD_t)	⇓ testicular inosine (HFD_t)
⇑ Body weight at weaning (HFD)	⇑ testicular ascorbate (HFD_t)
⇑ Normal sperm (HFD_t)	
⇓ Head defects (HFD_t)	
⇑ testicular glycine (HFD/HFD_t)	
⇑ testicular leucine (HFD)	
⇑ testicular acetate (HFD_t)	

been reported to improve sperm parameters in hypercholesterolemic rats (Gofur & Lestari, 2016). Decreased glycine levels have been associated with decreased cell permeability, mitochondrial membrane potential and the expression of blood-testis-barrier factors in the TM4 cell line (Xu et al., 2014), which derives from murine SCs. The increase in testicular glycine content could be due to an overcompensation mechanism to overcome the damage caused by HFD in the ancestors. Indeed, despite the increased body weight at weaning of the sons of HFD mice, there were no differences in sperm parameters between mice in this generation (F_1), and glycine positively correlated with sperm concentration. The sons of mice fed with HFD until early adulthood displayed changes in the testicular content of leucine, a branched-chain amino acid, compared to sons of CTRL. Branched-chain amino acids have been implicated in the onset of metabolic syndrome and insulin resistance (Lu et al., 2013). Increased serum branched-chain amino acid levels are associated with obesity and diabetes in humans, in response to insulin resistance (Adeva et al., 2012; Cummings et al., 2018). There are several testis-specific or abundant leucine-rich proteins (R. Wang & Sperry, 2008; Xue & Goldberg, 2000). Though the role for these proteins is not well characterized, accumulation of leucine may impact upon the assembly and function of these testicular leucine-rich proteins. Testicular acetate levels were increased in the sons of HFD_t mice and in the grandsons of HFD mice, compared to CTRL mice of the corresponding generation. Acetate is exported by SCs to developing germ cells, where it is used as a membrane lipid precursor (Alves et al., 2012). It was previously described that leptin promotes the excretion of acetate in isolated human SCs (Martins et al., 2015). Considering the reduced sperm counts of the grandsons of mice fed HFD, compared to the grandsons of CTRL, our data suggests that the turnover of germ cells is increased in these mice. Testicular glutamine levels were decreased in the grandsons of mice temporarily exposed to HFD. Glutamine is a crucial energy substrate for SCs,

whose metabolism is similar to the Warburg effect described in cancer cells (P. F. Oliveira et al., 2015). Grandsons of HFD_t mice also had a decrease in testicular inosine, compared to grandsons of CTRL and grandsons of HFD. Inosine is a precursor of purines. In rat testis, high-energy diets decrease inosine levels, with concomitant increase in hypoxanthine, which promotes a pro-inflammatory environment (Rato et al., 2014), due to increased xanthine oxidase activity. Indeed, testicular ascorbate levels are decreased in the grandsons of HFD mice, compared to the grandsons of HFD_t mice. Therefore, the decrease in glutamine may impair SC ability to provide nutritional support to developing germ cells. The inhibition of the rate-limiting reaction converting inosine to guanylyl metabolites has been used to arrest meiosis in mice oocytes (Wigglesworth et al., 2013). Thus, lower testicular inosine availability may inhibit the differentiation of germ cells, notably by inhibiting meiosis. This is reflected by our data that shows that the grandsons of HFD_t mice had lower sperm counts than the grandsons of CTRL. However, the association between transgenerational epigenetic and metabolomic fingerprints is scarce in the literature. Yet, a study in rats has demonstrated that early life exposure to di-n-butyl phthalate, an endocrine disruptor, caused an increase in testicular betaine levels for three generations, with concomitant decrease in sperm counts and DNA hypomethylation (Yuan et al., 2017). Although the ancestral exposure in this study was an indirect *in utero* exposure (embryonic exposure), it illustrates that metabolic fingerprints are likely the result of epigenetic factors that persist in the paternal lineage for several generations.

PCA was used in conjugation with PERMANOVA to evaluate the discriminant power of testicular metabolite content and sperm parameters as a function of diet and the male ancestor's diet (Figure 5.6, Table 5.S6). Indeed, this approach showed that animals fed with HFD, even transiently, share testicular metabolite signatures and sperm parameters, significantly different from animals fed a standard diet. Notably, sons of HFD_t mice display an intermediate phenotype between the sons of CTRL and the sons of HFD mice. However, the grandsons of HFD and HFD_t present specific testicular metabolome traits compared to CTRL grandsons, which are related to their poorer sperm parameters. Taken together, these results suggest that HFD, even transiently, causes a significant impact on sperm parameters and testicular metabolism in the diet-challenged generation (F_0), but also has intergenerational (F_1) and transgenerational (F_2) effects on the offspring. Our findings corroborate previous studies implicating deleterious effects caused by ancestral HFD on sperm parameters in rodents (Fullston et al., 2012; McPherson et al., 2014). Our data also support the theory that testicular metabolome is involved in this phenomenon, though the mechanism

underlying the inheritance of this phenotype is still unknown. The epigenetic signatures carried by spermatozoa is one of the most plausible mechanisms involved in this inheritance. They have been recently associated to the inheritance of the acquired phenotypes, notably in the male lineage and in response to ancestral exposure to HFD (Pereira et al., 2020). The DNA methylation pattern is one of the mechanisms previously implicated in the epigenetic transmission of traits. Previous studies have demonstrated that dietary and environmental exposures can alter the extent and the *loci* of methylation of the DNA carried by spermatozoa, thus conditioning gene expression as early as during embryo development (Skinner et al., 2018; Watkins et al., 2018). Yet, the extent to which DNA imprinting due to methylation can significantly affect development and health outcomes in the offspring is still debatable (Baxter & Drake, 2019; Sharp et al., 2021). Nevertheless, the influence of sperm DNA methylation and sperm sncRNA content in the inheritance of testicular metabolomic traits should be considered, and it must be a focus of further studies.

Here we show that sperm parameters are correlated with testicular metabolome, which corroborates our earlier findings (**Crisóstomo** et al., 2019). Hence, spermatozoa may carry signatures sensitive to HFD that encode the "metabolic memory" of testicular cells in the offspring, which drive the inter- and transgenerational phenotypes of sperm parameters. "Metabolic memory" was coined with the metabolic adaptations occurring at the cellular level in progressive stages of metabolic syndrome, and as a response to hyperglycemic states (Ihnat et al., 2007). This term is, however, too narrow in the context of intergenerational and transgenerational inheritance. "Inherited Metabolic Memory" refers to the inheritance of acquired metabolic signatures in response to environmental variables by the progeny which has not been exposed to the original stimuli. In summary, we suggest that an ancestral exposure to HFD, even if transiently, causes "inherited metabolic memory" in testicular tissue of the offspring up to two generation. This "inherited metabolic memory" is likely transmitted by epigenetic markers carried by the sperm, and is characterized by changes in the testicular metabolome, testicular function, and sperm parameters of the nonexposed progeny. Further research must focus on the epigenetic mechanisms underlying the "inherited metabolic memory".

5.5 Author contributions

Luís Crisóstomo, Pedro F. Oliveira, Marco G. Alves and Rachel L. Batterham contributed to study design, analysis and interpretation of data. Luís Rato, Ivana Jarak and **Luís Crisóstomo** performed experimental work. **Luís Crisóstomo** edited the images and tables, performed the

statistics and contributed to analysis and interpretation of data. Rachel L. Batterham and João F. Raposo critically reviewed the manuscript and suggested modifications. All the authors contributed to manuscript writing/editing and approved the final version.

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5.7 Supplementary Material

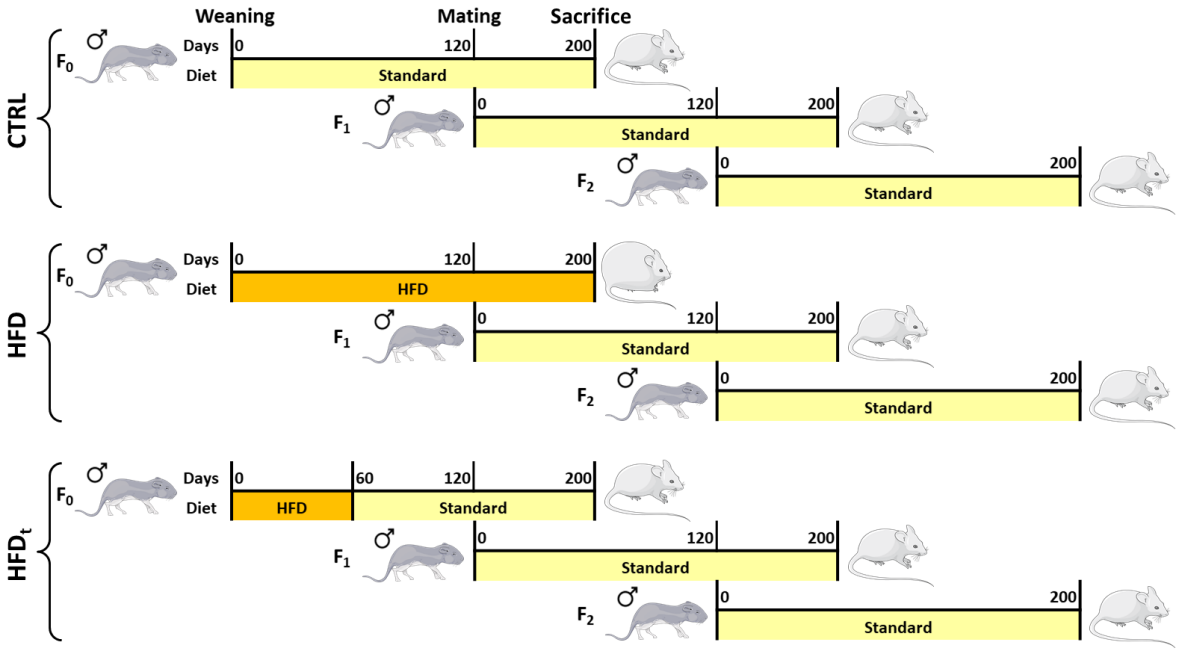


Figure 5.S1: Schematic representation of the experimental setting of the animal model. After weaning, mice generated from normoponderal males and females were weighed and randomly assigned to an experimental group: CTRL – standard diet; HFD – high-fat diet; HFD_t – transient high-fat diet (F_0 Generation) 60 days after weaning, HFD_t mice diet was reverted from high-fat diet to standard diet. 120 days after weaning, mice from all the groups were assigned to normoponderal females to generate the next generation (F_1 Generation). Mice were euthanized 200 days after weaning. Mice from the F_1 Generation were exclusively fed with standard diet. Similarly, they were mated with normoponderal females 120 days after weaning to generate the F_2 Generation, and were euthanized 200 days post-weaning. Mice from the F_2 Generation were fed with standard diet and euthanized at 200 days post-weaning.

Table 5.S1: Reproductive parameters. Matings were performed until a sufficient number of pups to continue the experiment was achieved (12 pups per group in each generation). The total number of matings performed per group in each generation is stated in the table. Results are expressed as the mean (%) ± SEM. Data was tested for independence using χ^2 test, whereas family-wise comparisons were performed by Z-test for column proportions with Bonferroni correction.

Generation	Parameter	CTRL	HFD	HFD_t	χ^2
F_0	*Matings*	23	18	18	-
	Success rate (%)	48 ± 10	56 ± 12	47 ± 12	0.32
	Litter size[a]	5.8 ± 2.0	4.9 ± 1.1	4.3 ± 1.4	-
	Male pups (%)	53 ± 9	48 ± 9	48 ± 11	0.22
F_1	*Matings*	11	12	12	-
	Success rate (%)	18 ± 12	25 ± 13	17 ± 11	0.29
	Litter size[a]	7.8 ± 1.3	8.2 ± 2.1	8.3 ± 1.2	-
	Male pups (%)	51 ± 8	58 ± 7	35 ± 8	4.31

[a]Tested by one-way ANOVA, Tukey's HSD, and expressed as the mean (liveborn pups) ± SD.

Table 5.S2: Resonance assignment in the ^{1}H-NMR spectra of polar testicular extracts (s: singlet, d: doublet, dd: doublet of doublets, t: triplet, q: quartet, m: multiplet).

	Compound[a]	δ^1H ppm (multiplicity, assigment)
1	acetate	1.92 (s, αCH_3)
2	alanine	1.48 (d, βCH_3), 3.78 (t, αCH)
3	ascorbate	4.52 (s, CH_4)
4	aspartate	2.66 (dd, βCH), 2.81 (dd, 'CH), 3.86 (m, αCH)
5	betaine	3.26 (s, αCH_3), 3.90 (s, αCH_2)
6	creatine	3.03 (s, αCH_3), 3.93 (s, CH_2)
7	glutamate	2.07 (m, βCH), 2.14 (m, β'CH), 2.35 (m, γCH_2), 3.76 (m, αCH)
8	glutamine	2.13 (m, βCH_2), 2.45 (m, γCH_2), 3.75 (m, αCH)
9	glutathione	2.16 (m, βCH_2 Glu), 2.56 (m, γCH_2 Glu), 2.95 (m, βCH_2 Cys), 3.79 (m, αCH Gly), 4.59 (q, αCH_2 Cys)
10	glycine	3.55 (s, CH_2)
11	isoleucine	0.94 (t, δCH_3), 1.01 (d, β'CH_3), 1.26 (m, γCH), 1.49 (m, γ'CH), 1.98 (m, βCH), 3.67 (m, αCH)
12	inosine	8.30 (s, CH), 2.81 (dd, 'CH), 3.86 (m, αCH)
13	lactate	1.33 (d, βCH_3), 4.11 (q, αCH)
14	leucine	0.96 (t, δCH_3), 1.73 (m, $\beta\delta CH_2$), 3.73 (m, αCH)
15	*myo*-inositol	3.26 (t, C5H), 3.52 (dd, C1H, C3H), 3.62 (m, C4H, C6H), 4.06 (t, C2H)
16	succinate	2.40 (s, CH_2)
17	taurine	3.26 (t, NCH_2), 3.43 (t, SCH_2)
18	valine	0.99 (d, γCH_3), 1.04 (d, γ'CH_3), 2.26 (m, βCH), 3.62 (m, αCH)

[a]all metabolites are putatively annotated (level 2 of identification according to Chemical Analysis Working Group (CAWG) Metabolomics Standards Initiative recommendations).

Table 5.S3: Testicular sperm metabolites across generations, quantified by semi-quantitative ^{1}H-NMR (n = 6 per group in each generation). Results are expressed as the mean ($\log_2$ FC) ± standard deviation. Results were analysed by Univariate ANOVA, corrected for FDR by Benjamini-Hochberg method, and with Tukey's HSD correction for pairwise comparisons. Two-way ANOVA with Šidak correction was used to compare each diet group in F_1 Generation against its F_2 counterparts. FDR cut-off was set to $p < 0.1$. [a] Generation F_0; [b] Generation F_1; [c] Generation F_2. Family-wise significance was considered when $p < 0.05$. * vs. CTRL; # vs. HFD; § vs. F_1 Generation. * p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001. Abbreviations: FC – Fold Change (to CTRL); SD – standard deviation.

	Generation F_0			**Generation F_1**			**Generation F_2**		
Metabolite	**CTRL**	**HFD**	**HFD$_t$**	**CTRL**	**HFD**	**HFD$_t$**	**CTRL**	**HFD**	**HFD$_t$**
Valine	0.00 ± 1.11	0.51 ± 0.18	0.64 ± 0.25	0.00 ± 0.13	0.27 ± 0.17*	0.15 ± 0.18	0.00 ± 0.17	0.24 ± 0.30	0.21 ± 0.28
Isoleucine	0.00 ± 0.19	−0.56 ± 0.76	0.01 ± 0.11	0.00 ± 0.17	0.29 ± 0.20	0.20 ± 0.26	0.00 ± 0.17	0.14 ± 0.25	0.15 ± 0.26
Leucine	0.00 ± 0.24	−0.29 ± 0.22	−0.08 ± 0.13	0.00 ± 0.07	0.26 ± 0.10**	0.13 ± 0.16	0.00 ± 0.10	0.03 ± 0.21§	0.01 ± 0.14
Alanine	0.00 ± 0.29	−0.14 ± 0.15	0.05 ± 0.19	0.00 ± 0.07	0.09 ± 0.15	0.02 ± 0.13	0.00 ± 0.27	−0.21 ± 0.20	−0.17 ± 0.08
Glutamate[a]	0.00 ± 0.06	−0.08 ± 0.09	0.04 ± 0.06#	0.00 ± 0.02	0.04 ± 0.03	0.01 ± 0.03	0.00 ± 0.09	0.02 ± 0.07	0.04 ± 0.06
Glutamine[a]	0.00 ± 0.06	0.16 ± 0.14	0.23 ± 0.06**	0.00 ± 0.09	−0.03 ± 0.11	0.03 ± 0.14	0.00 ± 0.12	−0.16 ± 0.09	−0.18 ± 0.07* §§
Glycine[b]	0.00 ± 0.11	−0.03 ± 0.10	−0.02 ± 0.06	0.00 ± 0.03	0.14 ± 0.06**	0.10 ± 0.06*	0.00 ± 0.07	−0.07 ± 0.04§§§§	−0.08 ± 0.06§§§§
Aspartate	0.00 ± 0.49	−0.20 ± 0.22	−0.21 ± 0.21	0.00 ± 0.19	0.11 ± 0.22	0.08 ± 0.05	0.00 ± 0.19	0.08 ± 0.25	0.11 ± 0.07
Acetate[a c]	0.00 ± 0.13	−0.64 ± 0.15****	−0.71 ± 0.09****	0.00 ± 0.15	0.46 ± 0.31	0.48 ± 0.35*	0.00 ± 0.29	1.33 ± 0.16** §§	0.27 ± 0.78#
Lactate	0.00 ± 0.16	−0.22 ± 0.17	−0.24 ± 0.23	0.00 ± 0.06	0.06 ± 0.12	0.01 ± 0.11	0.00 ± 0.16	0.15 ± 0.29	−0.13 ± 0.17
Succinate[a]	0.00 ± 0.36	−0.58 ± 0.59	1.00 ± 0.92##	0.00 ± 0.33	−0.48 ± 0.27	−0.19 ± 0.36	0.00 ± 0.22	−0.28 ± 0.31	0.15 ± 0.22§
Creatine[a]	0.00 ± 0.09	−0.18 ± 0.03***	−0.06 ± 0.04#	0.00 ± 0.05	0.00 ± 0.02	0.01 ± 0.02	0.00 ± 0.02	0.00 ± 0.03	0.01 ± 0.03
Ascorbate[a]	0.00 ± 0.27	0.45 ± 0.15**	0.41 ± 0.18*	0.00 ± 0.12	−0.15 ± 0.18	−0.09 ± 0.39	0.00 ± 0.36	−0.30 ± 0.53	0.42 ± 0.36§§
Glutathione[a]	0.00 ± 0.79	0.97 ± 0.27*	0.97 ± 0.24*	0.00 ± 0.04	−0.38 ± 0.38	−0.53 ± 0.78	0.00 ± 0.34	−0.31 ± 0.30	−0.43 ± 0.55
Betaine[a]	0.00 ± 0.04	0.15 ± 0.12	−0.08 ± 0.14#	0.00 ± 0.02	0.03 ± 0.05	0.00 ± 0.08	0.00 ± 0.04	0.03 ± 0.04	0.00 ± 0.05
Taurine	0.00 ± 0.15	−0.04 ± 0.13	0.10 ± 0.09	0.00 ± 0.09	0.05 ± 0.03	0.09 ± 0.06	0.00 ± 0.03	−0.01 ± 0.06	−0.05 ± 0.08§§§
Inosine[a c]	0.00 ± 0.31	−0.68 ± 0.22*	−0.44 ± 0.45	0.00 ± 0.04	−0.01 ± 0.13	−0.02 ± 0.06	0.00 ± 0.15	−0.09 ± 0.28	−0.68 ± 0.40** # §§§
***myo*-Inositol**[a]	0.00 ± 0.24	0.08 ± 0.06	−0.19 ± 0.10#	0.00 ± 0.02	0.00 ± 0.05	−0.01 ± 0.07	0.00 ± 0.02	−0.05 ± 0.05	0.00 ± 0.04

Table 5.S4: Biometric parameters across generations (n = 12 per group in each generation). Results are represented as the mean (g) ± SD. Data was tested with one-way ANOVA with Tukey's Honest Significant Differences. Significance was considered when $p < 0.05$. * vs. CTRL; # vs. HFD. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

	Group	Generation F_0	Generation F_1	Generation F_2
Body mass (g, at sacrifice)	**CTRL**	30.34 ± 1.80	28.92 ± 2.36	29.21 ± 3.58
	HFD	$47.10 \pm 3.48^{****}$	28.93 ± 2.42	29.50 ± 3.19
	HFD_t	$27.99 \pm 2.59^{\#\#\#\#}$	27.22 ± 2.20	27.51 ± 2.90
Left testis (g)	**CTRL**	0.11 ± 0.01	0.13 ± 0.01	0.13 ± 0.02
	HFD	0.12 ± 0.01	$0.11 \pm 0.02^{*}$	0.12 ± 0.01
	HFD_t	0.11 ± 0.01	$0.10 \pm 0.02^{***}$	0.12 ± 0.01
Right testis (g)	**CTRL**	0.11 ± 0.01	0.12 ± 0.01	0.13 ± 0.01
	HFD	0.11 ± 0.01	0.11 ± 0.01	0.13 ± 0.03
	HFD_t	0.11 ± 0.01	$0.10 \pm 0.01^{*}$	0.11 ± 0.01
Right epididymis (g)	**CTRL**	0.06 ± 0.01	0.06 ± 0.02	0.07 ± 0.02
	HFD	0.06 ± 0.01	0.05 ± 0.02	0.07 ± 0.01
	HFD_t	0.06 ± 0.01	0.05 ± 0.01	0.07 ± 0.02
Liver (g)	**CTRL**	1.15 ± 0.08	1.16 ± 0.17	1.16 ± 0.19
	HFD	$1.38 \pm 0.29^{*}$	1.13 ± 0.14	1.19 ± 0.17
	HFD_t	$1.08 \pm 0.09^{\#\#}$	1.03 ± 0.16	1.11 ± 0.13
Seminal vesicles (g)	**CTRL**	0.25 ± 0.05	0.28 ± 0.05	0.29 ± 0.08
	HFD	0.31 ± 0.04	0.31 ± 0.06	0.31 ± 0.04
	HFD_t	0.27 ± 0.09	0.27 ± 0.06	0.31 ± 0.03
Epididymal fat (g)	**CTRL**	0.73 ± 0.23	0.76 ± 0.20	1.00 ± 0.43
	HFD	$2.21 \pm 0.79^{****}$	0.73 ± 0.24	1.09 ± 0.44
	HFD_t	$0.63 \pm 0.29^{\#\#\#\#}$	$0.55 \pm 0.14^{*}$	0.82 ± 0.36
Perirenal fat (g)	**CTRL**	0.29 ± 0.14	0.29 ± 0.10	0.33 ± 0.22
	HFD	$1.56 \pm 0.37^{****}$	0.30 ± 0.13	0.38 ± 0.20
	HFD_t	$0.23 \pm 0.13^{\#\#\#\#}$	$0.21 \pm 0.07^{\#}$	0.31 ± 0.20
Retroperitoneal fat (g)	**CTRL**	0.32 ± 0.12	0.38 ± 0.11	0.38 ± 0.18
	HFD	$2.52 \pm 0.71^{****}$	0.36 ± 0.12	0.39 ± 0.21
	HFD_t	$0.35 \pm 0.19^{\#\#\#\#}$	0.29 ± 0.09	0.29 ± 0.18
Brown adipose tissue (g)	**CTRL**	0.14 ± 0.02	0.12 ± 0.03	0.16 ± 0.04
	HFD	$0.18 \pm 0.04^{**}$	0.12 ± 0.03	0.18 ± 0.06
	HFD_t	0.16 ± 0.04	0.11 ± 0.04	$0.12 \pm 0.05^{\#}$
Fat mass (%)	**CTRL**	4.83 ± 1.35	5.32 ± 1.01	6.22 ± 1.72
	HFD	$13.70 \pm 1.51^{****}$	5.12 ± 1.14	8.82 ± 2.38
	HFD_t	$4.78 \pm 1.83^{\#\#\#\#}$	4.19 ± 0.96	5.56 ± 2.11
Gonadosomatic index (%)	**CTRL**	0.75 ± 0.08	0.85 ± 0.10	0.90 ± 0.11
	HFD	$0.48 \pm 0.05^{****}$	0.77 ± 0.09	0.86 ± 0.11
	HFD_t	$0.79 \pm 0.06^{\#\#\#\#}$	0.74 ± 0.09	0.84 ± 0.10

Table 5.S5: Serum glycemia during the ipGTT and during the ipGTT (n = 12 per group in each generation). Data is represented as the mean glycemia (mg dL^{-1}) ± SD. The tests were performed at 196- and 198-days post-weaning age. ipGTT was performed after overnight fast, whereas ipGTT was performed after a 4-hour fast during the morning. Results were analysed by Repeated Measures ANOVA with Šidak correction for pairwise comparisons within timepoints, and Greenhouse-Geisser correction for sphericity. Significance was considered when $p < 0.05$. * vs. CTRL; # vs. HFD. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Gen	Group	ipGTT time (minutes)				
		0	**30**	**60**	**90**	**120**
F_0	CTRL	93 ± 10	325 ± 36	232 ± 30	167 ± 24	132 ± 15
	HFD	$128 \pm 30^{*}$	$409 \pm 32^{***}$	$384 \pm 80^{****}$	$267 \pm 57^{****}$	$226 \pm 38^{****}$
	HFD_t	$97 \pm 34^{\#}$	$317 \pm 57^{\#\#\#\#}$	$249 \pm 38^{\#\#\#\#}$	$171 \pm 23^{\#\#\#\#}$	$141 \pm 29^{\#\#\#\#}$
F_1	CTRL	82 ± 16	346 ± 54	293 ± 34	217 ± 36	158 ± 17
	HFD	$87 \pm 9^{*}$	331 ± 65	275 ± 63	192 ± 40	161 ± 43
	HFD_t	87 ± 13	363 ± 86	280 ± 72	196 ± 38	157 ± 23
F_2	CTRL	88 ± 7	345 ± 49	287 ± 29	216 ± 39	162 ± 13
	HFD	91 ± 11	338 ± 45	281 ± 52	196 ± 41	161 ± 45
	HFD_t	93 ± 9	333 ± 25	273 ± 64	205 ± 45	150 ± 22

Gen	Group	ipGTT time (minutes)				
		0	**30**	**60**	**90**	**120**
F_0	CTRL	129 ± 14	79 ± 11	81 ± 13	99 ± 21	114 ± 24
	HFD	$145 \pm 9^{*}$	$106 \pm 19^{**}$	$109 \pm 15^{**}$	$132 \pm 27^{**}$	$161 \pm 35^{**}$
	HFD_t	$121 \pm 14^{\#\#\#}$	$79 \pm 26^{\#\#}$	$77 \pm 30^{\#\#}$	$99 \pm 11^{\#\#}$	$119 \pm 32^{\#\#}$
F_1	CTRL	137 ± 12	78 ± 12	54 ± 34	83 ± 41	119 ± 28
	HFD	144 ± 23	81 ± 8	83 ± 30	$127 \pm 31^{**}$	$154 \pm 27^{**}$
	HFD_t	136 ± 14	81 ± 19	61 ± 15	$71 \pm 16^{\#\#}$	$96 \pm 14^{\#\#}$
F_2	CTRL	135 ± 21	80 ± 9	56 ± 7	84 ± 10	98 ± 6
	HFD	140 ± 20	83 ± 6	50 ± 9	87 ± 7	104 ± 9
	HFD_t	137 ± 25	87 ± 8	53 ± 12	82 ± 7	98 ± 5

Table 5.S6: Comparison of groups according to the 2-factor PCA in the two-dimensional Euclidean space, using permANOVA (R package vegan). Multiple comparisons corrected for the FDR by the Benjamini-Hochberg method, using the R package RVAideMemoire. Groups were considered significantly different when p.adj < 0.1.

Gen	Factor	Df	SumOfSqs	R^2	F	P	Comparison	P.adj
	Diet	2	20.87	0.75	17.56	0.001	*CTRL vs. HFD*	0.006
F_0	*Residual*	12	7.13	0.25			*CTRL vs. HFD_t*	0.006
	Total	14	28.00	1.00			*HFD vs. HFD_t*	0.006
	Diet	2	8.91	0.28	2.70	0.036	*CTRL vs. HFD*	0.018
F_1	*Residual*	14	23.09	0.72			*CTRL vs. HFD_t*	0.224
	Total	16	32.00	1.00			*HFD vs. HFD_t*	0.430
	Diet	2	14.51	0.52	6.45	0.001	*CTRL vs. HFD*	0.006
F_2	*Residual*	12	13.49	0.48			*CTRL vs. HFD_t*	0.006
	Total	14	28.00	1.00			*HFD vs. HFD_t*	0.089

	Factor	Df	SumOfSqs	R^2	F	P
	Diet	2	10.44	0.11	7.00	0.001
Transgenerational	*Generation*	2	30.07	0.33	20.19	0.001
	*Generation*Diet*	4	23.20	0.25	7.79	0.001
	Residual	38	28.29	0.30		
	Total	46	92.00	1.00		

Chapter 6

Inherited metabolic memory of high-fat diet impairs testicular fatty acid content and sperm parameters

This chapter was reproduced from the published work:

Crisóstomo, **L.**, Videira, R. A., Jarak, I., Starčević, K., Mašek, T., Rato, L., Raposo, J. F., Batterham, R. L., Oliveira, P. F., & Alves, M. G. (2022). Inherited metabolic memory of high-fat diet impairs testicular fatty acid content and sperm parameters. *Molecular Nutrition and Food Research*, *66*(5), 2100680. https://doi.org/10.1002/mnfr.202100680

Abstract

Scope

Exposure to a high-fat diet (HFD) from early-life is associated with a testicular metabolic signature linked to abnormal sperm parameters up to two generations after exposure in mice. Hereby, we describe a testicular lipid signature associated with "inherited metabolic memory" of exposure to HFD, persisting up to two generations in mice.

Methods & Results

Diet-challenged mice (n = 36) were 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) were fed with chow diet. Mice were euthanized 200 days post-weaning. Glucose homeostasis, serum hormones, testicular bioenergetics and antioxidant enzyme activity were evaluated. Testicular lipid-related metabolites and fatty acids were 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 ω3/ω6 ratio towards a pro-inflammatory environment. Grandsons of HFD_t 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.

Conclusions

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

6.1 Introduction

The epidemic proportions of acquired metabolic disorders, namely overweight/obesity and 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 WHO and other non-governmental organizations predict an increase in their prevalence in the decades to come (Saeedi et al., 2019; WHO, 2014). This trend promotes the early-onset of obesity and T2D (Saeedi et al., 2019; WHO, 2014), raising concerns regarding the possible consequences of these disorders to the sexual health of men of reproductive age (Imani et al., 2021; Salas-Huetos et al., 2021). 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) (Craig et al., 2017; Pavlinková et al., 2017). These effects have even been reported in humans (Kaati et al., 2002), suggesting that the signature of ances-

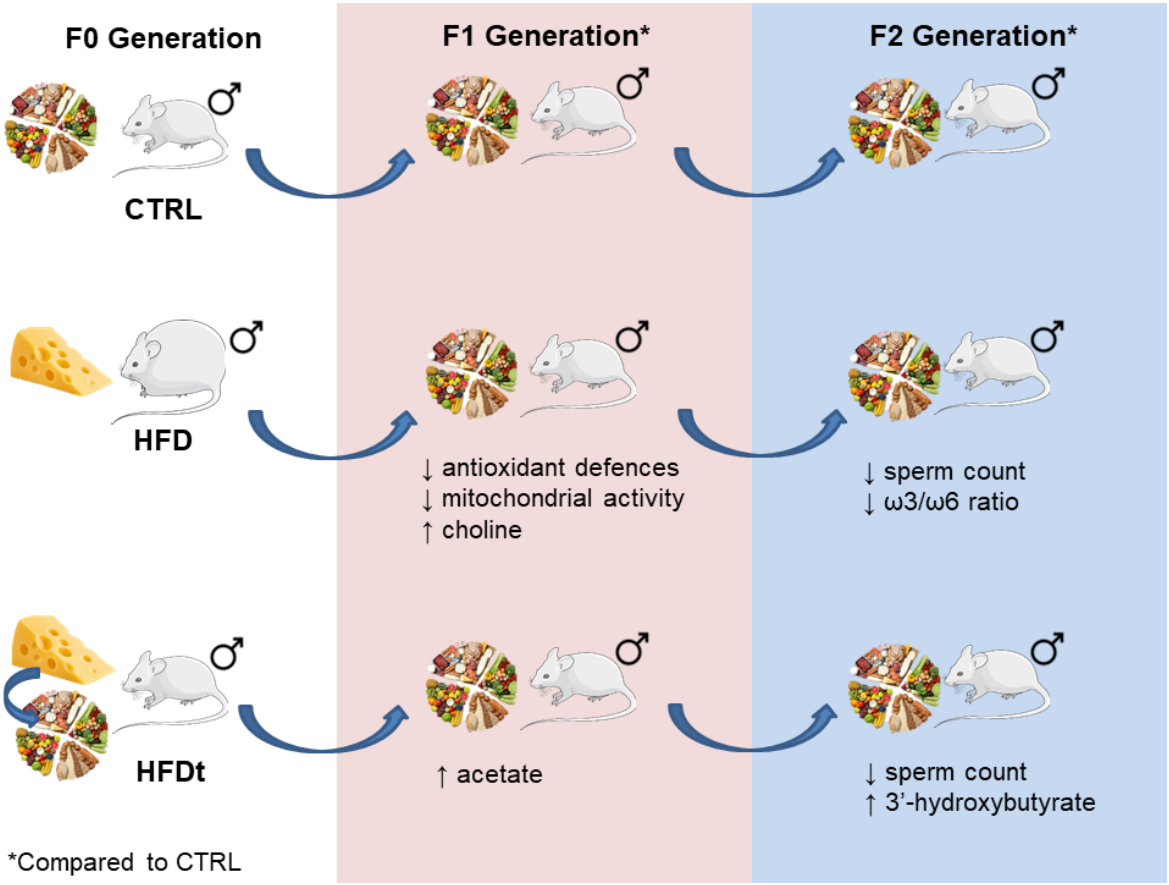


Figure 6: Graphical abstract. Ancestral exposure to high-fat diet, even if transient, causes detrimental effects in testicular lipid composition and function up to two generations. Sons of HFD mice had abnormal testicular choline metabolism, mitochondrial activity and antioxidants. Grandsons of HFD had a testicular ω3/ω6 ratio favorable to a pro-inflammatory environment, whereas grandsons of HFD_t had elevated testicular 3-hydroxybutyrate. Both grandsons of HFD and HFD_t presented lower sperm counts than grandsons of CTRL.

tral 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 (Carone et al., 2010; Chamorro-García et al., 2013). 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 (Masuyama et al., 2016). 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 (**Crisóstomo**, Jarak, et al., 2021). 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) (**Crisóstomo**, Jarak, et al., 2021). In this study, we further explore the effects caused by an ancestral exposure to an HFD in the testicular content of FAs and lipid-related metabolites. We apply a bottom-up Exploratory Data Analysis (EDA) strategy (Pinu et al., 2019) 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.

6.2 Material and Methods

Animal Model

This study was performed in three generations of *Mus musculus* C57BL6/J mice as described (**Crisóstomo**, Jarak, et al., 2021). 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, New Jersey, USA), then switched to standard chow (#F4031, BioServ, New Jersey, USA). Despite different fatty acid content, the proportion of fatty acids families to the total fatty acid content is the same between diets. The age of dietary switch has chosen to restrict the influence of this lifestyle intervention to adult, sexually mature mice (**Crisóstomo** et al., 2019). By this age mice have already completed 2–3 spermatogenic cycles (Whittingham & Wood, 1983), corresponding to a young man (≈ 20 years old) (Dutta & Sengupta, 2016). 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 hours each day, without water or food supply. After weaning, F_1 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); HFDt_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 is compliant with the ARRIVE guidelines and was licensed by the Portuguese Veterinarian and Food Department (0421 /000 /000 /2016).

Endocrine and metabolic function

Glucose tolerance and insulin resistance were assessed by the ipGTT and ipGTT protocols (McGuinness et al., 2009), 196 days 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, 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).

Evaluation of epididymal sperm parameters

Epididymides were isolated immediately after sacrifice (200 days post-weaning age) and placed in pre-warmed (37 °C) 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 (**Crisóstomo** et al., 2019; Rato et al., 2013). 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 (Kvist et al., 2002), 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) (Kvist et al., 2002).

NMR spectroscopy

A combined extraction of polar and nonpolar metabolites from testicular tissue was performed as previously described (Alves et al., 2011; **Crisóstomo** et al., 2019). Testes were not perfused with saline before extraction, as contamination by serum lipids and metabolites is negligible (Kaliss & Pressman, 1950; Napoli & McCormick, 1981), 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 (Jarak et al., 2018). 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 MSI guidelines (Sumner et al., 2007). Recorded spectra were compared to reference spectra of the HMDB (Wishart et al., 2007). Identification levels are indicated in the Supplementary Table 6.S1. ^{1}H were processed using previously described methods (Jarak et al., 2018). Peak areas were obtained using AMIX software (Bruker BioSpin GmbH, Rheinstetten, Germany) and normalized to the total spectral area.

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 length, SGE, Austin, TX). 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 up to 170 °C, 4 °C min up to 220 °C and 40 °C min 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 m/z and event time: 0.3 s. All of the experimental measurements were repeated three times, and the average values were reported. Fatty acids were identified by retention time and fragmentation profile and were quantified by the internal standard procedure. Results were expressed as a percentage of total fatty acids.

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 inhibitor cocktail (#B14001, Bimake, Munich, Germany) (1:10 $\frac{p}{v}$) using a glass-Teflon Potter Elvejhem (Kimble, Millville, NJ) as described previously (**Crisóstomo** et al., 2020). The resulting homogenate was split into two fractions: one was used for adenine nucleotides (ATP, ADP, and AMP) extraction and quantification by HPLC, as described (Monteiro-Cardoso et al., 2015); the other was fractionated by differential centrifugation to obtain a mitochondria-free cytosolic fraction and a mitochondria-enriched fraction, as described (**Crisóstomo** et al., 2020). The final protein content of each fraction was determined by the BCA (P. K. Smith et al., 1985). 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, 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) (del Río et al., 1977), and expressed in $\mathrm{nmol_{O_2}\,min^{-1}\,mg^{-1}_{protein}}$.

SOD, GPx and GSR activities were evaluated in 96-well plates, at 37 °C, as described (**Crisóstomo** et al., 2020; Neves et al., 2019). SOD activity was evaluated as the reduction of NBT and expressed as $\mathrm{U\,min^{-1}\,mg^{-1}_{protein}}$, 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 $\mathrm{nmol\,min^{-1}\,mg^{-1}_{protein}}$. GPx activity was evaluated by the rate of NADPH oxidation associated with H_2O_2 reduction, expressed in $\mathrm{nmol\,min^{-1}\,mg^{-1}_{protein}}$.

Testicular lipid peroxidation levels were evaluated by the production of TBARS assay. Shortly, 50 µL testicular cytosolic fraction were mixed with 600 µL of Reaction Solution [thiobarbituric acid 0.38 %$\frac{m}{v}$, trichloroacetic acid 37 %$\frac{m}{v}$ and 2,6-ditertbutyl-4-methylphenol 0.02 %$\frac{m}{v}$ (Mendes et al., 2018)] and incubated at 95 °C for 30 min. MDA formation was measured by colorimetric methods (ε= 156 $\mathrm{mmol^{-1}\,cm^{-1}}$), and results were expressed in $\mathrm{nmol_{MDA}\,mg^{-1}_{protein}}$.

Activity of mitochondrial enzyme complexes

Activities of the mitochondrial complexes CI, CII, CIV, and CS were assessed at 37 °C, in a 96-well plate by adapting previously described protocols (**Crisóstomo** et al., 2020; Monteiro-

Cardoso et al., 2015). CS activity was measured spectrophotometrically and calculated by the reduction rate DTNB ($\varepsilon = 13.6\,\mathrm{mM}^{-1}\,\mathrm{cm}^{-1}$), determined in the linear range of the plot, and expressed as $\mathrm{nmol}_{\mathrm{DTNB}}\,\mathrm{mg}^{-1}_{\mathrm{protein}}$.

CI 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 $\mathrm{nmol}_{\mathrm{NADH}}\,\mathrm{mg}^{-1}_{\mathrm{protein}}$.

CII activity was assessed monitoring DCPIP reduction at 600 nm ($\varepsilon = 20.7\,\mathrm{mM}^{-1}\,\mathrm{cm}^{-1}$). CII activity was expressed as the DCPIP reduction rate (corrected for spontaneous reduction) in $\mathrm{nmol}_{\mathrm{DCPIP}}\,\mathrm{mg}^{-1}_{\mathrm{protein}}$.

CIV activity was calculated by the CytC ($\varepsilon = 29.5\,\mathrm{mM}^{-1}\,\mathrm{cm}^{-1}$) oxidation rate, corrected by the spontaneous oxidation rate obtained in the KCN-inhibited wells, and expressed as $\mathrm{nmol}_{\mathrm{CytC}}\,\mathrm{mg}^{-1}_{\mathrm{protein}}$.

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-λAbsorbance Detector. ATP, ADP, and AMP were separated on a reverse-phase chromatography column (Lichrospher® RP-18 HPLC Column, 5 µm particle size, length *times* inner diameter 25 cm cm *times* 4.6 mm), using a gradient mobile phase that consisted of Phase A (100 mM KH_2PO_4 buffer with 1.2 $\%\frac{\mathrm{v}}{\mathrm{v}}$ methanol, pH = 7.0) and Phase B (100 mM KH_2PO_4 buffer with 10 $\%\frac{\mathrm{v}}{\mathrm{v}}$ methanol, pH = 7.0). The elution program was the following: 100 % of A from 0 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 $1\,\mathrm{mL\,min}^{-1}$, 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 $\mathrm{mol\,g}^{-1}_{\mathrm{wet\ tissue}}$.

Statistical analysis

Our bottom-up EDA design includes univariate and multivariate analysis (Figure 6.1). Phenotypic features were first compared between groups, within each Generation in the study, based on parametric univariate methods of analysis. The assumptions for parametric meth-

ods were tested by Kolmogorov-Smirnoff (KS) test with Lillefor's correction (normality) and by the Levene test (homoscedasticity). Data was 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 HSDs. 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 PCA based on the correlation matrix. Data were tested for normality using the KS test ($p > 0.05$) and for data reduction adequacy using the Kaiser-Meyer-Olkin Measure of Sample Adequacy (KMO) ($0.5 < KMO < 0.8$). Two 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).

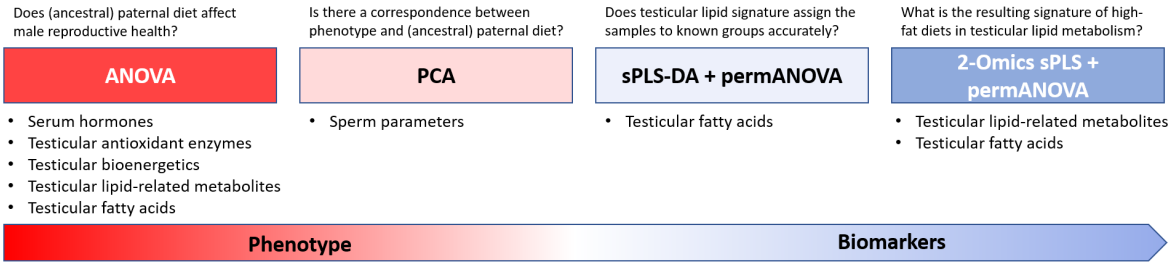


Figure 6.1: Bottom-up Exploratory Data Analysis workflow. We started by investigating whether serum hormones and testicular antioxidant enzymes, bioenergetics, lipid-related metabolite content and fatty acid content were different across groups, in each generation. This hypothesis was tested using Univariate ANOVA as previously (**Crisóstomo**, Jarak, et al., 2021; **Crisóstomo** et al., 2019; **Crisóstomo** et al., 2020). 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 (**Crisóstomo**, Jarak, et al., 2021; **Crisóstomo** et al., 2020). 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-sparse Projection of Latent Structures (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.

Testicular biomarkers associated with the phenotype were identified using multivariate analysis based on sPLS and sPLS-DA. These methods were implemented using the R package mixOmics (Rohart et al., 2017). The *"sparse"* version of these methods, implemented by

the mixOmics package introduces variable selection via implementation of the LASSO (ℓ_1) regularization (Tibshirani, 1996). 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 $\geq |0.2|$ were used to estimate the number of variables to be affected by the ℓ_1 parameter of the *"sparse"* method (Lê Cao et al., 2008). Samples were plotted in the space spanned by the extracted components. Group separation was evaluated using PERMANOVA, using the adonis2 function provided in the vegan R package (Oksanen et al., 2020). 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).

6.3 Results

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 (**Crisóstomo** et al., 2020), but differences were found in the descendants (Figure 6.2). In Generation F_1, sons of HFD_t fed mice show lower E_2 levels ($201 \pm 99\,pg\,mL^{-1}$) than CTRL ($33 \pm 73\,pg\,mL^{-1}$) and HFD fed mice sons ($341 \pm 97\,pg\,mL^{-1}$). Notably, in generation F_2 serum FSH levels were increased in grandsons of HFD_t fed mice ($84.2 \pm 55.7\,ng\,mL^{-1}$) comparing to the levels detected in CTRL and HFD fed grandsons (16.2 ± 5.6 and $35.8 \pm 24.3\,ng\,mL^{-1}$, respectively) while serum LH in grandsons of HFD_t fed mice presented decreased levels ($330 \pm 28\,ng\,mL^{-1}$) compared to the grandsons of HFD fed mice ($401 \pm 38\,ng\,mL^{-1}$). E_2 levels were increased in the grandsons of HFD ($309 \pm 132\,pg\,mL^{-1}$), comparing to other groups (CTRL: $151 \pm 53\,pg\,mL^{-1}$; HFD_t: $252 \pm 202\,pg\,mL^{-1}$, 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 minutes on the ipGTT (**Crisóstomo**, Jarak, et al., 2021). No changes were found in HOMA2 metrics in sons and grandsons of the HFD-challenged mice.

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 CAT and GSR in the testis of mice fed a life-long HFD (**Crisóstomo** et al., 2020). Here, we observed a decrease in the activity of catalase in testes of their offspring (generation F_1, HFD: $952 \pm 192\,\mathrm{nmol_{O_2}\,min^{-1}\,mg^{-1}_{protein}}$), comparing to other groups (Table 6.1). SOD activity was increased in sons of HFD_t-fed mice ($21.1 \pm 3.2\,\mathrm{U_{cat}\,mg^{-1}_{protein}}$) compared to the sons of HFD fed mice. Interestingly, testicular mitochondrial complex CI and CIV activities are reduced in the sons of HFD fed mice (generation F_1: $46.54 \pm 19.21\,\mathrm{nmol_{NADH}\,min^{-1}\,mg^{-1}_{protein}}$, and $29.32 \pm 9.03\,\mathrm{nmol_{CytC}\,min^{-1}\,mg^{-1}_{protein}}$, respectively) comparing to the sons of the other groups (Table 6.S2). 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 6.S3).

Table 6.1: 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 (HFD_t), and their progeny (Generation F_1 and F_2). 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 F_1 and F_2 were compared using two-way ANOVA corrected by Šidak method. Significance was considered when $p < 0.05$. * vs. CTRL; # vs. HFD; § vs. Generation F_1. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

	Parameter	CTRL	HFD	HFD_t
Generation F_0	*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 F_1	*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 ± 0.34	21.0 ± 3.2#
	CAT activity[d]	1356 ± 255	953 ± 192*	1220 ± 205
	GSR activity[e]	142.5 ± 7.5	149.2 ± 19.4	142.9 ± 6.0
Generation F_2	*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

Units: [a] $\mathrm{nmol_{MDA}\,mg^{-1}_{protein}}$; [b] $\mathrm{nmol_{NADH}\,min^{-1}\,mg^{-1}_{protein}}$; [c] $\mathrm{U_{cat}\,mg^{-1}_{protein}}$; [d] $\mathrm{nmol_{O_2}\,min^{-1}\,mg^{-1}_{protein}}$; [e] $\mathrm{nmol_{NADH}\,min^{-1}\,mg^{-1}_{protein}}$.

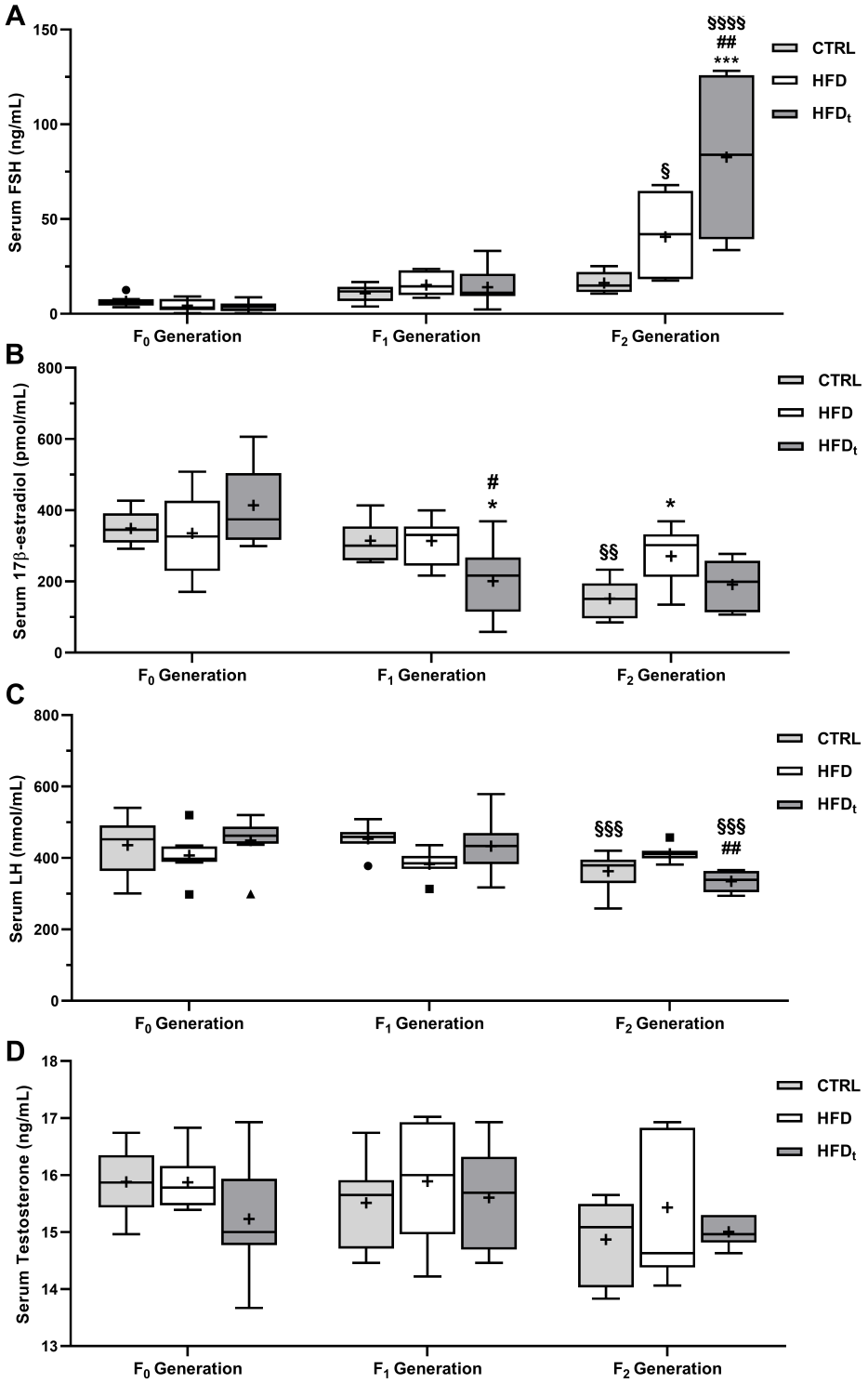


Figure 6.2: Serum hormones quantified in mice fed standard chow (CTRL) or life-long high-fat diet (HFD) or subjected to diet correction after 60 days (HFD_t), and in their progeny (Generations F_1 and F_2). Results are expressed as Tukey's whisker boxes (median, 25th to 75th percentiles ± 1.5 IQR). Extreme 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; * vs. CTRL; # vs. HFD. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$.

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 (**Crisóstomo** et al., 2020). 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 ($0.36 \pm 0.12\,\log_2$ FC) was the only intergenerational effect found in our study (Table 6.3A). 3OHBut was over-represented in testes of the grandsons (generation F_2) of HFD ($0.41 \pm 0.21\,\log_2$ FC) and HFD_t fed mice ($0.44 \pm 0.26\,\log_2$ FC) compared to the levels found in the testis of grandsons of CTRL mice (Table 6.3B). EtAm was increased in the testes of the grandsons of HFD fed mice (0.76 ± 0.59 $\log_2$ FC), comparing to CTRL (Table 6.3C). Similarly, grandsons of HFD fed mice had a higher testicular acetate concentration ($1.33 \pm 0.16\,\log_2$ FC) than grandsons of CTRL mice (Table 6.S4). Opposingly, grandsons of HFD fed mice had lower content of testicular PChol ($-0.08 \pm 0.06\,\log_2$ FC) and PEtAm ($-0.12 \pm 0.09\,\log_2$ FC) than grandsons of CTRL mice (Table 6.S4). Results are detailed in supplemental Table 6.S4. No intergenerational effects of ancestral HFD were found in testicular FA content (Figure 6.4). 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 PUFAs ($-0.24 \pm 0.16\,\log_2$ FC), ω3 PUFAs ($-0.50 \pm 0.32\,\log_2$ FC) and ω6 PUFAs ($-0.19 \pm 0.14\,\log_2$ FC). The grandsons of HFD-fed mice further showed reduced values for several lipid ratios: ω3/ω6 ($-0.31 \pm 0.18\,\log_2$ FC), C22:6n3/C20:4n6 ($-0.47 \pm 0.31\,\log_2$ FC) and C22:5n6/C20:4n6 ($-0.42 \pm 0.25\,\log_2$ 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 6.S5).

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 by PCA (Figure 6.5). To avoid multicollinearity, only four variables were considered for dimension reduction: sperm counts ($M\,mL^{-1}$), 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.

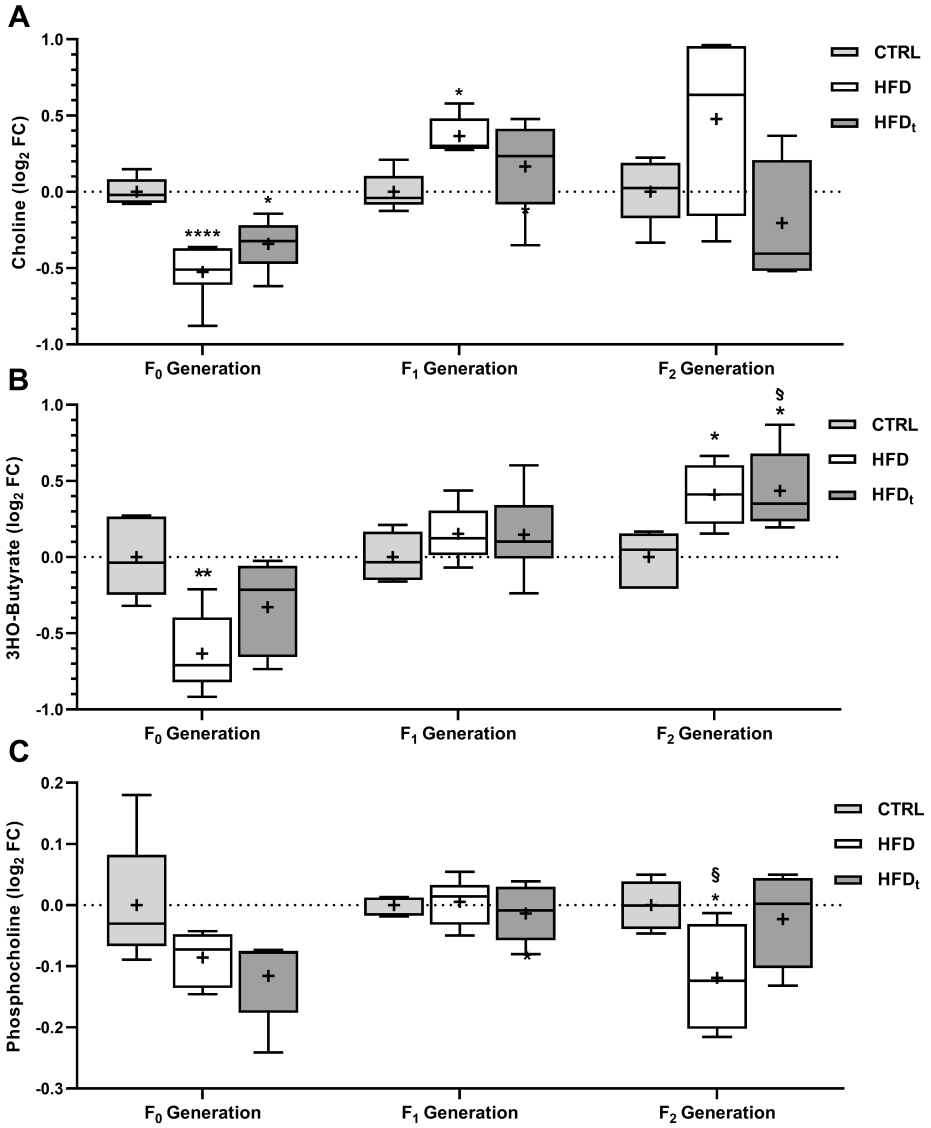


Figure 6.3: Testicular content in lipid-related metabolites quantified in mice fed standard chow (CTRL) or life-long high-fat diet (HFD) or subjected to diet correction after 60 days (HFD_t), and in their progeny (Generation F_1 and F_2). Results are expressed as Tukey's whisker boxes (median, 25th to 75th percentiles ± 1.5 IQR). Pairwise comparisons were tested using ANOVA corrected by Tukey's HSD (n per group disclosed in Supplemental Table 6.S4). 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; * vs. CTRL; # vs. HFD. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$. Abbreviations: FC – Fold Change.

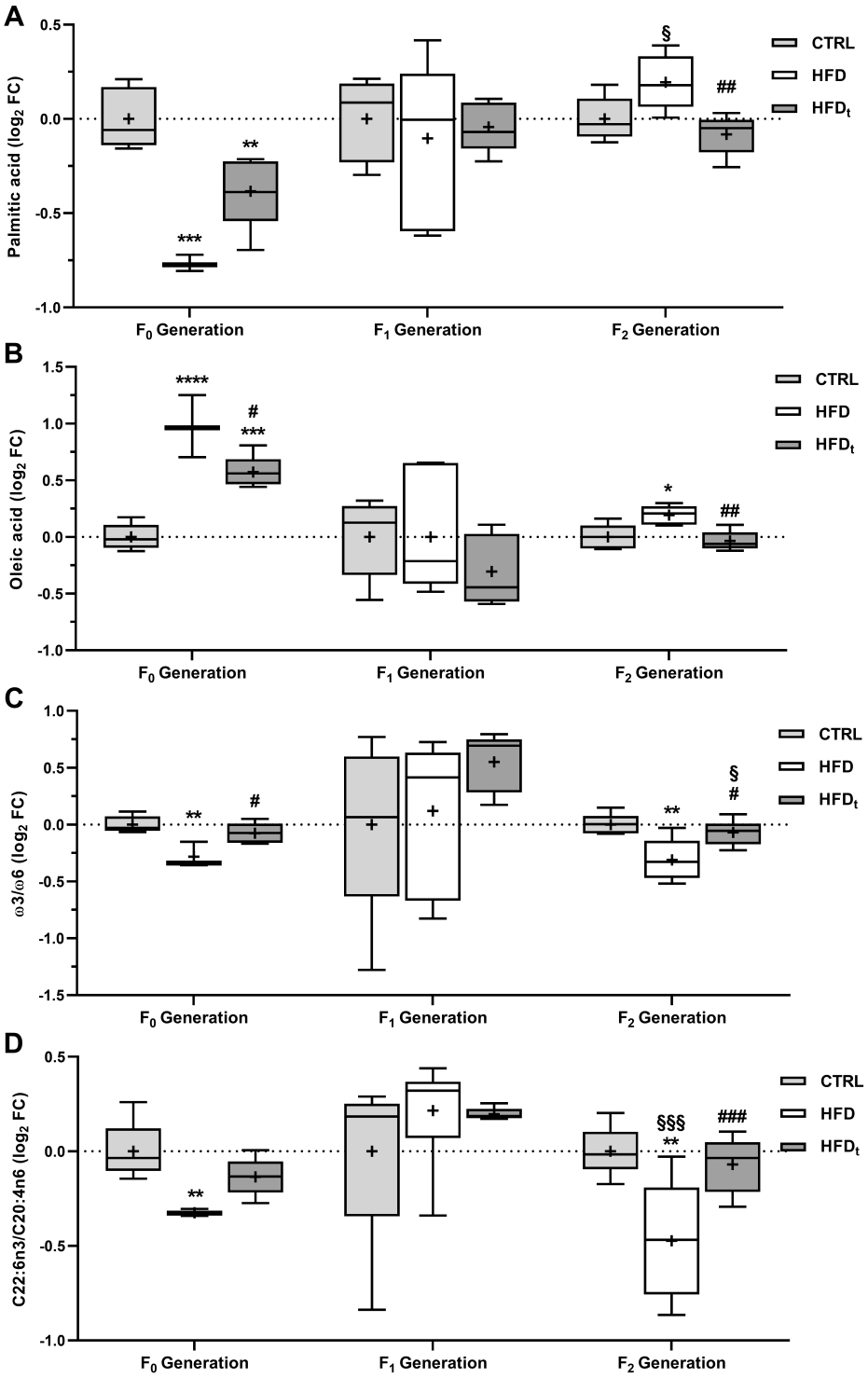


Figure 6.4: Testicular fatty acid content quantified in mice fed standard chow (CTRL) or life-long high-fat diet (HFD) or subjected to diet correction after 60 days (HFD_t), and in their progeny (Generation F_1 and F_2). Results are expressed as Tukey's whisker boxes (median, 25th to 75th percentiles ± 1.5 IQR). Pairwise comparisons were tested using ANOVA corrected by Tukey's HSD (n per group disclosed in Supplemental Table 6.S5). 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; * vs. CTRL; # vs. HFD. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$. Abbreviations: FC – Fold Change.

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.

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, Projection of Latent Structures with Discriminant Analysis (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 (supplemental Table 6.S6). 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, 9 and 5 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 (Table 6.6A). 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 (Table 6.6B). 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) (Table 6.6C). 10 variables were selected in component 1, and 7 in component 2. Although some samples are plotted over areas of another group and the overall discriminative power of the sPLS-DA is significant ($P < 0.001$).

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 Projection of Latent Structures (sPLS) method was performed before sPLS to estimate the number of meaningful variables in

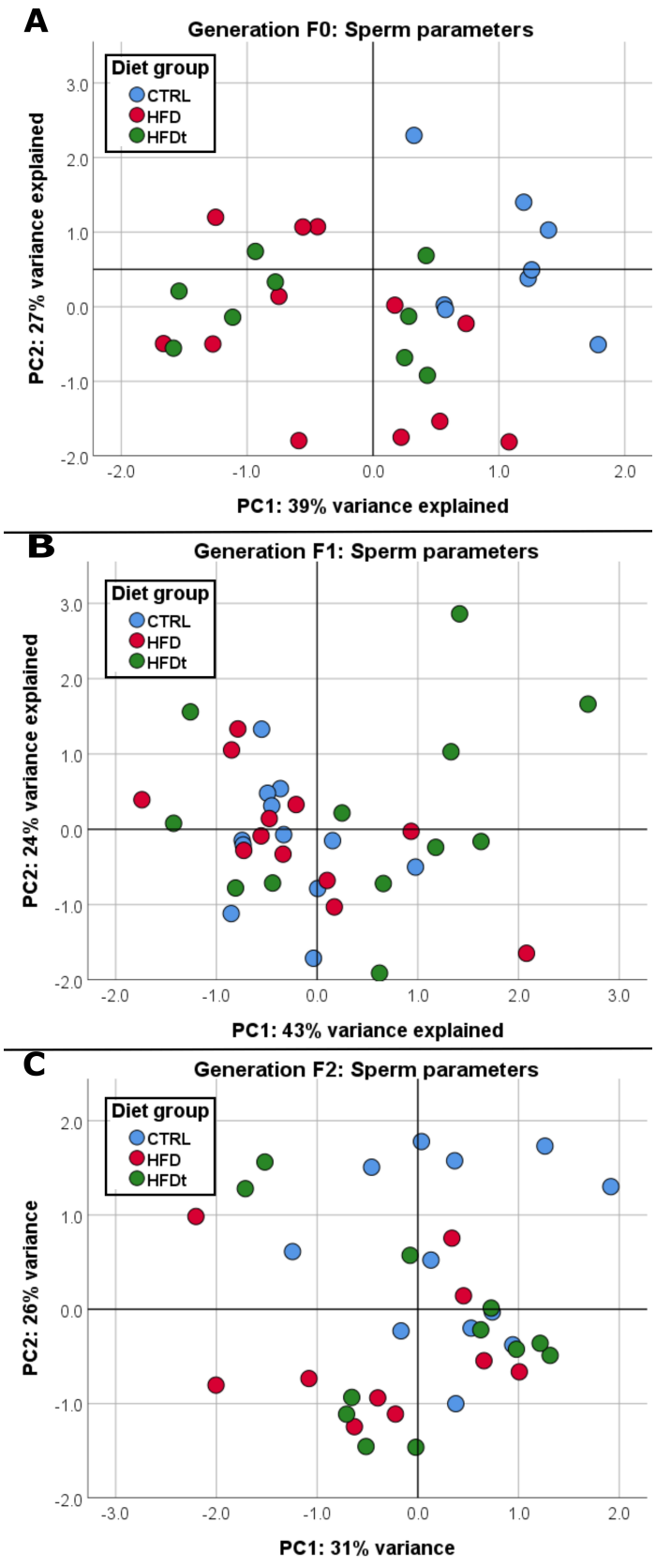


Figure 6.5: Sample distribution in the space defined by the two Principal Components extracted by PCA , based on sperm parameters. A) Generation F_0; B) Generation F_1; C) Generation F_2.

each component, for each dataset (supplemental Table 6.S7). The experimental design does not support the discrimination between fatty 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 6.6D). A molecular network was established with 3OHBut, choline, PChol, acetate and PEtAm taking a central role between SFAs, and MUFAs and PUFAs (Figure 6.6G). In generation F_1 (sons) no changes were found between groups ($P = 0.103$) (Figure 6.6E). The metabolic network (Figure 6.6H) of relevant FAs and lipid-related metabolites is divided into two nuclei, eliciting triacylglycerol and phospholipid tail composition. Choline and PChol 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 6.6F). The resulting metabolic network (Figure 6.6I) evidences that choline and PChol (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 figures 6.6G, H and I for visualization purposes.

6.4 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 (Eisenberg et al., 2016a; D. Guo et al., 2017b; Palmer, Bakos, Fullston, et al., 2012). Although the biochemical cues linking these phenomena are still debatable, evidence has brought a new layer of complexity into this equation: transgenerational inheritance (Baxter & Drake, 2019; Sharma & Rando, 2017). 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 (**Crisóstomo** et al., 2019; **Crisóstomo** et al., 2020). 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 (**Crisóstomo**, Jarak, et al., 2021; **Crisóstomo** et al., 2019). Those deleterious effects persist not only in sons (intergenerational effects) but also in grandsons (transgenerational effects) (**Crisóstomo**, Jarak, et al., 2021). 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 6.2). Moreover, we characterize the molecular fingerprints of this metabolic memory, relating the testicular content in FAs

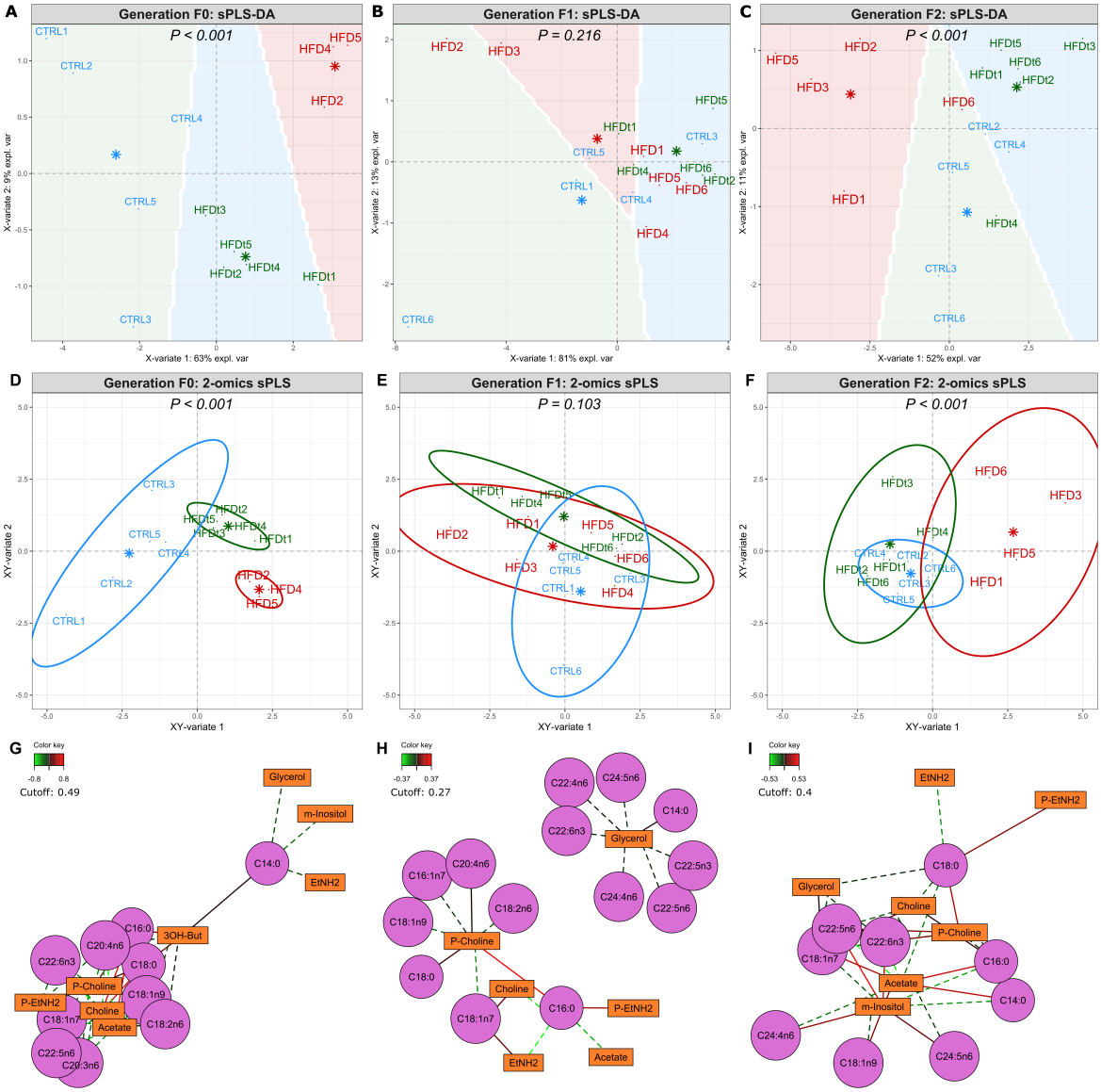


Figure 6.6: Metabolite and lipid profile of mice testis in different generations. A, B and C) Sample distribution in the space defined by the two Principal Components extracted by sPLS-DA of testicular FAs. The background color was calculated according to the Euclidean distance to the group's centroid. 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 vs. 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.

and lipid-related metabolites employing a bottom-up EDA design (Figure 6.1).

Table 6.2: Intergenerational and transgenerational effects of HFDs in the paternal lineage. The affected lineage is reported in brackets, comparing to the CTRL group of the corresponding generation.

Intergenerational effects	Transgenerational effects
⇓ serum E_2 (HFD_t)	⇑ serum FSH (HFD_t)
⇓ testicular CAT activity (HFD)	⇑ serum E_2 (HFD)
⇓ testicular CI activity (HFD)	⇑ testicular 3OHBut (HFD/HFD_t)
⇓ testicular CIV activity (HFD)	⇑ testicular acetate (HFD)
⇓ testicular choline (HFD)	⇑ testicular EtAm (HFD)
	⇓ testicular PChol (HFD)
	⇓ testicular PEtAm (HFD)
	⇑ testicular C18:1n9 (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 PUFAs (HFD)
	⇓ testicular C22:6n3/C20:4n6 PUFAs (HFD)
	⇓ testicular C22:5n6/C20:4n6 PUFAs (HFD)

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 6.6). 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 (A. R. Johnson et al., 2010). Nevertheless, we have not observed such an effect (**Crisóstomo**, Jarak, et al., 2021). 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 catalase and decreased activity of mitochondrial complexes CIs and CIVs, 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, a SFA (Figure 6.6G) but positively correlated to the pro-inflammatory ω6 PUFAs (Figure 6.6I). Inositol-containing phospholipids commonly contain AA, particularly in the C2 position of glycerol (Holub, 1986). Also, the ω6 elongation pathway from arachidonic acid is highly active in testicular cells, where ω6 FAs play signaling and structural roles (Zadravec et al., 2011). 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. AA (C20:4n6), ω6 DPA (C22:5n6) and DHA (C22:6n3) 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 (Harayama & Riezman, 2018; Zadravec et al., 2011). 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 (**Crisóstomo**, Jarak, et al., 2021), 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 (Stoffel et al., 2020; Zadravec et al., 2011).

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 $\approx 50\,\%$ higher than CTRL (**Crisóstomo** et al., 2020). The decrease in PUFAs proportion does not improve sperm morphological quality; sperm head defects are more prevalent in this group (**Crisóstomo** et al., 2020). 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, PChol, EtAm and PEtAm found in the grandsons of HFD-fed mice are correlated to depletion of C18 lipids, and to the inhibition of ω3 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 3OHBut, a ketone body that, in testis, is produced by SCs as a nutritional source for germ cells (Regueira et al., 2017). 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

(**Crisóstomo** et al., 2018). 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 (P. F. Oliveira, Sousa, et al., 2017), which in turn inhibits the secretion of inhibin B (Robeva et al., 2012), and decreases the expression of FSH and E_2 receptors (Martins et al., 2013; Pitetti et al., 2013). 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 (**Crisóstomo**, Jarak, et al., 2021). Besides, FSH is essential for SC proliferation before sexual maturation, which is a limiting factor for sperm concentration in adulthood (Oduwole et al., 2018). 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 (Levine et al., 2017). 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 (**Crisóstomo** et al., 2019; **Crisóstomo** et al., 2020) 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 sncRNA carried by spermatozoa have been implicated in the transmission of acquired traits to progeny, including in humans (Gluckman et al., 2007; King & Skinner, 2020). Besides, it has been shown that sperm sncRNA content dynamically changes according to diet (Nätt et al., 2019) and that it drives transgenerational epigenetic inheritance (King & Skinner, 2020; Nätt & Öst, 2020). 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 ω3/ω6 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 3OHBut 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.

6.5 Author contributions

Luís Crisóstomo, Pedro F. Oliveira, Marco G. Alves and Rachel L. Batterham contributed to study design, analysis and interpretation of data. Luís Rato, Ivana Jarak, Kristina Starčević, Tomislav Mašek, Romeu A. Videira and **Luís Crisóstomo** performed experimental work. **Luís Crisóstomo** edited the images and tables, performed the statistics and contributed to the analysis and interpretation of data. Rachel L. Batterham, João F. Raposo and Romeu A. Videira critically reviewed the manuscript and suggested modifications. All the authors contributed to manuscript writing/editing and approved the final version.

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6.7 Supplemental Data

Table 6.S1: Resonance assignment in the ^{1}H-NMR spectra of polar lipid-related metabolites extracted from testis (s: singlet, d: doublet, dd: doublet of doublets, t: triplet, m: multiplet).

	Compound[a]	δ^{1}H ppm (multiplicity, assigment)
1	choline	3.20 (s, CH_3), 3.51 (m, NCH_2), 4.06 (m, OCH_2)
2	ethanolamine	3.14 (m, NCH_2), 3.82 (m, CH_2)
3	glycerol	3.56 (dd, C1H_2), 3.65 (dd, C3H_2), 3.79 (m, C2H)
4	GPC	3.23 (s, CH_3), 3.68 (m, NCH_2), 3.88 (s, αCH_2), 4.33 (m, OCH_2)
5	3OHBut	1.20 (d, γCH_3, 2.24 (dd, αCH), 4.15 (m, βCH))
6	*myo*-inositol	3.26 (t, C5H), 3.52 (dd, C1H, C3H), 3.62 (m, C4H, C6H), 4.06 (t, C2H)
7	PChol	3.22 (s, CH_3), 3.91 (m, NCH_2), 4.17 (m, OCH_2)
8	PEtAm	3.21 (m, NCH_2), 3.97 (m, OCH_2)

[a]all metabolites are putatively annotated (level 2 of identification according to Chemical Analysis Working Group (CAWG) Metabolomics Standards Initiative recommendations).

Table 6.S2: Enzymatic activity of mitochondrial complexes in testes of mice fed standard chow (CTRL), life-long high-fat diet (HFD), and those subjected to diet correction after 60 days (HFD_t) and their progeny (Generations F_1 and F_2). 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 F_1 and F_2 were compared using two-way ANOVA corrected by Šidak method. Significance was considered when $p < 0.05$. * vs. CTRL; # vs. HFD. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

	Parameter	CTRL	HFD	HFD_t
Generation F_0	*Citrate Synthase*[a]	193.47 ± 50.11	175.42 ± 67.62	165.00 ± 18.78
	Complex I[b]	35.43 ± 10.59	30.65 ± 9.57	32.14 ± 6.65
	Complex II[c]	49.58 ± 8.47	40.23 ± 15.53	46.84 ± 9.47
	Complex IV[d]	84.22 ± 15.60	69.31 ± 22.38	64.98 ± 16.41
	CI/CS ratio	0.19 ± 0.05	0.18 ± 0.04	0.19 ± 0.03
	CII/CS ratio	0.26 ± 0.04	0.23 ± 0.06	0.29 ± 0.07
	CIV/CS ratio	0.46 ± 0.14	0.42 ± 0.10	0.40 ± 0.12
Generation F_1	*Citrate Synthase*[a]	235.15 ± 44.43	211.91 ± 37.94	229.15 ± 38.79
	Complex I[b]	83.43 ± 27.48	46.54 ± 19.21*	61.54 ± 20.18
	Complex II[c]	46.42 ± 5.23	38.24 ± 6.33	44.58 ± 7.54
	Complex IV[d]	49.91 ± 15.39	29.32 ± 9.03	36.95 ± 9.88
	CI/CS ratio	0.36 ± 0.11	0.23 ± 0.13	0.28 ± 0.13
	CII/CS ratio	0.20 ± 0.03	0.18 ± 0.01	0.20 ± 0.03
	CIV/CS ratio	0.21 ± 0.03	0.14 ± 0.05	0.17 ± 0.05
Generation F_2	*Citrate Synthase*[a]	222.03 ± 45.83	212.18 ± 54.78	217.87 ± 47.21
	Complex I[b]	59.74 ± 39.08	49.25 ± 17.91	47.16 ± 11.41
	Complex II[c]	38.13 ± 10.47	37.29 ± 8.92	32.49 ± 6.40
	Complex IV[d]	42.82 ± 17.04	24.99 ± 11.11	33.96 ± 13.87
	CI/CS ratio	0.29 ± 0.22	0.24 ± 0.07	0.23 ± 0.07
	CII/CS ratio	0.22 ± 0.14	0.19 ± 0.08	0.17 ± 0.07
	CIV/CS ratio	0.19 ± 0.07	0.13 ± 0.06	0.16 ± 0.08

Units: [a] $nmol_{DTNB}\,min^{-1}\,mg^{-1}_{protein}$; [b] $nmol_{NADH}\,min^{-1}\,mg^{-1}_{protein}$; [c] $nmol_{DCPIP}\,min^{-1}\,mg^{-1}_{protein}$; [d] $nmol_{CytC}\,min^{-1}\,mg^{-1}_{protein}$.

Table 6.S3: Testicular bioenergetics of mice fed standard chow (CTRL), life-long high-fat diet (HFD), and those subjected to diet correction after 60 days (HFD_t), and their offspring (Generation F_1 and F_2). 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 F_1 and F_2 were compared using two-way ANOVA corrected by Šidak method. Significance was considered when $p < 0.05$. * vs. CTRL; # vs. HFD; § vs. Generation F_1. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

	Parameter	CTRL	HFD	HFD_t
Generation F_0	*ATP* ($nmol\,mg^{-1}_{fresh\ tissue}$)	1355.05 ± 409.86	1242.37 ± 305.59	1381.14 ± 215.19
	ADP ($nmol\,mg^{-1}_{fresh\ tissue}$)	898.80 ± 339.79	510.97 ± 184.30	828.81 ± 718.97
	AMP ($nmol\,mg^{-1}_{fresh\ tissue}$)	595.85 ± 340.32	467.75 ± 294.22	478.63 ± 217.89
	Adenosine Nucleotide Pool[a]	2849.70 ± 938.94	2221.09 ± 520.29	2688.57 ± 751.63
	ATP/ADP ratio	1.60 ± 0.63	2.80 ± 1.28	2.62 ± 1.54
	AMP/ATP ratio	0.41 ± 0.18	0.36 ± 0.16	0.34 ± 0.12
	Energy charge	0.65 ± 0.05	0.68 ± 0.05	0.68 ± 0.04
Generation F_1	*ATP* ($nmol\,mg^{-1}_{fresh\ tissue}$)	334.03 ± 189.67	258.38 ± 89.58	377.88 ± 227.18
	ADP ($nmol\,mg^{-1}_{fresh\ tissue}$)	229.28 ± 140.28	156.80 ± 70.66	245.07 ± 139.48
	AMP ($nmol\,mg^{-1}_{fresh\ tissue}$)	189.25 ± 30.78	253.95 ± 25.98	278.71 ± 95.38
	Adenosine Nucleotide Pool[a]	752.56 ± 313.37	669.13 ± 156.27	901.66 ± 438.18
	ATP/ADP ratio	1.72 ± 0.58	1.51 ± 0.17	1.55 ± 0.06
	AMP/ATP ratio	0.45 ± 0.26	0.76 ± 0.30	0.54 ± 0.18
	Energy charge	0.64 ± 0.09	0.56 ± 0.07	0.61 ± 0.05
Generation F_2	*ATP* ($nmol\,mg^{-1}_{fresh\ tissue}$)	482.27 ± 121.03	424.71 ± 173.32	431.88 ± 136.53
	ADP ($nmol\,mg^{-1}_{fresh\ tissue}$)	193.13 ± 40.21	209.94 ± 74.26	235.33 ± 52.69
	AMP ($nmol\,mg^{-1}_{fresh\ tissue}$)	252.33 ± 88.39	$143.88 \pm 110.29^{\S}$	203.11 ± 84.95
	Adenosine Nucleotide Pool[a]	927.73 ± 131.59	778.52 ± 266.21	870.32 ± 182.73
	ATP/ADP ratio	$2.58 \pm 0.77^{\S\S}$	1.98 ± 0.18	1.87 ± 0.51
	AMP/ATP ratio	0.56 ± 0.27	$0.40 \pm 0.29^{\S}$	0.49 ± 0.23
	Energy charge	0.62 ± 0.09	$0.67 \pm 0.10^{\S}$	0.63 ± 0.07

Units: [a] $nmol\,mg^{-1}_{fresh\ tissue}$.

Table 6.S4: Testicular lipid-related metabolites across generations, quantified by semi-quantitative ^{1}H-NMR. Results are expressed as the mean (log2 FC) ± SD. Results were analysed by Univariate ANOVA, corrected for FDR by Benjamini-Hochberg method, and with Tukey's HSD correction for pairwise comparisons. Two-way ANOVA with Šidak correction was used to compare each diet group in Generation F_1 against its F_2 counterparts. FDR cut-off was set to $p < 0.1$. [a] Generation F_0; [b] Generation F_1; [c] Generation F_2. Family-wise significance was considered when $p < 0.05$. * vs. CTRL; # vs. HFD; § vs. F_1 Generation. * p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001. Abbreviations: FC – Fold Change (to CTRL); SD – standard deviation.

	Generation F_0			Generation F_1			Generation F_2		
Metabolite	**CTRL**	**HFD**	**HFD$_t$**	**CTRL**	**HFD**	**HFD$_t$**	**CTRL**	**HFD**	**HFD$_t$**
3OHBut[a]	0.00 ± 0.26	$-0.63 \pm 0.26^{**}$	-0.33 ± 0.31	0.00 ± 0.16	0.15 ± 0.18	0.15 ± 0.28	0.00 ± 0.17	$0.41 \pm 0.21^{*}$	$0.44 \pm 0.26^{*,\S}$
Acetate[a,c]	0.00 ± 0.13	$-0.64 \pm 0.15^{****}$	$-0.71 \pm 0.09^{****}$	0.00 ± 0.15	0.46 ± 0.31	$0.48 \pm 0.35^{*}$	0.00 ± 0.29	$1.33 \pm 0.16^{**,\S\S}$	$0.27 \pm 0.78^{\#}$
Choline[a]	0.00 ± 0.09	$-0.52 \pm 0.08^{****}$	$-0.34 \pm 0.17^{*}$	0.00 ± 0.12	$0.36 \pm 0.12^{*}$	0.17 ± 0.30	0.00 ± 0.22	0.48 ± 0.61	-0.20 ± 0.40
Ethanolamine[a]	0.00 ± 0.14	0.06 ± 0.12	$-0.17 \pm 0.12^{\#}$	0.00 ± 0.05	0.24 ± 0.28	0.33 ± 0.38	0.00 ± 0.14	$0.76 \pm 0.59^{*,\S}$	0.34 ± 0.43
Glycerol[a]	0.00 ± 0.27	0.21 ± 0.03	-0.05 ± 0.13	0.00 ± 0.14	-0.08 ± 0.16	-0.16 ± 0.06	0.00 ± 0.16	0.04 ± 0.09	-0.21 ± 0.13
***myo*-Inositol**[a]	0.00 ± 0.24	0.08 ± 0.06	$-0.19 \pm 0.10^{\#}$	0.00 ± 0.02	0.00 ± 0.04	-0.01 ± 0.07	0.00 ± 0.02	-0.05 ± 0.03	0.00 ± 0.04
Phosphocholine	0.00 ± 0.10	-0.09 ± 0.04	-0.12 ± 0.07	0.00 ± 0.02	0.01 ± 0.04	-0.01 ± 0.05	0.00 ± 0.03	$-0.08 \pm 0.06^{*,\S}$	-0.02 ± 0.05
PEtAm	0.00 ± 0.23	-0.10 ± 0.08	-0.22 ± 0.06	0.00 ± 0.05	-0.06 ± 0.09	-0.09 ± 0.12	0.00 ± 0.04	$-0.12 \pm 0.09^{*,\S}$	-0.02 ± 0.08

Table 6.S5: Fatty acid composition of the apolar fraction of testicular extracts of mice fed a standard chow (CTRL) or life-long high-fat diet (HFD) or subjected to diet correction after 60 days (HFD_t). FAs are presented as: Common name (Lipid numbers). Results are expressed as the mean (log2 FC) ± SD. Results were analysed by Univariate ANOVA, corrected for FDR by Benjamini-Hochberg method, and with Tukey's HSD correction for pairwise comparisons. Two-way ANOVA with Šidak correction was used to compare each diet group in Generation F_1 against its F_2 counterparts. FDR cut-off was set to $p < 0.1$. [a] Generation F_0; [b] Generation F_1; [c] Generation F_2. Family-wise significance was considered when $p < 0.05$. * vs. CTRL; # vs. HFD; § vs. F_1 Generation. * p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001. Abbreviations: FC – Fold Change (to CTRL); SD – standard deviation.

	Generation F_0			**Generation F_1**			**Generation F_2**		
Metabolite	**CTRL**	**HFD**	**HFD_t**	**CTRL**	**HFD**	**HFD_t**	**CTRL**	**HFD**	**HFD_t**
Myristic acid (C14:0)[a,c]	0.00 ± 0.40	-0.62 ± 0.32*	$0.01 \pm 0.13^{\#}$	0.00 ± 0.73	-0.29 ± 0.55	-0.73 ± 0.27	0.00 ± 0.40	$0.33 \pm 0.28^{\#}$	$-0.27 \pm 0.17^{\#}$
Palmitic acid (C16:0)[a,c]	0.00 ± 0.16	-0.77 ± 0.04***	-0.38 ± 0.19**	0.00 ± 0.22	-0.10 ± 0.42	-0.04 ± 0.13	0.00 ± 0.12	$0.19 \pm 0.15^{\S}$	$-0.08 \pm 0.10^{\#\#}$
Stearic acid (C18:0)[a]	0.00 ± 0.21	-1.03 ± 0.04***	$-0.49 \pm 0.32^{\#\#}$	0.00 ± 0.46	-0.05 ± 0.73	0.44 ± 0.24	0.00 ± 0.12	-0.07 ± 0.04	0.02 ± 0.05
Palmitoleic acid (C16:1n-7)	0.00 ± 0.58	-0.35 ± 0.06	-0.50 ± 1.12	0.00 ± 1.26	-0.41 ± 1.48	-1.31 ± 1.17	0.00 ± 0.32	-0.30 ± 0.42	-0.54 ± 0.59
Vaccenic acid (C18:1n-7)[a,c]	0.00 ± 0.40	0.46 ± 0.22	0.58 ± 0.27*	0.00 ± 0.04	0.10 ± 0.20	0.00 ± 0.06	0.00 ± 0.08	0.06 ± 0.07	$-0.10 \pm 0.04^{\#\#}$
Oleic acid (C18:1n-9)[a,c]	0.00 ± 0.11	0.97 ± 0.27****	0.57 ± 0.14***,#	0.00 ± 0.35	0.00 ± 0.52	-0.31 ± 0.31	0.00 ± 0.11	0.19 ± 0.08*	$-0.04 \pm 0.08^{\#\#}$
Linoleic acid (C18:2n-6)[a]	0.00 ± 0.20	0.88 ± 0.14**	0.44 ± 0.29*	0.00 ± 0.98	-0.27 ± 1.16	-0.85 ± 0.93	0.00 ± 0.16	0.08 ± 0.39	0.00 ± 0.34
Dihomo-γ-linolenic acid (C20:3n-6)[a]	0.00 ± 0.63	0.90 ± 0.24	0.80 ± 0.35	0.00 ± 0.60	-0.03 ± 0.68	0.42 ± 0.26	0.00 ± 0.16	0.03 ± 0.10	0.11 ± 0.17
Arachidonic acid (C20:4n-6)[a,c]	0.00 ± 0.37	0.68 ± 0.20*	0.43 ± 0.09	0.00 ± 0.55	-0.06 ± 0.85	0.52 ± 0.26	0.00 ± 0.12	-0.04 ± 0.04	$0.11 \pm 0.07^{\#}$

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Table 6.S5 Continued from previous page

	Generation F_0			Generation F_1			Generation F_2		
Metabolite	**CTRL**	**HFD**	**HFD$_t$**	**CTRL**	**HFD**	**HFD$_t$**	**CTRL**	**HFD**	**HFD$_t$**
Adrenic acid (C22:4n-6)	N/A	N/A	N/A	0.00 ± 0.80	0.01 ± 0.75	0.64 ± 0.32	0.00 ± 0.12	-0.19 ± 0.21	0.05 ± 0.16
ω-6 Docosapentaenoic acid (C22:5n-6)[c]	0.00 ± 0.45	0.46 ± 0.16	0.27 ± 0.11	0.00 ± 0.96	0.18 ± 0.86	0.79 ± 0.27	0.00 ± 0.10	$-0.45 \pm 0.27^{**}$	$0.10 \pm 0.09^{\#\#\#,\S}$
ω-3 Docosapentaenoic acid (C22:5n-3)	N/A	N/A	N/A	0.00 ± 0.95	-0.02 ± 0.41	0.71 ± 0.35	0.00 ± 0.37	-0.27 ± 0.28	$-0.18 \pm 0.28^{\S\S}$
Docosahexaenoic acid (C22:6n-3)[a,c]	0.00 ± 0.25	0.36 ± 0.21	0.30 ± 0.18	0.00 ± 0.97	0.16 ± 0.74	0.71 ± 0.27	0.00 ± 0.06	$-0.51 \pm 0.33^{**,\S}$	$0.04 \pm 0.16^{\#\#,\S}$
Tetracosatetraenoic acid (C24:4n-6)[c]	N/A	N/A	N/A	0.00 ± 1.42	0.35 ± 0.72	1.03 ± 0.28	0.00 ± 0.17	$-0.55 \pm 0.44^{*,\S}$	$0.28 \pm 0.13^{\#\#\#}$
Tetracosapentaenoic acid (C24:5n-6)[c]	N/A	N/A	N/A	0.00 ± 0.97	0.25 ± 0.76	0.82 ± 0.26	0.00 ± 0.18	$-0.47 \pm 0.31^{*,\S}$	$0.16 \pm 0.14^{**}$
SFA[a,c]	0.00 ± 0.17	$-0.88 \pm 0.02^{***}$	$-0.42 \pm 0.23^{**,\#}$	0.00 ± 0.20	-0.10 ± 0.45	0.04 ± 0.15	0.00 ± 0.08	0.14 ± 0.12	$-0.06 \pm 0.09^{\#}$
MUFA[a,c]	0.00 ± 0.09	$0.80 \pm 0.23^{***}$	$0.49 \pm 0.21^{*}$	0.00 ± 0.41	-0.04 ± 0.59	-0.38 ± 0.34	0.00 ± 0.10	0.14 ± 0.08	$-0.07 \pm 0.09^{\#\#}$
PUFA[a,c]	0.00 ± 0.27	$0.59 \pm 0.12^{**}$	$0.36 \pm 0.11^{*}$	0.00 ± 0.29	0.05 ± 0.17	0.24 ± 0.06	0.00 ± 0.07	$-0.24 \pm 0.16^{*,\S\S}$	$0.09 \pm 0.08^{\#\#\#}$
ω3-PUFA[a,c]	0.00 ± 0.25	0.36 ± 0.21	0.30 ± 0.18	0.00 ± 0.96	0.15 ± 0.72	0.71 ± 0.27	0.00 ± 0.06	$-0.50 \pm 0.32^{**}$	$0.03 \pm 0.16^{\#\#,\S}$
ω3-PUFA[a,c]	0.00 ± 0.28	$0.64 \pm 0.10^{**}$	$0.37 \pm 0.10^{*}$	0.00 ± 0.21	0.03 ± 0.10	0.16 ± 0.03	0.00 ± 0.09	$-0.19 \pm 0.14^{*,\S\S}$	$0.10 \pm 0.07^{\#\#\#}$
ω3/ω6[a,c]	0.00 ± 0.07	$-0.28 \pm 0.11^{**}$	$-0.08 \pm 0.09^{\#}$	0.00 ± 0.78	0.12 ± 0.67	0.55 ± 0.26	0.00 ± 0.09	$-0.31 \pm 0.18^{**}$	$-0.07 \pm 0.11^{\#,\S}$

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Table 6.S5 Continued from previous page

	Generation F_0			Generation F_1			Generation F_2		
Metabolite	**CTRL**	**HFD**	**HFD$_t$**	**CTRL**	**HFD**	**HFD$_t$**	**CTRL**	**HFD**	**HFD$_t$**
C22:6n-3/C20:4n-6[a,c]	0.00 ± 0.15	-0.32 ± 0.02**	-0.13 ± 0.10	0.00 ± 0.47	0.22 ± 0.28	0.20 ± 0.03	0.00 ± 0.13	-0.47 ± 0.31**,§§§	-0.07 ± 0.15###
C20:4n-6/C18:2n-6	0.00 ± 0.54	-0.20 ± 0.32	-0.01 ± 0.27	0.00 ± 1.45	0.21 ± 1.98	1.37 ± 1.19	0.00 ± 0.19	-0.11 ± 0.39	0.11 ± 0.38
C22:5n-6/C20:4n-6[c]	0.00 ± 0.21	-0.23 ± 0.04	-0.16 ± 0.02	0.00 ± 0.47	0.23 ± 0.17	0.28 ± 0.12	0.00 ± 0.12	-0.42 ± 0.25**,§§§§	-0.01 ± 0.07##

Table 6.S6: Variable loadings calculated by Discriminant Analysis of Projection of Latent Structures (PLS-DA) of testicular FAs. This method was used to estimate the number of meaningful variables for each component to be accounted by the sparse version method (sPLS-DA). The variable cut-off was set to: component load $\geq |0.2|$. Abbreviations: Comp – Components; # variables – Number of selected variables for the sparse version of the method. *Component load above the cut-off value.

Method: PLS-DA (Lipidomics)						
	Generation F_0		**Generation F_1**		**Generation F_2**	
Variable	**Comp. 1**	**Comp. 2**	**Comp. 1**	**Comp. 2**	**Comp. 1**	**Comp. 2**
C14:0	−0.201	−0.662	−0.318	−0.302	−0.289	−0.128*
C16:0	−0.376	−0.148*	0.004*	−0.447	−0.306	0.020*
C18:0	−0.364	−0.185*	0.264	−0.179*	0.187*	−0.099*
C16:1n7	−0.109*	−0.234	−0.261	−0.140*	−0.065*	−0.576
C18:1n7	0.258	−0.525	−0.109*	0.695	−0.310	−0.283
C18:1n9	0.395	0.036*	−0.233	0.121*	−0.316	0.189*
C18:2n6	0.357	0.174*	−0.221	−0.090*	−0.049*	0.050*
C20:3n6	0.294	−0.288	0.244	−0.173*	0.079*	0.376
C20:4n6	0.329	0.053*	0.266	−0.180*	0.245	0.370
C22:4n6	n.d.	n.d.	0.288	−0.095*	0.239	−0.049*
C22:5n3	n.d.	n.d.	0.336	−0.065*	0.068*	−0.387
C22:5n6	0.239	0.023*	0.282	0.070*	0.351	−0.161*
C22:6n3	0.268	−0.239	0.272	0.058*	0.320	−0.247
C24:4n6	n.d.	n.d.	0.285	0.208	0.343	0.013*
C24:5n6	n.d.	n.d.	0.291	0.157*	0.337	−0.079*
# variables	10	5	13	4	10	6

Table 6.S7: Variable loadings calculated by Projection of Latent Structures (sPLS) of testicular FAs and lipid-related metabolites. This method was used to estimate the number of meaningful variables for each variable block and component to be accounted by the sparse version method (sPLS). The variable cut-off was set to: component load $\geq |0.2|$. Abbreviations: Block 1 – Variable block 1 (testicular lipids); Block 2 – Variable block 2 (testicular lipid precursors); Comp – Components; # variables – Number of selected variables for the sparse version of the method. Abbreviations: Comp – Components; GPC – Glycerophosphocholine; P-Chol – Phosphocholine; EtNH2 – Ethanolamine; P-EtNH2 – Phosphoethanolamine; m-Inositol – myo-Inositol; 3HO-But – 3-hydroxy-Butyrate; n.d. – not detected (by GC-MS); # variables – Number of selected variables for the sparse version of the method. *Component load above the cut-off value.

Method: Two-omics sPLS (fatty acids + lipid-related metabolites)						
	Generation F_0		**Generation F_1**		**Generation F_2**	
Variables – Block 1	**Comp. 1**	**Comp. 2**	**Comp. 1**	**Comp. 2**	**Comp. 1**	**Comp. 2**
C14:0	−0.077	0.639	−0.157*	−0.375	0.287	−0.149*
C16:0	−0.345	0.191	0.422	−0.386	0.271	−0.190*
C18:0	−0.317	0.269	0.242	0.070*	−0.378	−0.525
C16:1n7	−0.112	−0.154	−0.395	−0.034*	−0.049*	−0.324
C18:1n7	0.364	0.368	−0.352	0.324	0.256	−0.098*
C18:1n9	0.335	−0.234	−0.363	0.011*	0.407	0.244
C18:2n6	0.279	−0.370	−0.397	0.017*	0.003*	−0.019*
C20:3n6	0.338	0.270	0.240	0.077*	0.058*	0.521
C20:4n6	0.379	0.158	0.217	0.115*	−0.264	0.080*
C22:4n6	n.d.	n.d.	0.176*	0.207	−0.153*	0.326
C22:5n3	n.d.	n.d.	0.093*	0.318	0.021*	0.126*
C22:5n6	0.285	0.138	0.093*	0.292	−0.354	0.052*
C22:6n3	0.318	0.125	0.103*	0.290	−0.313	0.098*
C24:4n6	n.d.	n.d.	0.029*	0.384	−0.268	0.226
C24:5n6	n.d.	n.d.	0.060*	0.346	−0.275	0.168*
# variables	10	5	13	4	10	6
Variables – Block 2	**Comp. 1**	**Comp. 2**	**Comp. 1**	**Comp. 2**	**Comp. 1**	**Comp. 2**
Glycerol	0.023*	−0.544	−0.429	−0.227	0.368	0.314
Choline	−0.502	0.021*	−0.405	0.409	0.372	0.355
Acetate	−0.533	−0.114*	−0.233	0.489	0.452	0.318
PChol	−0.412	−0.223	0.604	−0.234	−0.385	−0.406
EtAm	0.000*	−0.332	−0.291	0.500	0.269	0.464
PEtAm	−0.366	−0.414	0.348	−0.355	−0.305	−0.427
***myo*-Inositol**	−0.132*	−0.536	−0.090*	−0.274	−0.435	−0.244
3OHBut	−0.377	0.268	−0.139*	0.189*	0.137*	0.228
# variables	5	6	6	7	7	8

Chapter 7

Testicular "inherited metabolic memory" of ancestral high-fat diet is associated with sperm sncRNA content

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Crisóstomo, **L.**, Bourgery, M., Rato, L., Raposo, J. F., Batterham, R. L., Kotaja, N., & Alves, M. G. (2022). Testicular *"inherited metabolic memory"* of ancestral high-fat diet is associated with sperm sncrna content. *Biomedicines*, *10*(4), 909. https://doi.org/10.3390/biomedicines10040909

Abstract

Excessive adiposity caused by high-fat diets (HFD) is associated with testicular metabolic and functional abnormalities up to grand-offspring, but the mechanisms of this epigenetic inheritance are unclear. Here we describe an association of sperm sncRNA with testicular *"inherited metabolic memory"* of ancestral HFD, using a transgenerational rodent model. Male founders were fed a standard chow for 200 days (CTRL), HFD for 200 days, (HFD) or HFD for 60 days followed by standard chow for 140 days (HFD_t). The male offspring and grand-offspring were fed by standard chow for 200 days. The sncRNA sequencing from epididymal spermatozoa revealed signatures associated with testicular metabolic plasticity in HFD-exposed mice and in the unexposed progeny. Sperm tsRNA and repRNA content were specially affected by HFD_t and in the offspring of HFD and HFD_t mice. The grand-offspring of HFD and HFD_t mice showed lower sperm counts than CTRL descendants, whereas sperm miRNA content was affected. Although the causality between sperm sncRNAs content and transgenerational epigenetic inheritance of HFD-related traits remains elusive, our results suggest that sperm sncRNA content is influenced by ancestral exposure to HFD, contributing to the sperm epigenome up to the grand-offspring.

7.1 Introduction

The adoption of HFDs associated with increased adiposity, often from early-life, is considered one of the major contributors for the present "fat pandemic". Concurrently, the prevalence of non-communicable diseases, such as T2D has increased proportionally. The most recent figures by the WHO and by the International Diabetes Federation (IDF) estimate that more than 500 million people worldwide have obesity, and 463 million suffer from T2D (IDF, 2019; WHO, 2014). These reports also highlight the earlier age of onset of obesity-related diseases, raising concerns about its consequences to adults in active and reproductive age. In addition, reports suggest an influence between unhealthy diets, as promoters of overweight and obesity, and health outcomes in progeny, particularly the onset of metabolic disease (Kaati et al., 2002; Lumey et al., 2007). Therefore, there is a rising concern regarding the long-term consequences of excess adiposity associated with the adoption of HFD to prospective parents and their offspring.

The role of sperm sncRNA in epigenetic inheritance has been suggested by several authors (Baxter & Drake, 2019; Perez & Lehner, 2019). Recent findings show that tsRNA, the most abundant sncRNA biotype in sperm, can induce an acquired pathology in non-exposed mice (Q. Chen et al., 2016), without the influence of any other epigenetic mechanism. Sperm miRNA and tsRNA content has also shown to be crucial for mammalian embryo development

(Conine et al., 2018). Besides these functions directly related to epigenetic inheritance, other tsRNA biotypes are crucial for spermatogenesis. For instance, piRNAs expressed by male germ cells during spermatogenesis and have a critical role in silencing transposable elements (Goh et al., 2015; X. Z. Li et al., 2013).

Obesity, T2D and diet alter sperm sncRNA content (Donkin et al., 2016; Nätt et al., 2019; Swanson et al., 2020), and those changes may persist in the offspring for several generations (Fullston et al., 2013; Pavlinková et al., 2017). Our group previously showed detrimental effects of HFD exposure to mice testicular metabolism and function, after feeding with HFD for 200 days from weaning or HFD for 60 days from weaning and then fed with standard chow for 140 days, comparing to littermates exclusively fed with standard chow (**Crisóstomo** et al., 2019; **Crisóstomo** et al., 2020). These changes included higher proportion of immotile, non-viable and morphologically abnormal spermatozoa; alterations in the testicular content of several metabolites and fatty acids; and differences in the relative testicular proportions of SFAs, MUFAs, ω3 PUFAs and ω6 PUFAs (**Crisóstomo** et al., 2019; **Crisóstomo** et al., 2020). We have further observed the inheritance of these changes in testis up to two generations, a phenomenon that we coined as "inherited metabolic memory" (**Crisóstomo**, Jarak, et al., 2021; **Crisóstomo**, Videira, et al., 2022). Particularly, we observed changes in the testicular content of metabolites involved with the energy-obtaining pathways and insulin resistance, and in the proportion of ω3 PUFAs and ω6 PUFAs in the grand-offspring of mice exposed to HFD, compared to the grand-offspring of mice fed with standard chow (**Crisóstomo**, Jarak, et al., 2021; **Crisóstomo**, Videira, et al., 2022). However, the mechanisms underlying this heritable metabolic fingerprint of ancestral exposure to HFD were unknown. In light of their response to increased adiposity due to diet and their potential to mediate embryo development, sperm sncRNAs are a potential vehicle of the "inherited metabolic memory" from fathers to sons and grandsons.

In this study we describe sperm sncRNA response to lifelong exposure to HFD and exposure to HFD until early adulthood. We further investigate if ancestral exposure to HFD is reflected in sperm sncRNA profile in the offspring. This work was conducted using the same animal model as our recent studies (**Crisóstomo**, Jarak, et al., 2021; **Crisóstomo** et al., 2019; **Crisóstomo** et al., 2020; 2022), expanding our previous findings and providing the mechanistic evidence of *"inherited metabolic memory"* in the testis of mice.

7.2 Material and Methods

Animal Model

We designed a transgenerational ancestral exposure model based on three generations of C57BL6/J mice (**Crisóstomo**, Jarak, et al., 2021; **Crisóstomo**, Videira, et al., 2022). Normoponderal male and female mice, fed with a standard chow (#F4031, BioServ, Flemingtown, NJ, USA – Carbohydrate: 61.6 %, Protein: 20.5 %, Fat: 7.2 % - 16.3 $\%_{Kcal}$) and water *ad libitum*, were used to produce the exposed founders (Generation F_0). 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). Each group was assigned to a diet regimen: CTRL – standard chow (#F4031, BioServ, Flemingtown, NJ, USA) during 200 days; HFD – fat-enriched diet (#F3282, BioServ, Flemingtown, NJ, USA – Carbohydrate: 35.7 %, Protein: 20.5 %, Fat: 36.0 % - 59.0 $\%_{Kcal}$) during 200 days; HFD_t – fat-enriched diet (#F3282, BioServ, Flemingtown, NJ, USA) during 60 days, then switched to standard chow (#F4031, BioServ, Flemingtown, NJ, USA) during 140 days. When animals reached 120 days post-weaning age F_0 mice were mated with normoponderal, chow-fed, same-age, randomly selected females to generate the unexposed offspring (Generation F_1). Mating was performed in mating pairs without access to water and food for 6 hours a day. The process was repeated over 8 days. After weaning, the F_1 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); HFDt_F1 – Offspring of HFD_t (n=12), but all mice were fed with standard chow (#F4031, BioServ, Flemingtown, NJ, USA) during 200 days. F_1 mice were mated in the same scheme as their progenitors to generate the unexposed grand-offspring (Generation F_2). F_2 mice were assigned to experimental groups in the same way as their fathers (12 animals per group), and fed with standard chow for 200 days after weaning. Food and water were supplied to all generations 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 is compliant with the ARRIVE guidelines, was internally reviewed by the ORBEA and approved and licensed by the DGAV with the registration number 0421/000/000/2016. All animal experiments were conducted in the Animal Facility of the Research Center in Health Sciences (CICS)) of the University of Beira Interior (Covilhã, Portugal) according to the "Guide for the Care and Use of Laboratory Animals" published by The US National Institutes of Health (NIH Publication No. 85–23, revised 1996) and European rules for the care and handling of laboratory animals (Directive 86/609/EEC). None of the interventions performed on the animals required the

use of anesthetics.

Sperm collection and RNA extraction

Epididymides were isolated and placed in pre-warmed (37 °C) HBSS pH 7.4. Then small incisions were made using a scalpel blade and the suspension was incubated for 5 min (37 °C) to allow spermatozoa to swim out of the epididymis. The purity of the sperm solution was accessed in sperm swabs stained by eosin-nigrosin and observed by an optical microscope (Supplementary Figure 7.S1). The solution was then snap-frozen in liquid nitrogen and stored at −80 °C. This solution was later thawed, and total RNA was extracted based on the acid guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski & Sacchi, 1987). Before adding 1 mL of the ready-to-use reagent for total RNA isolation (EXTRAzol, Blirt S.A., Gdańsk, Poland), the sperm solution was centrifuged at 200 *g* for 5 min at room temperature, and the supernatant was discarded, according to the manufacturer's instructions (protocol for cells grown in suspension). RNA pellets were resuspended in 20 µL of DEPC-treated water and stored at −80 °C until library preparation.

cDNA library preparation

Before library preparation, RNA concentration and integrity were screened using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The RNA integrity number (RIN) was globally low (ranging 2–5), a normal value for RNA obtained from mature spermatozoa, which lacks 18S and 28S rRNA peaks (Goodrich et al., 2013). RNA concentration was considered acceptable between 1 ng and 2 µg. Library preparation was done with NEXTFLEX® Small RNA-Seq Kit v3 for Illumina (Bioo Scientific Corporation, Austin, TX, USA) according to the manufacturer instructions. Pooled libraries were sequenced using NGS, in single-end mode, on NextSeq 500 with NextSeq 500/550 High Output Kit version 2, 75 cycles (Illumina, San Diego, CA, USA). All pooled libraries passed Illumina's default quality control.

Processing and annotation of sequenced reads

Cutadapt version 2.10 (Martin, 2011) was used to trim the 3' adapter (TGGAATTCTCGGGTGCCAAGG) and four nucleotides upstream and downstream the adapter from sequenced reads. The adaptor is a nucleotide sequence added during library preparation to allow the amplification of sncRNAs. This sequence will also be sequenced as part of the fragments, therefore contaminating them, and hindering the correct annotation of the RNA

sequences. Only trimmed reads, containing adaptor sequence, and with 80 % of the nucleotides having Illumina quality scores (Q-scores) >20 were retained. Trimmed reads were mapped to small RNAs using SPORTS 1.1 (https://github.com/junchaoshi/sports1.1; accessed 1st June 2020), a perl-based analytical workflow tweaked for the annotation of sncRNAs (Shi et al., 2018). SPORTS does not presently annotate piRNAs, but it can identify sequences as piRNA. Representative sperm RNA diagnosis statistics obtained by SPORTS 1.1 are represented in Supplemental Figure 7.S2. We used the default settings and custom databases for the mouse genome: full genome from GRCm38/mm10 UCSC (https://hgdownload.soe.ucsc.edu/downloads.html#mouse; accessed 1st June 2020), miRNA from miRbase 22 (http://www.mirbase.org/; accessed 1st June 2020), rRNA-derived small RNA (rsRNA) from NCBI/Nucleotide (available at SPORTS repository), tsRNA from GtRNAdb (http://gtrnadb.ucsc.edu/genomes/eukaryota/Mmusc10/; accessed June 2020) and piRNA from pirBase 2.0 (http://www.regulatoryrna.org/database/piRNA/download.html; accessed 1st June 2020). Fragment counts for specific sequences and for sncRNA biotypes (miRNA, rsRNA, tsRNA, piRNA), per sample, were based on the default annotations in SPORTS result output files.

Annotation of piRNA clusters and repeats

SPORTS 1.1 is unable to annotate piRNAs and repRNAs, transcripts from transposable elements like Long Interspersed Nuclear Elements (LINEs), Short Interspersed Nuclear Elements (SINEs) among others. Therefore, an alternative annotation using HISAT2 (Kim et al., 2015), SAMtools (H. Li et al., 2009) and featureCounts (Liao et al., 2013) was applied. The annotation of individual piRNA sequences is not consensual due to their ambiguity and multiplicity of targets, thus a reliable alternative is the annotation of sequences according to the pachytene piRNA cluster from which they are transcribed (Betel et al., 2007; Rosenkranz, 2015). Trimmed samples were annotated against the total mouse genome from GRCm38/mm10 UCSC database using HISAT2. The output files containing the fragment reads were then indexed using SAMtools. Eventually, index files were used to map piRNAs to their precursor cluster in the mouse piRNA cluster database (Rosenkranz, 2015) (https://www.smallrnagroup.uni-mainz.de/piCdb/; accessed 3rd June 2020), and interspersed repeats to their genomic region in to Dfam 3.1 database (Hubley et al., 2015) (https://www.dfam.org/home; accessed 3rd June 2020), using featureCounts to aggregate the final sequence counts.

Term enrichment analysis

Differently expressed sncRNAs were selected for term enrichment analysis based on GO terms (Ashburner et al., 2000; The Gene Ontology Consortium [Consortium], 2020). First, miRNA and tsRNA targets were estimated using sRNAtools (https://bioinformatics.caf.ac.cn/sRNAtools; accessed 15th August 2020) (Q. Liu et al., 2021), based on the union of the potential gene targets calculated by miRanda (Enright et al., 2003) and RNAhybrid (Rehmsmeier et al., 2004) interaction algorithms. miRanda parameters were set to Minimal free energy cutoff = -20 and miRanda score cutoff = 160, whereas RNAhybrid parameters were set to energy cutoff = -20 and P-value cutoff = 0.01. Differently expressed piRNA clusters were added to the list of potential miRNA and tsRNA targets. This list was uploaded to R 4.1.0 (R Core Team, 2021a) and annotated to GO terms using the packages topGO 2.42.0 (Alexa & Rahnenfuhrer, 2021) and GO.db 3.8.2 [Mus Musculus] (Carlson, 2021). Target genes were coalesced, and unique targets were filtered out. GO terms were annotated as for Biological Process, Molecular Function and Cellular Component. Terms with less than 10 gene annotations were excluded. Term enrichment analysis was performed using the "weight01" algorithm included in topGO.

Statistics

Raw sequence counts obtained by SPORTS and featureCounts were imported as dataframes into R 4.1.0 (R Core Team, 2021a). A nonconservative coverage filter was applied to discarded fragments with <17 nucleotides, and <0.01 Reads per Million (RPM) per group. All statistics were performed using DESeq2 package v1.28.1 (Love et al., 2014). Samples were normalized by the package's code, based on a Generalized Linear Model (GLM) and Bayesian shrinkage (Love et al., 2014). The differential expression analysis is based on the Negative Binomial distribution, using Wald statistics. Multiple hypotheses are corrected for the FDR based on the Benjamini-Hochberg method. The proportion of sncRNA families was considered changed if adjusted p <0.1. A fragment was considered differently expressed whenever log2 FC > |1.5| and adjusted p <0.1. GO term enrichment analysis was tested by Fisher Exact test. GO term annotations were sorted according to their "weight01" score. GO terms were considered significant when p <0.1, regardless of the statistical test. Whenever more than 10 GO terms met the criteria, only the top 10 terms were considered.

7.3 Results

Dietary changes affect sperm sncRNA content

To understand how sperm sncRNA profile respond to chronic (200 days) exposure to high-fat initiated at weaning, we collected mature spermatozoa from chow-fed (CTRL) and HFD-fed mice (HFD) for small RNA sequencing. We also analyzed sperm from mice on transient HFD (HFD_t, 60 days of HFD started at weaning followed by 140 days of chow before sacrifice) to study if the exposure to HFD and adiposity cause irreversible changes in sperm sncRNA content. To get a general view on the mapping of sperm RNA reads into different RNA biotypes, annotated RNA sequences were categorized as miRNA, tsRNA, mitochondrial tRNA-derived small RNA (mtRNA), piRNA, rsRNA, repRNA and Y RNA, and their total counts were normalized using DESeq2 based on total genome-matched sequences. We have only compared animals which have lived during the same period, to limit the influence of uncontrollable variables that may affect sperm sncRNA content, such as seasonal and environmental variation. This analytical strategy has been commonly adopted in transgenerational inheritance models (Carone et al., 2010; Gapp et al., 2014).

HFD or HFD_t did not cause major changes in the relative fraction of the different RNA biotypes in sperm in any generations (F_0-F_2). Only rsRNA reads were slightly increased in F_0 sperm after transient HFD_t compared to CTRL (1.16 $\log_2$ FC, p = 0.0013) and compared to HFD (0.76 $\log_2$ FC, p = 0.0945) (Figure 7.1A). Contrary to their F_0 progenitors, the F_1 offspring of HFD_t mice displayed reduced levels of rsRNA reads in sperm than the offspring of CTRL mice (Figure 7.1A,B) (−1.18 $\log_2$ FC, p = 0.0128). The size distribution of small RNA reads was as expected (Supplementary Figure 7.S2) and was not affected by the different diets.

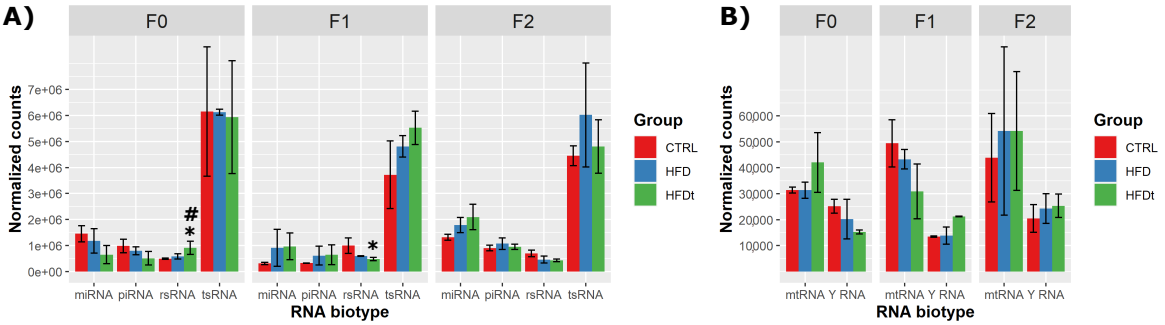


Figure 7.1: Distribution of sncRNA reads between different RNA biotypes across groups and generations. Results are expressed as the mean of normalized counts ± standard deviation. Generation F_0 and F_2: n = 3 per group; Generation F_1: n = 2 per group. A) Most representative sperm sncRNA biotypes; B) mtRNA and Y RNA. Data was tested by Wald's test, corrected by the Benjamini-Hochberg method, using DESeq2. Significance was considered when p <0.1. * vs. CTRL; # vs. HFD.

Next, we performed differential expression (DE) analysis between groups to identify any

changes in the levels of individual RNAs. We analyzed separated miRNAs and two distinct groups of tsRNAs: transcription initiation RNAs (tiRNAs), also known as rRNA halves, and tRNA-derived fragments (tRFs). tiRNAs are 29–50 nucleotide sequences resulting from the cleavage of mature rRNAs at the anticodon loop by angiogenin, thus producing a 3'-rRNA half and a 5'-rRNA half. tiRNA is involved in the post-transcriptional gene expression regulation via base complementarity to transcription start sites, to RNAse II recognition patterns, and to the ribosome (Gonskikh et al., 2020; Ivanov et al., 2011; Taft et al., 2009). Notably, tiRNA expression is triggered in response to oxidative stress to inhibit protein translation and assemble stress granules (Gonskikh et al., 2020; Ivanov et al., 2011). tRFs are shorter (16–28) nucleotide sequences resulting from the cleavage of mature rRNA or pre-rRNA by angiogenin and DICER. These sequences are often classified according to the mature rRNA loop they derive from. tRFs are associated with the silencing of transposable elements (Park et al., 2020; Schorn et al., 2017) but their expression is not restricted to male germline cells. We also mapped sncRNA reads to the 214 genomic clusters known to produce piRNAs during spermatogenesis (Betel et al., 2007; Rosenkranz, 2015), as well as interspersed repeat sequences, including transposable elements (Betel et al., 2007; Smalheiser & Torvik, 2005) to study if the piRNA clusters and repRNAs are affected. Earlier reports have shown that HFD affects sperm sncRNA levels, particularly tsRNAs but also miRNAs (Donkin et al., 2016; Raad et al., 2021). Interestingly, in our experimental setup in which HFD was initiated already at puberty, we did not see any changes in sperm RNA levels in HFD vs. CTRL mice (Figure 7.2A,C). In contrast, HFD_t induced changes in tsRNAs and repRNA levels compared to CTRL. All the differently expressed tsRNA sequences (8 up and 2 down) were tiRNAs, 9 of which are encoded by the mitochondrial genome (Figure 7.2B). Regarding repRNAs, 7 sequences were upregulated and 2 downregulated (Figure 7.2D): 2 LINEs, 3 spliceosomal small nuclear RNAs (snRNAs), 1 SINE, 1 signal recognition particle RNA (sprRNA) and 2 simple repeats. Our results show that changes in the diet (from chow to HFD and back to chow) induce more changes in sperm RNA profile than constant long-term HFD starting at weaning, suggesting that mice and their sperm RNA profile can adapt to the chronic HFD.

Dietary changes affect sperm sncRNA content of the offspring of exposed mice

To study the intergenerational effect of exposure to HFD in sperm sncRNA content, mice founders (Generation F_0) were randomly assigned to a normoponderal female in mating pairs, 120 days after weaning. The resulting male offspring (Generation F_1) were exclusively fed with chow diet for 200 days, independently of the diet regimen of its male progenitor. Littermates

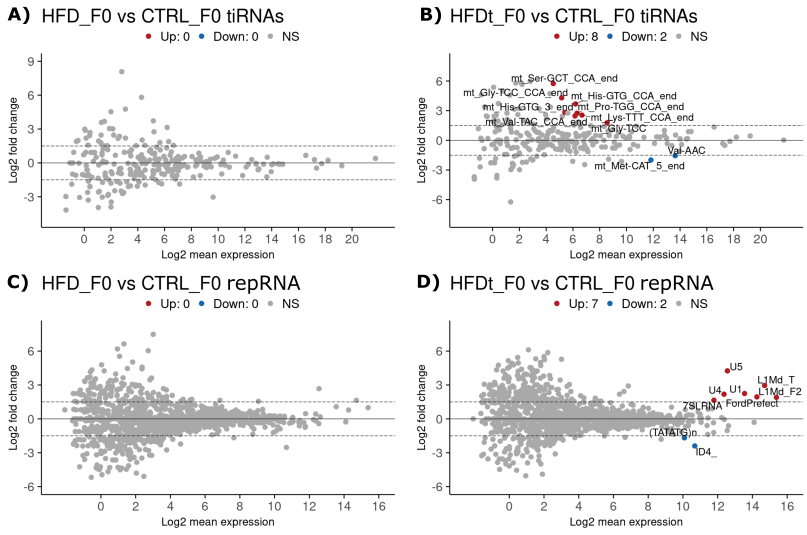


Figure 7.2: Differently expressed sperm sncRNAs in F_0 generation. Sperm sncRNA levels were compared between mice (Generation F_0, n = 3 per group) fed with standard diet (CTRL), high-fat diet (HFD), or HFD for 60 days then replaced by standard diet (HFD_t). Results are presented as MA plots (mean $\log_2$ FC vs. log2 mean expression). Differently expressed sequences are highlighted and annotated. Data was tested by Wald's test, corrected by the Benjamini-Hochberg method, using DESeq2. Significance was considered when p <0.1. A) tiRNA: HFD vs. CTRL; B) tiRNA: HFD_t vs. CTRL; C) repRNA: HFD vs. CTRL; D) repRNA: HFD_t vs. CTRL.

were assigned to the same experimental group (lineage) as its progenitor.

DE analysis revealed that ancestral HFD and HFD_t affect sperm RNA levels in the offspring. Interestingly, different biotypes were affected in HFD and HFD_t F_1 offspring compared to offspring of CTRL group (Figure 7.3A-I). HFD F_1 offspring sperm had drastically lower levels of two miRNAs (215-5p: $-23.96\,\log_2$ FC, p <0.0001; and 3068-5p: $-6.78\,\log_2$ FC, p = 0.0701) and one tiRNA (mt_Gly-TCC_3_end: $-8.65\,\log_2$ FC, p = 0.046) compared to the CTRL F_1 offspring (Figure 7.3A,D,G). In contrast, HFD_t F_1 offspring tRF levels were affected with 5 upregulated and 8 downregulated tRFs compared to the CTRL F_1 offspring (Figure 7.3B,E,H).

Interestingly, the DE analysis of piRNA clusters revealed altogether 18 clusters that were downregulated in HFD F_1 offspring sperm compared to CTRL offspring (Figure 7.4A). Only one of them was downregulated in HFD_t F_1 offspring sperm (Figure 7.4B). This suggests that, although father's exposure to chronic HFD does not affect sperm piRNA levels in the exposed animal, it does induce intergenerational changes in F_1 offspring spermatogenesis that are reflected as differential levels of piRNAs in the offspring sperm. In addition to piRNAs, a large number of differently expressed sperm repRNAs were found between the HFD F_1 offspring and the CTRL F_1 offspring (29 up and 51 down) (Figure 7.4D). 21 sperm repRNAs were also more abundant in the offspring of HFD_t compared to the offspring of CTRL (Figure 7.4E). The different levels of repRNAs in F_1 offspring sperm may also originate from father's HFD-induced changes in offspring spermatogenesis that affect the expression of repRNAs.

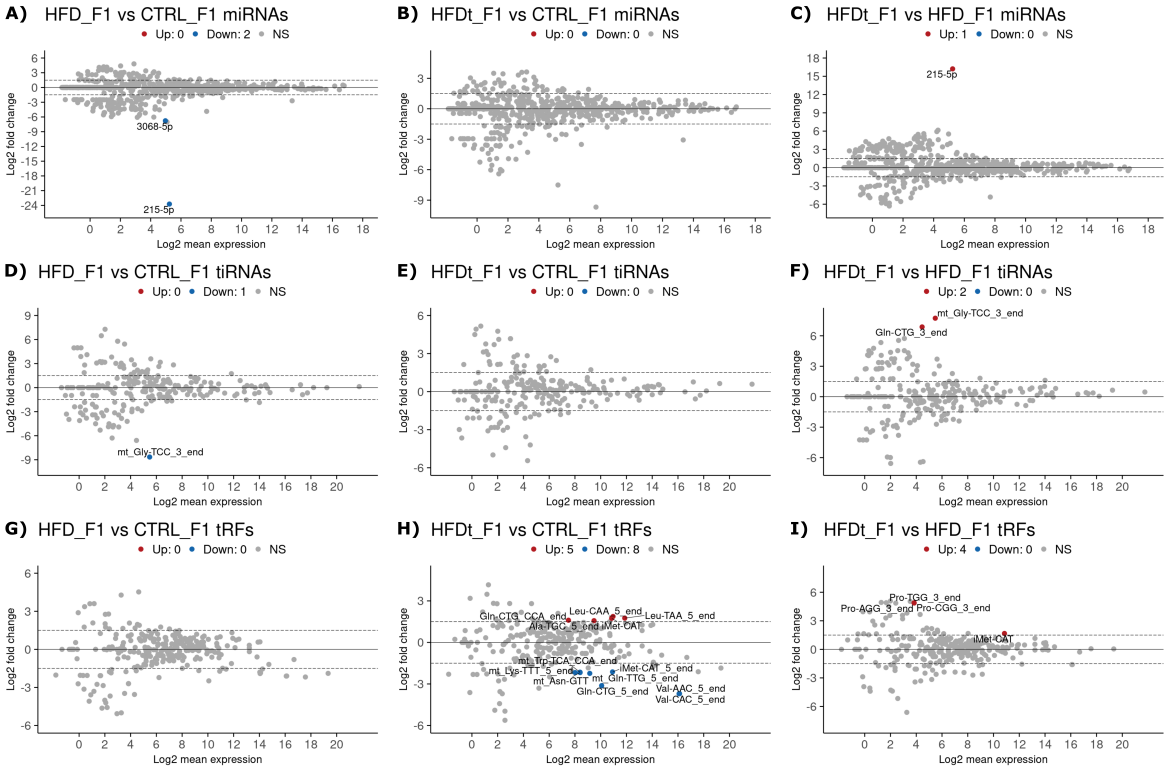


Figure 7.3: Differently expressed sperm miRNAs, transcription initiation RNAs (tiRNAs) and tRNA-derived fragments (tRFs) in F_1 generation. Sperm sncRNA content was compared between the offspring (n = 2 per group) of mice fed with standard diet (CTRL), high-fat diet (HFD), or HFD for 60 days then replaced by standard diet (HFD_t). Results are presented as MA plots (mean $\log_2$ FC vs. log2 mean expression). Differently expressed sequences are highlighted and annotated. Data was tested by Wald's test, corrected by the Benjamini-Hochberg method, using DESeq2. Significance was considered when p <0.1. A) miRNA: HFD vs. CTRL; B) miRNA: HFD_t vs. CTRL; C) miRNA: HFD_t vs. HFD; D) tiRNA: HFD vs. CTRL; E) tiRNA: HFD_t vs. CTRL; F) tiRNA: HFD_t vs. HFD; G) tRF: HFD vs. CTRL; H) tRF: HFD_t vs. CTRL; I) tRF: HFD_t vs. HFD.

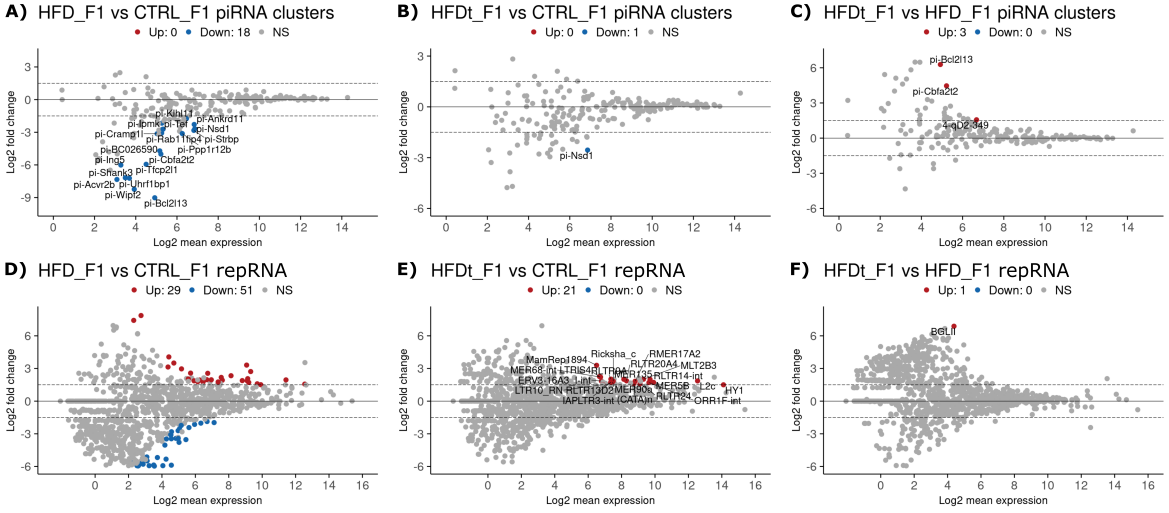


Figure 7.4: Differently expressed sperm piwi-interacting RNA (piRNA) and repeat-derived RNA (repRNA) sequences in Generation F_1. Sperm sncRNA content was compared between the offspring (n = 2 per group) of mice fed with standard diet (CTRL), high-fat diet (HFD), or HFD for 60 days then replaced by standard diet (HFD_t). Results are expressed as MA plots (mean $\log_2$ FC vs. log2 mean expression). Differently expressed sequences are highlighted and annotated. Data was tested by Wald's test, corrected by the Benjamini-Hochberg method, using DESeq2. Significance was considered when p <0.1. A) piRNA: HFD vs. CTRL; B) piRNA: HFD_t vs. CTRL; C) piRNA: HFD_t vs. HFD; D) repRNA: HFD vs. CTRL; E) repRNA: HFD_t vs. CTRL; F) repRNA: HFD_t vs. HFD.

Ancestral dietary changes can be detected in sperm of the grand-offspring

To study the transgenerational effect of ancestral exposure to HFD in sperm sncRNA content, the mice of Generation F_1 were mated in the same conditions as their progenitors. The resulting male offspring were exclusively fed with chow diet for 200 days and constitute the grand-offspring (Generation F_2) of the male founders.

Ancestral chronic HFD did not leave any detectable RNA signatures in F_2 sperm since no differentially expressed RNAs were identified (Figure 7.5A). However, we identified some changes the F_2 offspring of mice exposed to transient HFD_t compared to CTRL. Majority of sncRNAs were not affected, but two miRNAs were upregulated in HFD_t F_2 offspring sperm compared to CTRL F_2 offspring sperm (196a-5p and 196b-5p) and three were downregulated (144-3p, 34c-5p and 471-5p) (Figure 7.5B). 196a-5p and 196b-5p were also upregulated when HFD_t F_2 offspring was compared to HFD F_2 offspring (Figure 7.5C). In addition, we identified two other upregulated miRNAs (127-3p and 145a-5p) and two downregulated (135b-5p and 32-5p) miRNAs between these groups (Figure 7.5C), possibly contributing to the testicular and sperm phenotype differences that have been earlier reported between these groups (**Crisóstomo**, Jarak, et al., 2021; **Crisóstomo**, Videira, et al., 2022).

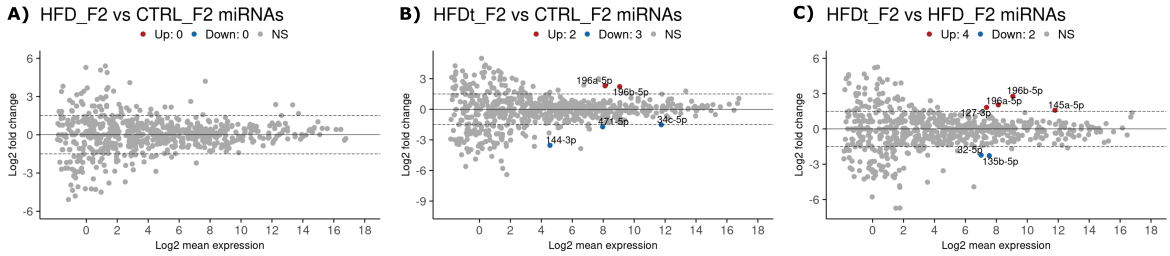


Figure 7.5: Differently expressed sperm sncRNA sequences in Generation F_2. Sperm sncRNA content was compared between the grand-offspring (Generation F2, n = 3 per group) of mice fed with standard diet (CTRL), high-fat diet (HFD), or HFD for 60 days then replaced by standard diet (HFD_t). Results are expressed as MA plots (mean $\log_2$ FC vs. log2 mean expression). Differently expressed sequences are highlighted and annotated. Data was tested by Wald's test, corrected by the Benjamini-Hochberg method, using DESeq2. Significance was considered when $p < 0.1$. A) miRNA: HFD vs. CTRL; B) miRNA: HFD_t vs. CTRL; C) miRNA: HFD_t vs. HFD.

Targets of differently expressed sperm sncRNAs are related with crucial biological processes for testicular function

To estimate the potential role of the sncRNA sequences in testicular function and sperm parameters, targets of differently expressed miRNAs and tsRNAs were predicted using bioinformatic tools. These targets and the differently expressed piRNA clusters were then annotated according to GO terms for biological processes (Figure 7.6), molecular function (Supplementary Figure 7.S3) and cellular component (Supplementary Figure 7.S4), and their

overall GO term impact calculated using the R package topGO. Briefly, the GO term impact is evaluated as a function of the pool of entered genes, the number of genes associated with a term, and the number of *"hits"* between the entered gene list and the term gene list.

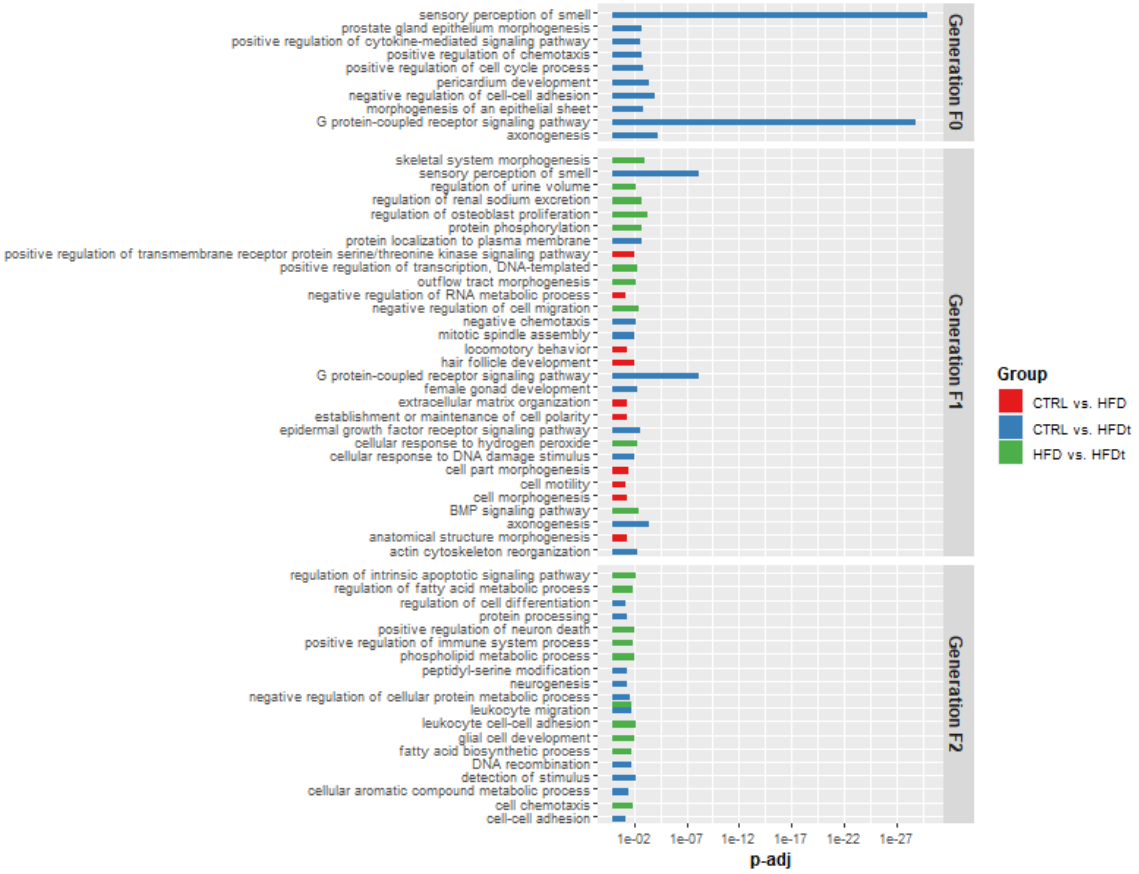


Figure 7.6: Gene ontology of the targets of differently expressed sperm sncRNAs, according to Biological Process. Targets of sncRNAs were estimated recurring to the online suite sRNAtools (https://bioinformatics.caf.ac.cn/sRNAtools/; accessed 15th August 2020). The output was then used for functional annotation based on the Gene Ontology Resource (http://geneontology.org/; accessed 17th August 2020). The annotation was performed by the topGO package ran in R 4.1.0. GO terms with less than 10 annotated genes and single gene targets were excluded from the analysis. CTRL - mice fed with standard diet and their descendants; HFD – mice fed with high-fat diet and their descendants; HFD_t – mice fed with high-fat diet for 60 days then replaced by standard diet and their descendants.

The most significant GO term resulting from the targets of the differently expressed sncRNAs between HFD_t and CTRL mice are the sensory perception of smell and the G protein-coupled receptor signaling pathway (Figure 7.6), indicating a possible impact in the chemotaxis of mature spermatozoa toward the oocyte.

Interestingly, this term is also prominent considering the differences between the offspring of HFD_t compared to the offspring of HFD. Compared to the offspring of CTRL, sperm sncRNAs from the offspring of HFD has several changes in GO terms of biological processes linked to cell motility, cytoplasm projections and even sperm activation (cell morphogenesis, establishment or maintenance of cell polarity, locomotory behavior and positive regulation of

transmembrane receptor protein serine/threonine kinase pathway). Comparing the potential impact of differently expressed sperm sncRNAs between the offspring of HFD and the offspring of HFD_t, the most relevant biologic process terms are related to antioxidant response and cell proliferation, especially of bone-related cells. Yet, in this comparison, relevant molecular function terms are related to ATP binding and energy-sensing receptors (Supplementary Figure 7.S3), whereas relevant cellular component terms are related to the cytoskeleton and lipid membranes (Supplementary Figure 7.S4). Taken together these results suggest an impact in the plasticity of Sertoli cell membrane, crucial for spermatogenesis, but also for sperm morphology and motility.

The targets of the differently expressed sperm sncRNAs between the grand-offspring of HFD_t and grand-offspring of CTRL mice are mostly associated to metabolic regulation, cell adhesion and cell differentiation (Figure 7.6). The estimated impacts of the differently expressed sperm sncRNA of the grand-offspring of HFD compared to the grand-offspring of HFD_t are linked to apoptosis, lipid metabolism, chemotaxis and inflammation (Figure 7.6). These findings suggest that the maintenance of blood-testis barrier is affected by ancestral excess adiposity caused by HFD, while the reversion to standard chow promotes changes in testicular metabolism, reflected even in the grand-offspring.

7.4 Discussion

The increasing prevalence of obesity and associated comorbidities is primary associated with risk behaviors, such as excessive caloric intake and physical inactivity. However, several studies have suggested a modulation of the predisposition to weight gain and the onset of non-communicable diseases due to ancestral exposure HFD (Grandjean et al., 2016; Pavlinková et al., 2017; Sharma et al., 2016), even in humans (Kaati et al., 2002; Lumey et al., 2007). We have previously shown that ancestral exposure to HFD causes metabolic and functional changes in testes up to two generations, a phenomenon we have coined as *"inherited metabolic memory"* (**Crisóstomo**, Jarak, et al., 2021). In this work, we studied the role of sperm sncRNA content in the transmission of acquired testicular metabolic adaptions to HFD to the offspring and grand-offspring of mice exposed to HFD.

In previous works (**Crisóstomo** et al., 2019; **Crisóstomo** et al., 2020), we have shown in mice that the adoption of HFD from weaning led to an obese phenotype with markers of pre-diabetes and marked metabolic reprogramming of the testicular tissue associated with lower sperm quality. We have further reported that the reversion of the HFD during early adulthood prevented the pre-diabetic phenotype but could not revert the testicular meta-

Table 7.1: Number of differently expressed sncRNA sequences grouped by biotype.The detailed annotation of the differently expressed sequences, sorted by biotype, is provided as Supporting Information, Table 7.S1. Abbreviations: miRNA – micro RNA; tsRNA - tRNA-derived small RNA; tRF - tRNA-derived fragment; tiRNA - transcription initiation RNA; piRNA - piwi-interacting RNA; repRNA - repeat-derived small RNA.

Generation	Comparison	miRNA	tsRNA		piRNA	repRNA
			tRF	tiRNA		
$\mathbf{F_0}$	*CTRL vs. HFD*	0	0	0	0	0
	CTRL vs. HFD_t	0	0	10	0	9
	HFD vs. HFD_t	0	0	0	0	0
$\mathbf{F_1}$	*CTRL vs. HFD*	2	0	1	18	80
	CTRL vs. HFD_t	0	13	0	1	21
	HFD vs. HFD_t	1	4	2	3	1
$\mathbf{F_2}$	*CTRL vs. HFD*	0	0	0	0	0
	CTRL vs. HFD_t	5	0	0	0	0
	HFD vs. HFD_t	6	0	0	0	0

bolic reprogramming and rescue sperm quality. Despite that, the replacement of HFD for chow diet promoted changes in testicular metabolism, notably the fatty acid metabolism and fatty lipid composition of the testes. Curiously, in this work, we exclusively found changes between the sperm sncRNA content of transient HFD mice (HFD_t) and controls (CTRL) (Figure 7.4 and 7.7A). The majority of differently expressed sncRNA sequences in sperm are tiRNAs of mitochondrial origin (Table 7.1). This finding supports our previous hypothesis that the reversion of diet promotes the mobilization of fatty acids in testes which are used by mitochondria as energy substrate (**Crisóstomo** et al., 2020). Besides, as previously reported (**Crisóstomo** et al., 2019; J. Guo et al., 2020), testicular metabolism is susceptible to environmental factors such as diet before sexual maturation. Hence, the changes observed in sperm sncRNA content of HFD_t compared to CTRL mice may reflect the limited metabolic reprogramming in response to a new environmental factor (chow diet). tiRNAs may also influence post-transcriptional regulation of gene transcripts via base pair complementarity with gene transcription start sites, RNA polymerase II binding motifs (Taft et al., 2009), and may inhibit gene translation via the assembly of stress granules (Gonskikh et al., 2020; Ivanov et al., 2011). We have estimated the potential targets of differently expressed tiRNAs and annotated then according to GO terms to predict their biological role (Figure 7.6). This analysis estimated a significant impact in GO terms related to sensory perception of smell. Odorant receptors have been identified in sperm for several years and have been implicated in sperm capacitation, acrosome reaction and oocyte fertilization (Ali et al., 2021; Spehr et al., 2003). However, as mature spermatozoa are transcriptionally silent, it is difficult to

associate the differently expressed tiRNAs with decreased expression of odorant receptors in sperm. Moreover, we have not observed differences in fertility rates among founder mice (**Crisóstomo**, Jarak, et al., 2021).

Another striking difference between the results of this study and our previous studies is related to the offspring of the mice exposed to HFD (**Crisóstomo**, Jarak, et al., 2021; **Crisóstomo**, Videira, et al., 2022). The intergenerational effects of HFD and HFD_t had a limited impact in the phenotype of the mice offspring, even on testicular metabolism. Comparing the testicular metabolic fingerprints of the offspring, it was not possible to segregate samples according to the diet of the founders. In contrast, in this study we have observed major differences in sperm sncRNA content according to the diet of the founder mice (Figure 7.4, Figure 7.5 and Figure 7.7A). Both the offspring of HFD and the offspring of HFD_t had a large number of differently expressed sperm repRNAs compared to the offspring of CTRL (Figure 7.4D and E). Notably, all the differently expressed sperm repRNAs between HFD_t and CTRL were upregulated. The expression of genomic transposable elements must be repressed throughout meiosis to secure the integrity of the DNA. piRNAs have a central role in this function and are required to achieve a normal spermatogenesis (Aravin et al., 2007; Ernst et al., 2017; X. Z. Li et al., 2013). tRFs have been suggested to play a similar role (Park et al., 2020; Schorn et al., 2017) but, contrary to piRNAs, their expression is not restricted to male germline cells. Curiously, we have found differently expressed sperm piRNAs in the offspring of HFD compared to CTRL (Figure 7.4A), and differently expressed tRFs in the offspring of HFD_t compared to CTRL (Figure 7.3H). Therefore, our data suggests that control over the transcription of transposable elements is destabilized by paternal HFD via the piRNA-directed cleavage of transcripts in the chromatoid body of spermatocytes and spermatids (Goh et al., 2015), and by paternal HFD_t via Angiogenin/DICER cleavage of mature rRNAs of germline cells (Yadav et al., 2020).

An interesting finding from our previous transgenerational studies (**Crisóstomo**, Jarak, et al., 2021; **Crisóstomo**, Videira, et al., 2022) was the reappearance of the defective sperm phenotype, characterized by decreased sperm concentration, in the grand-offspring of the mice fed with HFD or HFD_t (**Crisóstomo**, Jarak, et al., 2021). Besides, the testicular metabolic profile had clear differences according to the ancestral expo-sure to HFD. In this study, and similarly to mice founders, the grand-offspring of mice fed with HFD_t had a limited number of differently expressed sperm sncRNA compared to CTRL mice, and the grand-offspring of HFD had no changes compared to CTRL (Figure 7.5). Moreover, all the differently expressed sperm sncRNAs in this generation are miRNAs. Interestingly, the disruption of miRNA in

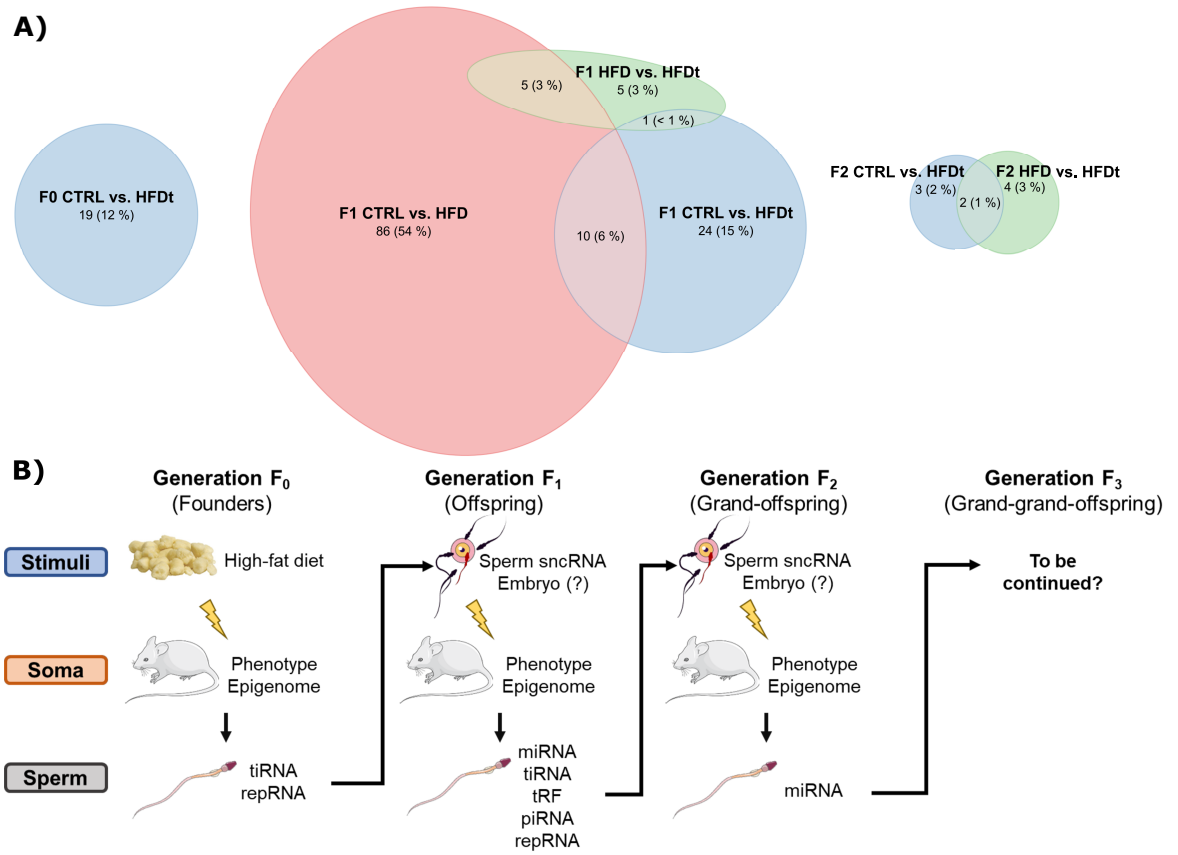


Figure 7.7: The dynamics of sperm sncRNA content across generations. The number of differently expressed sperm sncRNA sequences varies significantly in each generation and in each lineage of ancestral exposure to HFD. A) Euler diagram illustrating the scale of differently expressed sperm sncRNA sequences in every generation and considering all the comparisons: HFD vs. CTRL, HFD_t vs. CTRL, and HFD_t vs. HFD. The breakdown of these numbers according to sncRNA biotype is provided in Table 1 Table 7.1. B) Schematic representation of "inherited metabolic memory" of testicular cells via sperm sncRNA. The exposure of the founder mice to HFD is the stimulus driving epigenetic changes in somatic cells, including Sertoli cells and spermatocytes. These changes are reflected in the sperm of the founders, which presented alterations in the number of tiRNAs and repRNAs, compared to CTRL. This epigenetic fingerprint will be the stimulus for epigenomic change in the offspring, specially during embryo development. Thus, the soma of the offspring will have a different epigenetic configuration that will in its turn influence the epigenome and the sncRNA content of produced sperm, distinct from the ancestors. This sperm sncRNA fingerprint will be the stimulus for the next generation, influencing the embryo development and the epigenome of the grand-offspring of the exposed mice.

mouse germline due to conditional knock-out of DROSHA, an enzyme involved in miRNA processing, decreased sperm counts (Wu et al., 2012). In mammals, miRNAs are involved in the post-transcriptional regulation of mRNA, inhibiting the translation of gene transcripts by base-pair complementarity binding (Lewis et al., 2003). The biological impact can therefore be estimated by the complementary to gene transcripts – targets. Due to this mechanism, sperm miRNAs have been particularly associated with embryonic development, although the size of this effect in mammals is controversial. Also, sperm miRNA content is influenced by

the transcription during spermatogenesis. Similarly to previous studies (Donkin et al., 2016), we have estimated the potential targets of the differently expressed miRNAs to evaluate the potential biological impact according to GO annotation (Figure 7.6). Despite the limited number of differently expressed miRNAs, their targets are related to biological processes involved in spermatogenesis (regulation of cell differentiation), blood-testis barrier (cell-cell adhesion) and lipid metabolism (regulation of fatty acid metabolic process, phospholipid metabolic process). Nevertheless, the results of this analysis must be discussed critically, as we cannot predict the effects of the differently expressed miRNAs in the grand-grand-offspring, nor prove the expression of miRNA targets in the cell precursors of the collected epidydimal spermatozoa.

With this project we have demonstrated the conditional inheritability of phenotypes related to ancestral exposure to HFD (Figure 7.7B). The exposure to HFD and the reversion from HFD to chow are the stimuli driving phenotypic changes and, there-fore, epigenetic remodeling in somatic cells of the founder mice (Generation F_0). This remodeling will also impact the sncRNAs content of mature spermatozoa that will originate the offspring. Sperm of the HFD-exposed founder will likely carry other epigenetic signatures of HFD, such as changes in DNA methylation pattern, in protamine retention and in histone modification (Baxter & Drake, 2019; Perez & Lehner, 2019), that will contribute for the phenotype of the offspring from early embryonic development (Q. Chen et al., 2016; Conine et al., 2018). In this study we observed changes in sperm tiRNA and repRNA content, although those changes were unable to induce an evident phenotype in the offspring (**Crisóstomo**, Jarak, et al., 2021; **Crisóstomo**, Videira, et al., 2022). Yet, this stimulus has produced a response, likely in the epigenome of somatic cell, that caused several changes in sperm RNA content. Both the offspring of HFD and the offspring of HFD_t have differences in repRNA sequences, eliciting an impact of paternal HFD in the ability to silence transcripts from transposable elements of DNA. This inability may by its turn be a result of upstream dysfunction of the repeat-silencing mechanisms, namely piRNAs and tRFs. This sperm sncRNA signature, in addition to other epigenetic features carried by the offspring sperm, will be the first environmental stimulus of the grand-offspring of mice founders. This stimulus was able to induce a phenotype in sperm parameters and testicular metabolism (**Crisóstomo**, Jarak, et al., 2021; **Crisóstomo**, Videira, et al., 2022). As a result, sperm of grand-offspring carry another unique sncRNA signature, that will provide the first epigenetic stimulus of the following generation.

7.5 Conclusions

In summary, we have shown that HFD causes a *"rippling effect"* in HFD-related phenotypes of testicular function and sperm sncRNA content. The *"inherited metabolic memory"* of ancestral exposure HFD results from a complex interaction between the inherited epigenome and stochastic events that create a new epigenetic landscape that may not be associated with an evident phenotype, yet produces a new sperm epigenetic signature which will influence the epigenome of the following generation (Figure 7.7B). However, to support this inheritance mechanism, future studies must investigate other epigenetic factors carried by sperm in response to HFD and HFD-related adiposity, as well as the impact of HFD and adiposity on the epigenome of germ cells and testicular cells, notably Sertoli cells. Besides, the impact of altered sperm sncRNA content in embryo development could be further studied, for instance by injecting sperm sncRNAs isolated from HFD-exposed individuals into naïve zygotes (Q. Chen et al., 2016). Additionally, qRT-PCR could be used to validate our sncRNA candidates, increasing the number of biological replicates. Although NGS and qRT-PCR are distinct techniques, especially regarding normalization and statistical analyses, it has been demonstrated that the results are comparable, even for miRNA expression (Git et al., 2010). Despite these limitations, we describe the complexity of the epigenetic inheritance mechanisms in mammals, and we show the relevance of sperm sncRNAs to sperm parameters and testicular metabolism. For instance, transcripts from repeating elements have been implicated with success rates of Intracytoplasmatic Sperm Injection (ICSI) (Cui et al., 2018), thus knowing the family history of metabolic disease may help the decision-making process of the best suited ART of the infertile couple. Yet, further studies are needed to translate our findings to human health. Now, we suggest that sperm sncRNAs are another meaningful marker of metabolic and functional changes in testes due to ancestral exposure to HFD. Acknowledging these detrimental effects opens new therapeutical opportunities in idiopathic male infertility, to improve the efficiency of the ART, and to curb the perpetuation of acquired metabolic disease.

7.6 Author contributions

Conceptualization: **Luís Crisóstomo**, Rachel L. Batterham, Noora Kotaja and Marco G. Alves; Methodology: **Luís Crisóstomo**, Matthieu Bourgery and Luís Rato; Software: **Luís Crisóstomo** and Matthieu Bourgery; Validation: **Luís Crisóstomo** and Matthieu Bourgery; Formal analysis: **Luís Crisóstomo** and Matthieu Bourgery; Investigation: **Luís Crisóstomo** and Luís Rato; resources: Noora Kotaja and Marco G. Alves; Data curation: **Luís Crisóstomo** and

Matthieu Bourgery; Writing — original draft preparation: **Luís Crisóstomo**; Writing — review and editing: **Luís Crisóstomo**, Matthieu Bourgery, Luís Rato, João F. Raposo, Rachel L. Batterham, Noora Kotaja and Marco G. Alves; visualization: **Luís Crisóstomo** and Matthieu Bourgery; Supervision: João F. Raposo and Marco G. Alves; Project administration: Marco G. Alves; Funding acquisition: **Luís Crisóstomo** and Marco G. Alves. All authors have read and agreed to the published version of the manuscript.

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7.8 Supporting Information

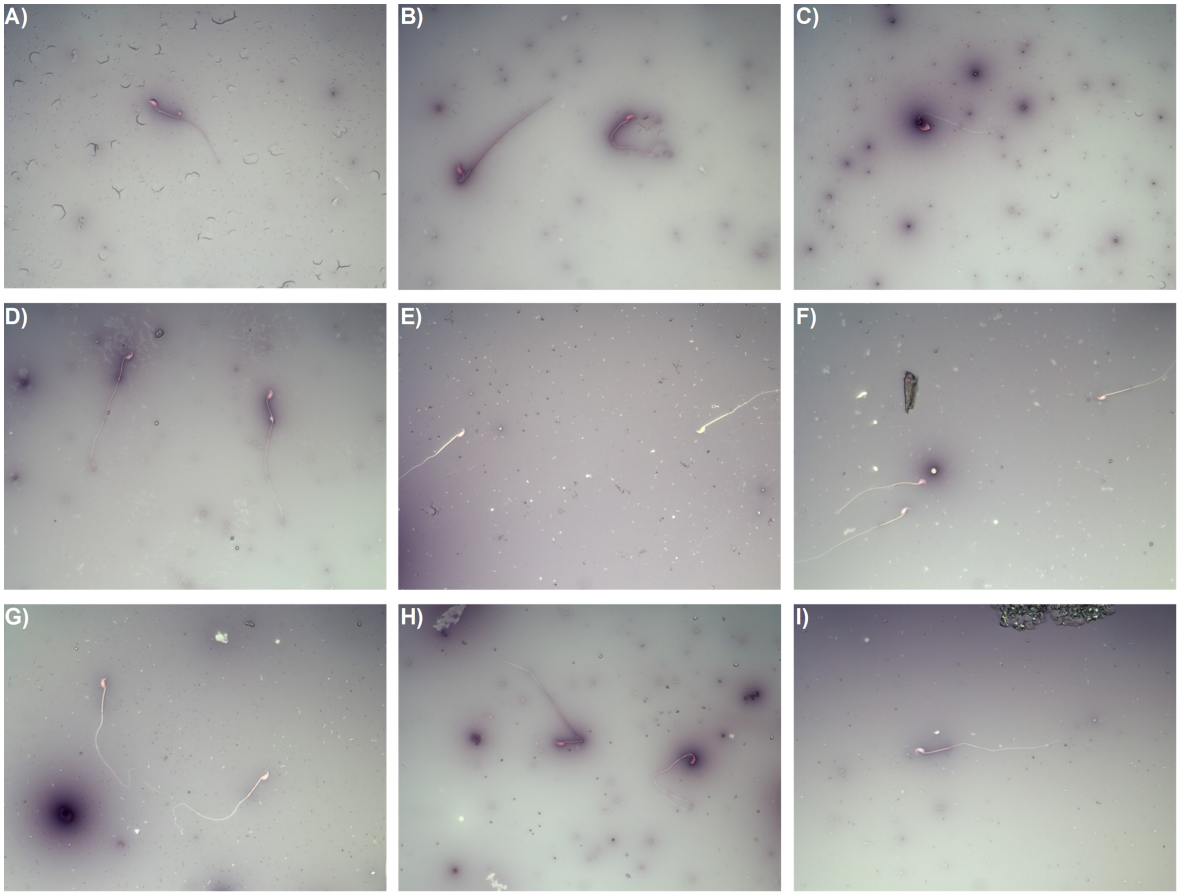


Figure 7.S1: Mice epididymal sperm swabs stained by eosin-nigrosin dye. Images obtained by ×400 magnification using an optical microscope. Epididymal spermatozoa were obtained immediately after the collection of the epididymis by swim-out. Shortly, the epididymis was collected in a warmed (37 °C) petri dish containing HBSS buffer and small excisions were made to release the sperm cells. After 5 min, to allow sperm cells to flow out of the epididymis to the HBSS buffer, 1 mL of the HBSS + sperm solution was collected for sperm parameter analysis and sperm RNA extraction. The amount of sperm debris and other cells observed in these sperm swabs was negligible. A) F_0-CTRL; B) F_0-HFD; C) F_0-HFD_t; D) F_1-CTRL; E) F_1-HFD; F) F_1-HFD_t; G) F_2-CTRL; H) F_2-HFD; I) F_2-HFD_t.

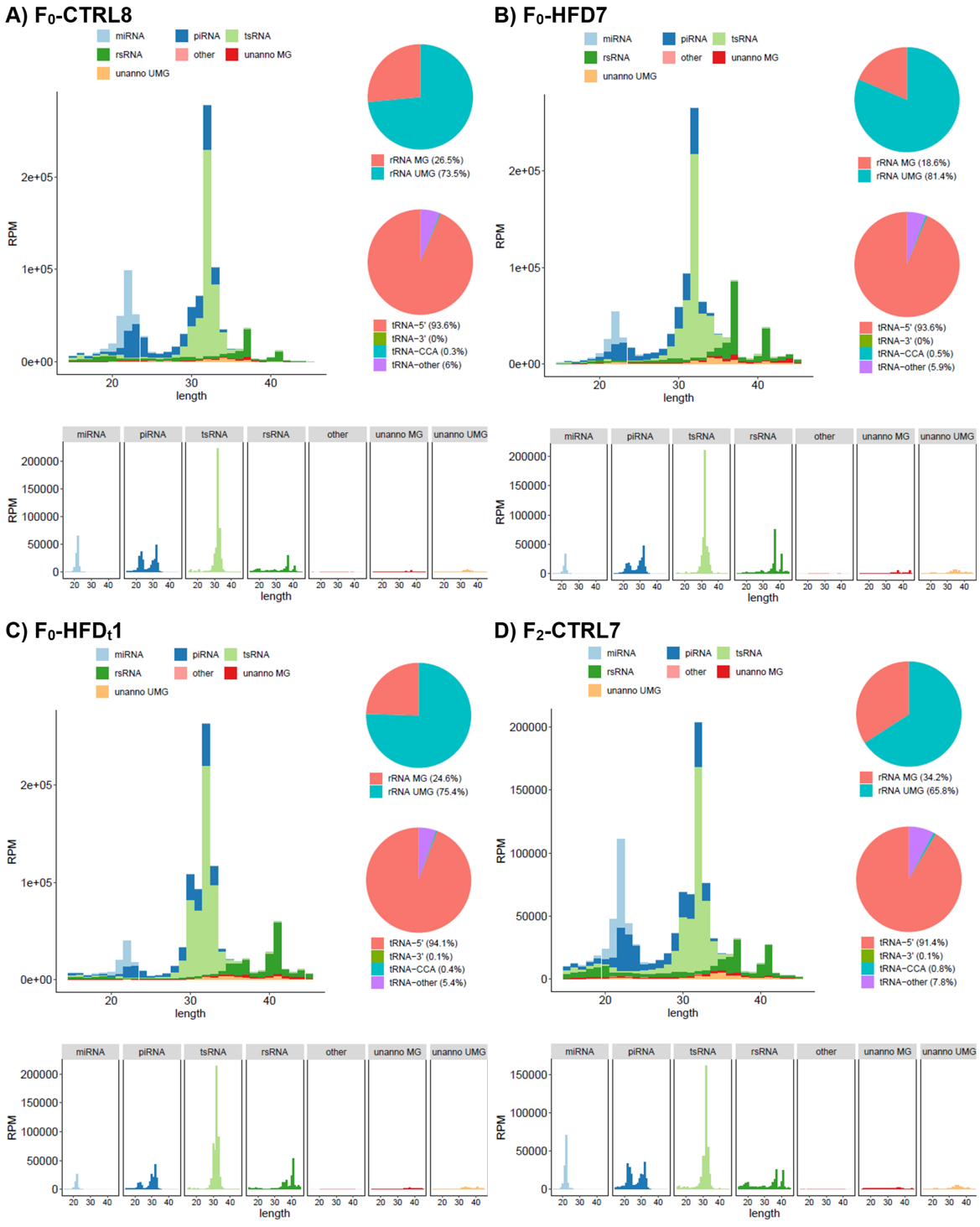


Figure 7.S2: Representative sperm RNA diagnosis statistics obtained by SPORTS 1.1. The most abundant sncRNA biotype in sperm RNA extracts was tsRNA, similarly as reported by similar studies. miRNA, piRNA and rsRNA were present in similar proportions across samples. As expected, there are two distinct piRNAs populations, one 20-23 nucleotide long and another 28–32 nucleotide long. rsRNA have a similar distribution at 35–38 nucleotides and 41–43 nucleotides. The proportion of rRNA matched to genome ranges from 20 %–35 %, eliciting a low degree of contamination by somatic cells. A) F_0-CTRL8; B) F_0-HFD7; C) F_0-HFD$_t$1; D) F_2-CTRL7.

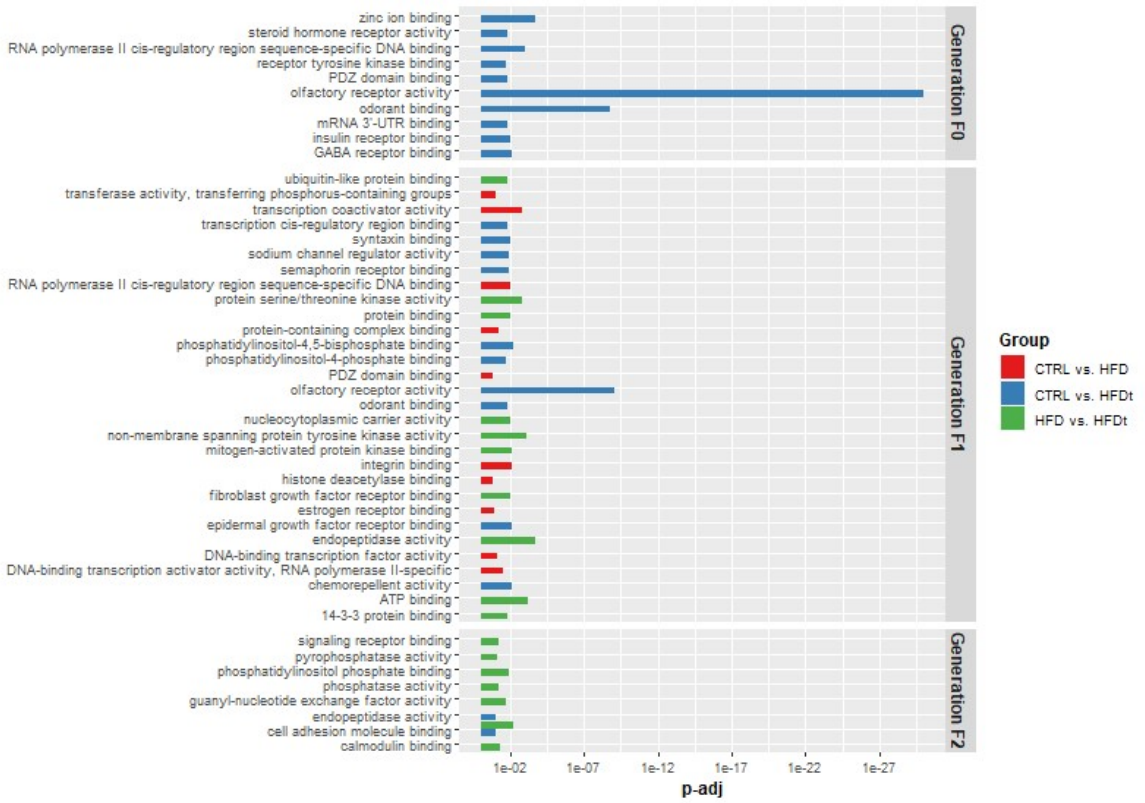


Figure 7.S3: Gene ontology of the targets of differently expressed sperm sncRNAs, according to Molecular Function. Targets of sncRNAs were estimated recurring to the online suite sRNAtools (https://bioinformatics.caf.ac.cn/sRNAtools/; accessed 15th August 2020). The output was then used for functional annotation based on the Gene Ontology Resource (http://geneontology.org/; accessed 17th August 2020). The annotation was performed by the topGO package ran in R 4.1.0. GO terms with less than 10 annotated genes and single gene targets were excluded from the analysis. CTRL - mice fed with standard diet and their offspring; HFD – mice fed with high-fat diet and their offspring; HFD_t – mice fed with high-fat diet for 60 days then replaced by standard diet and their offspring.

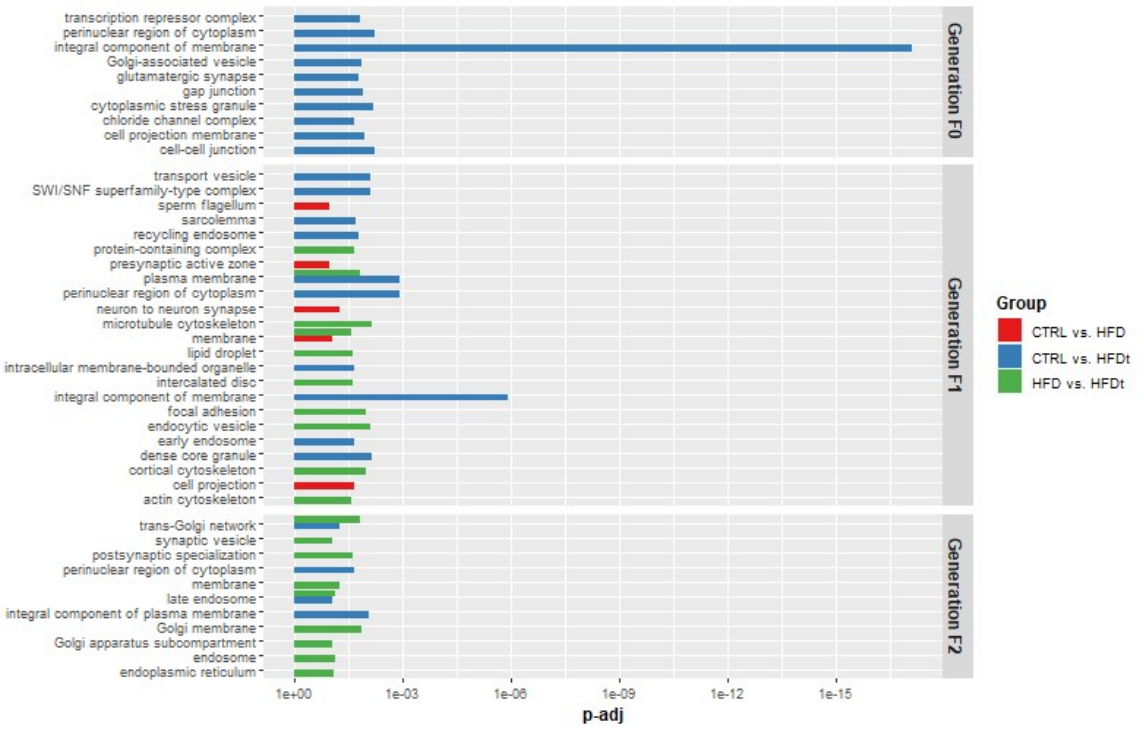


Figure 7.S4: Gene ontology of the targets of differently expressed sperm sncRNAs, according to Cellular Component. Targets of sncRNAs were estimated recurring to the online suite sRNAtools (https://bioinformatics.caf.ac.cn/sRNAtools/; accessed 15th August 2020). The output was then used for functional annotation based on the Gene Ontology Resource (http://geneontology.org/; accessed 17th August 2020). The annotation was performed by the topGO package ran in R 4.1.0. GO terms with less than 10 annotated genes and single gene targets were excluded from the analysis. CTRL - mice fed with standard diet and their offspring; HFD – mice fed with high-fat diet and their offspring; HFD_t – mice fed with high-fat diet for 60 days then replaced by standard diet and their offspring.

Table 7.S1: Detailed list of differently expressed sperm sncRNAs.

miRNA	tsRNA		piRNA	repRNA
	tRF	tiRNA		
3068-5p, 215-5p, 144-3p, 196a-5p, 196b-5p, 34c-5p, 471-5p, 127-3p, 135b-5p, 32-5p, 145a-5p	mt_Gln-TTG_5_end, mt_Lys-TTT_5_end, mt_Asn-GTT, iMet-CAT, mt_Trp-TCA_CCA_end, Ala-TGC_5_end, Gln-CTG_5_end, Gln-CTG_CCA_end, iMet-CAT_5_end, Leu-CAA_5_end, Leu-TAA_5_end, Val-AAC_5_end, Val-CAC_5_end, Pro-AGG_3_end, Pro-CGG_3_end, Pro-TGG_3_end	Val-AAC, mt_Gly-TCC, mt_Gly-TCC_CCA_end, mt_His-GTG_3_end, mt_His-GTG_CCA_end, mt_Lys-TTT_CCA_end, mt_Met-CAT_5_end, mt_Pro-TGG_CCA_end, mt_Ser-GCT_CCA_end, mt_Val-TAC_CCA_end	Acvr2b, Ankrd11, BC026590, Bcl2l13, Cbfa2t2, Cramp1l, Ing5, Ipmk, Klhl11, Ppp1r12b, Rab11fip4, Nsd1, Shank3, Strbp, Tef, Tfcp2l1, Uhrf1bp1, Wipf2, 4-qD2-349	L1Md_T, L1Md_F2, ID4_, (TATATG)n, 7SLRNA, U1, FordPrefect, U5, U4, RLTR10, L1MA5, L2c, MER5B, MLT2B4, (CAG)n, MTE2a-int, GC_rich, MLT1A1, RMER12, RLTR11B, MTD-int, (TCCA)n, MER33, RLTR24, Tigger15a, MARNA, BGLII, L1MA9, Charlie5, RMER6A, MLT2B3, RMER17C-int, MLT1J2, Tigger17c, Tigger13a, L1MEc, L1M4a2, MER113, MLT1F2, (ATTG)n, RMER6D, MuRRS4-int, LTR41B, MER115, MER58C, RMER17A2, RLTR14-int, RMER20A, Arthur1, RMER6B, MLT2F, IAPEY4_I-int, L1VL2, RLTR13G, AmnSINE1, MLT1H2, RLTR30B_MM, RLTR26D_MM, L1MCa, L1MB1, L1ME3, MMETn-int, L4_B_Mam, RLTR12E, MTEb-int, RLTR13D1, Charlie24, IAPEY2_LTR, ORR1A1-int, MamTip2, MER102b, IAP-d-int, ORR1C2-int, RLTR18, RLTR12D, MER94B, MamRep1894, MamGypLTR1d, RLTR13D6, MER135, L1MCb, ERVB2_1A-I_MM-int, MER68-int, LTRIS4B, BGLII_C, MER91C, LTRIS4, RLTR18-int, LTR86C, LTR28, IAPLTR3-int, (CATA)n, RLTR20A4, HY1, ERV3-16A3_I-int, MER90a, Ricksha_c, LTR10_RN, RLTR13D2, ORR1F-int, RLTR9A

Chapter 8

Conclusion

This chapter was reproduced from the unpublished work:

Crisóstomo, **L.**, Oliveira, P. F., & Alves, M. G. (2022). *Paternal epigenetic inheritance of metabolic traits: A scientometric and systematic review* (Journal Article)

8.1 Redundant publications in epigenetic inheritance: Are we missing the "full picture"?

The number of publications on paternal epigenetic inheritance are limited. The complexity of the experimental designs and the large datasets produced by the monitoring of the model and the different experiments are challenging to interpret and publish. To overcome the content limits of scientific journals and to simplify the research, often authors opt to publish research in paternal epigenetic inheritance as a series of papers addressing a specific aspect of the model. For this reason, the real number of studies in paternal epigenetic inheritance is even lower than the number retrieved by a systematic bibliographic search on the subject. This practice might be considered close to "salami publishing" (Abraham, 2000), although the hypothesis and the methods are not shared across the several publications. Still, we consider that the characteristics of transgenerational studies and publishing policies justify the data slicing in this context. However, with the development of complex multivariate statistical methods, the publication of these data across different publications hinders conclusions that could be drawn otherwise if it adequate multivariate statistical methods have been applied.

We have conducted a systematic analysis of available bibliography in paternal epigenetic inheritance of metabolic traits (**Crisóstomo**, Oliveira, et al., 2022), having retrieved 232 publications. A metanalysis based upon the publications meeting our inclusion criteria would lack power due to its limited size. Nevertheless, it is possible to combine, integrate and extract information from publications referring to the same set of samples and individuals. Future metanalyses on the subject must acknowledge and apply strategies to tackle the bias created by closely related publications. Hereby we demonstrate a strategy based on multiomics integration applied to a set of published papers of our own authorship (**Crisóstomo**, Bourgery, et al., 2022; **Crisóstomo**, Jarak, et al., 2021; **Crisóstomo** et al., 2019; **Crisóstomo** et al., 2020; 2022).

8.2 Multiomics integration of our previously published data

We have designed a mouse model of paternal ancestral exposure to HFD consisting in 3 generations (exposed mice – unexposed offspring – unexposed grand-offspring). Summarily, we have assigned 36 male mice to 3 different diet regimens, after weaning (exposed F_0 Generation): standard chow for 200 days (CTRL), high-fat diet for 200 days (HFD) and HFD for 60 days, then replaced by standard chow (HFD_t). At 120 days post-weaning age males were mated with same age, normoponderal, non-exposed females to generate the F_1 Generation.

The process was repeated with F_1 males to generate the F_2 Generation. Both F_1 and F_2 mice were fed standard chow for 200 days from weaning. More details of the model are available in our earlier publications (**Crisóstomo**, Bourgery, et al., 2022; **Crisóstomo**, Jarak, et al., 2021; **Crisóstomo** et al., 2019; **Crisóstomo** et al., 2020; 2022).

Based on this model, we have obtained data from testicular metabolomics (**Crisóstomo**, Jarak, et al., 2021; **Crisóstomo** et al., 2019), testicular lipidomics (**Crisóstomo** et al., 2020; 2022) and sperm small RNA transcriptomics (**Crisóstomo**, Bourgery, et al., 2022). To integrate data originated from different *omics*, raw peak areas of ^{1}H-NMR (metabolites) and GC-MS (fatty acids), and normalized counts of Next Generation RNA-seq (sncRNA sequences) were normalized using linear regression (Callister et al., 2006). Normalized counts were obtained using DESeq2 (Love et al., 2014). Multiomics integration was performed using PLS-DA, available in the mixOmics package (Rohart et al., 2017). The first 2 latent structures were estimated and the top 90 % variables with highest loading in each component were selected. The methods applied for RNA-seq sequence count normalization, linear regression normalization and multiomics integration were run in R 4.1.2 for Windows 64-bit (R Core Team, 2021b).

We have exclusively selected publicly available data from our publications (**Crisóstomo**, Bourgery, et al., 2022; **Crisóstomo**, Jarak, et al., 2021; **Crisóstomo** et al., 2019; **Crisóstomo** et al., 2020; 2022) for multiomics integration, although we have used raw data to apply a different normalization method prior to integration, as described. However, multiomics integration could be performed relying solely in the means, standard deviations and number of biological replicates reported in the publications. With those statistical descriptors it is easy to generate a dataset with similar characteristics using R, SPSS or other statistical software. In this example, we have selected data from testicular metabolites obtained by ^{1}H-NMR, testicular fatty acids obtained from GC-MS, and various fractions of sperm sncRNAs obtained from Next Generation RNA-seq. The multivariate analysis of selected datasets was performed using sPLS-DA. This method is a supervised tool based on machine learning to highlight sources of variability between different groups (Ruiz-Perez et al., 2020). The "sparse" component improves the discriminant power by reducing the weight of variables with low influence on the model (Lê Cao et al., 2008). A drawback of the method is the need of equal number of samples across blocks (datasets). In this example we had to exclude samples with metabolomic and lipidomic data to meet the biological replicates of sperm sncRNA sequencing ($n = 3$ per group in Generation F_0 and F_2, $n = 2$ per group in Generation F_1).

8.3 Multiomics integration highlights sources of variation between mice exposed to different diets and their progeny

We have previously demonstrated that exposure to HFD, even after reversion to standard chow, is associated with a testicular metabolic and lipidomic signature that is transgenerationally inherited via the paternal lineage up to the grand-offspring (**Crisóstomo**, Jarak, et al., 2021; **Crisóstomo** et al., 2019; **Crisóstomo** et al., 2020; 2022). We have then reported that sperm sncRNA content has also affected by the exposure to HFD and HFD_t, but it was not possible to relate differently expressed sperm sncRNA with the testicular metabolic phenotype (transcriptome). Using multiomics integration to combine the datasets obtained using the different techniques and tissues (testis and sperm), it was possible to obtain a wider view over testis-spermatozoa interactions that support the phenotypic observations previously reported (Figure 8.1). The projection of the two first latent structures obtained for each dataset in Generation F_0 (Figure 8.1a) is coherent to our previous reports, highlighting the differences in testicular metabolome and lipidome, but few differences in sperm sncRNA content. This is further evidenced by the block correlation plot of the first latent structure (Figure 8.1d), where all sncRNA fractions achieve very high correlation levels, therefore not contributing for group separation. Opposingly, lipids have low correlation coefficients with all other datasets, thus is the major source of difference between groups. The most interesting finding in this integration is the clear separation of samples belonging to different groups in generation F_1 (Figure 8.1b and e). Previously, we have found an evident overlap between the testicular metabolomic and lipidomic signature of the offspring of HFD, HFD_t and CTRL mice. Despite the large number of differently expressed sperm sncRNAs found between the offspring of HFD and HFD_t, we have not found a clear separation between groups either. But when integrating together, the samples of the offspring of HFD are always plotted together in a cluster well-separated from the HFD_t and CTRL clusters (Figure 8.1b). Analyzing the correlation plots (Figure 8.1e) it is further evidenced that HFD_t samples do not correlate well with CTRL, despite their partial overlap. Regarding Generation F_2, the multiomics analysis corroborates our previous findings, but highlights a total separation between groups in terms of testicular metabolite content, whereas testicular fatty acid content is more similar between grand-offspring of CTRL and HFD_t. Interestingly, sperm miRNA content is similar in CTRL and HFD_t groups, despite we have only identified differently expressed miRNAs between those groups in Generation F_2 (**Crisóstomo**, Bourgery, et al., 2022). The block correlation plot (Figure 8.1f) suggests that testicular metabolome and lipidome are the major sources of variation between grand-offspring of CTRL, HFD and HFD_t.

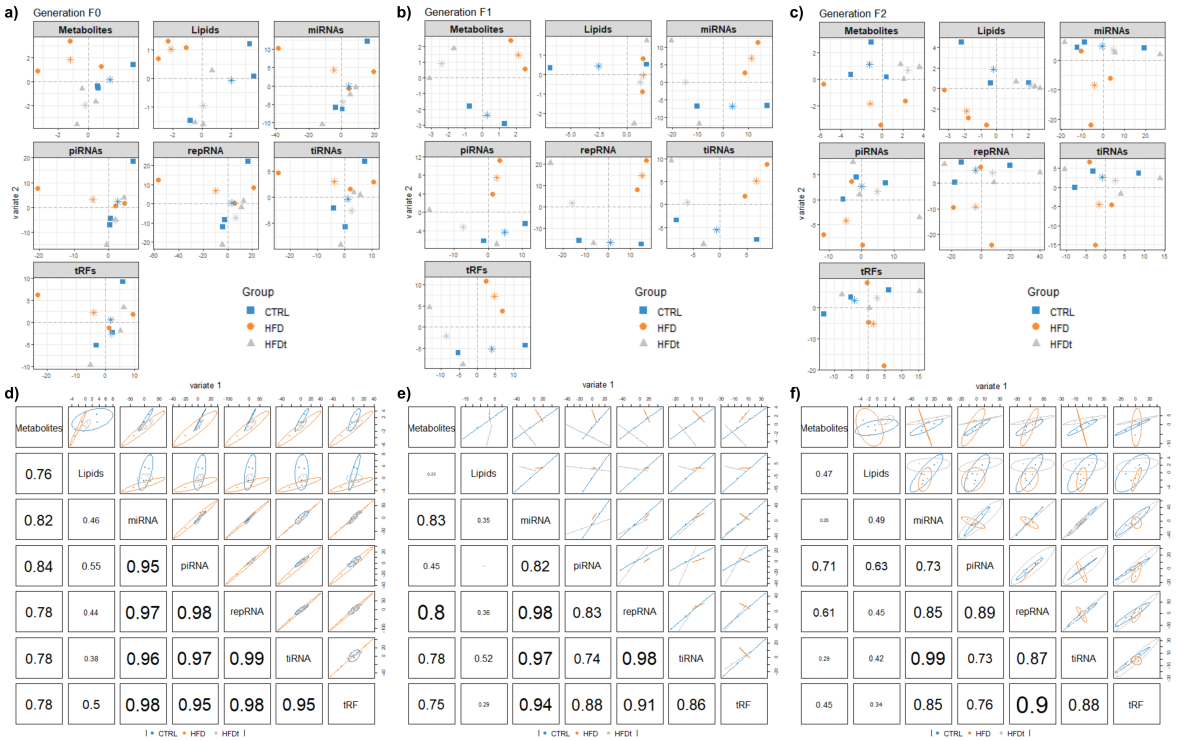


Figure 8.1: Multiomics integration of data from different papers based on the same animal model using sPLS-DA. Individual block plots of samples in the two-dimensional space spawned by the 2 first Latent Structures of a) Generation F_0, b) Generation F_1 and c) Generation F_2. sPLS-DA requires an equal number of samples in each block (dataset), thus the most representative samples according to metabolomic and lipidomic profile were selected using 2-omics sPLS. The closest samples to the centroid of the group were selected to match the number of sncRNA-sequenced samples. Due to the integration of more datasets, the clustering of samples belonging to the same group (CTRL, HFD and HFD_t) is enhanced, particularly in the F_1 generation. The block correlation plots of d) Generation F_0, e) Generation F_1 and f) Generation F_2 highlight each datasets share most similarities among each other, based on the first latent structure. Low correlation values suggest sources of differences among samples from different groups.

The multiomics integration of data from 5 different publications obtained from the same animal model has provided a broader view of the interactions between metabolome, lipidome and sncRNAs. We have reported that exposure to HFD and HFD_t affects testicular metabolism and consequently sperm parameters, and that those traits were partly inherited by the grand-offspring (**Crisóstomo**, Jarak, et al., 2021; **Crisóstomo**, Videira, et al., 2022). However, the weak phenotype expressed by the F_1 offspring and the unclear separation of samples by groups according to their sperm sncRNA content challenged the hypothesis of epigenetic inheritance of the metabolic phenotype via the male gamete. Observing the "full picture" by integrating all datasets, the results suggest that a silent epigenetic fingerprint is inherited by the F_1 generation and it is reflected in sperm sncRNA content. This complex epigenetic signature will contribute for the reappearance of the phenotype in the following F_2 generation, but the mechanisms responsible for this epigenetic regulation are not fully understood. This method has some limitations, especially the small number of samples that could be selected to perform

the multivariate profiling. Nevertheless, multiomics integration provides a reliable method to combine multiple papers and reconstruct the "full picture" of a transgenerational study.

8.4 Concluding remarks

Phenotype results from a complex interaction of genome, epigenome and environment, as postulated by Conrad Hal Waddington even before the "DNA Era". There is now mounting evidence that acquired traits can be inherited or influence traits for several generations downstream an environmental exposure. However, the concept of epigenetic inheritance still faces challenges among the scientific community. The initial focus on intergenerational inheritance studies targeted gestation, due to the direct influence over the environment of the developing embryo. Due to this assumption, the number of publications in epigenetic inheritance is still biased towards the study of the matrilinear effects (**Crisóstomo**, Oliveira, et al., 2022). However, it is now accepted that the male gamete can also transmit key epigenetic markers with the potential to transmit traits acquired (or modulated) by the environment.

After these findings, the number of publications in paternal epigenetic inheritance has fast expanded, particularly in the context of the metabolic outcomes in the offspring (**Crisóstomo**, Oliveira, et al., 2022). There are, however, other challenges to be addressed. Despite the variety of experimental designs, exposures and outcomes, present literature is still dominated by a limited number of research groups which have found in the epigenetic inheritance their research niche. The collaboration between the most active groups in paternal epigenetic inheritance research while may expand the scope of their research, might also increase the risks of "scientific inbreeding". Current literature exhibits already a bias towards studies of exposure to environmental toxicants and high-fat diets, and towards the outcomes in DNA methylation of onset of the disease (**Crisóstomo**, Oliveira, et al., 2022). This bias may be aggravated if the few research groups working on the subject start working in the same projects. Another matter of caution is the insufficient number of mechanistic studies, comparing to ecological studies, regardless of the experimental design adopted. Besides these issues, as demonstrated by the multiomics integration, the widespread practice of publishing multiple papers from the same experimental model can conceal important information about the interaction of the different factors acting towards the expression of the phenotype. In future metanalyses on the subject, authors must consider this practice before pooling the extracted data together to avoid overestimation of the number of biological replicates.

It is crucial to ensure the highest quality standards from the research in paternal epigenetic inheritance of metabolic traits. As the prevalence of non-communicable diseases continues to

increase despite all the efforts for its prevention, it is urgent to understand other mechanisms influencing the individual susceptibility to disease. The inter-, trans- and multigenerational effects of environmental exposures transmitted by epigenetic factors likely play a relevant part in this susceptibility, as we have demonstrated on the course of the present dissertation (**Crisóstomo**, Bourgery, et al., 2022; **Crisóstomo**, Jarak, et al., 2021; **Crisóstomo** et al., 2019; **Crisóstomo** et al., 2020; 2022). Our findings highlight the importance of environmental variables, notably lifestyle choices, in the health of future generations. More research on the subject, particularly in the paternal influence to health of the progeny, may help raise awareness beyond the scientific community, and build new policies and habits that may prevent disease. The health of future generations depends on our lifestyle choices and on a toxicant-free environment.

8.5 Author Contributions

Luís Crisóstomo performed the bibliography search, screened the selected publications, performed the analyses, and wrote the manuscript. Pedro F. Oliveira and Marco G. Alves have critically reviewed the manuscript and conceptualized the review. All the authors contributed to manuscript writing/editing and approved the final version.

8.6 References

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Annexes

Appendix A

Integral manuscripts

A.1 Molecular Mechanisms and Signaling Pathways Involved in the Nutritional Support of Spermatogenesis by Sertoli Cells

Abstract

SCs play a central role in spermatogenesis. They maintain the blood-testis barrier, an essential feature of seminiferous tubules which creates the proper environment for the occurrence of the spermatogenesis. However, this confinement renders germ cells almost exclusively dependent on SCs' nursing function and support. Throughout spermatogenesis, differentiating sperm cells become more specialized, and their biochemical machinery is insufficient to meet their metabolic demands. Although the needs are not the same at all differentiation stages, SCs are able to satisfy their needs. In order to maintain the seminiferous tubule energetic homeostasis, SCs react in response to several metabolic stimuli, through signaling cascades. The AMP-activated kinase, sensitive to the global energetic status; the hypoxia-inducible factors, sensitive to oxygen concentration; and the peroxisome proliferator-activated receptors, sensitive to fatty acid availability, are pathways already described in SCs. These cells' metabolism also reflects the whole-body metabolic dynamics. Metabolic diseases, including obesity and T2D, induce changes that, both directly and indirectly, affect Sertoli cell function and, ultimately, (dys)function in male reproductive health. Insulin resistance, increased estrogen synthesis, vascular disease, and pubic fat accumulation are examples of metabolic-related conditions that affect male fertility potential. On the other hand, malnutrition can also induce negative effects on male sexual function. In this chapter, we review the molecular mechanisms associated with the nutritional state and male sexual (dys)function and the central role played by the SCs.

A.1.1 Introduction

The reproductive system is the only system unnecessary for the survival of an individual of any species. Nevertheless, a considerable amount of energy is directed toward the reproductive functions. Under an evolutionary perspective, this fact is easy to understand, since (sexual) reproduction is the engine for any species to prevail and succeed, inducing genetic variance and the natural selection of the most advantageous characteristics

Known as "nurse cells" (Rato, Alves, Socorro, Duarte, et al., 2012), the Sertoli Cells play a decisive role on the nutritional support of the male germline during the processes of differentiation, particularly to fulfill their energetic needs. To achieve this, SC metabolism is tightly regulated by a complex network of endocrine and paracrine stimuli, as well as by environmental cues. In addition, the role of SCs is not restricted to the nutritional support of spermatogenesis; they also provide physical support for the differentiating germ cells, regulate and maintain the BTB, and ensure the homeostasis of the tubular fluid in the lumen of the SeT.

The SCs were firstly described by Enrico Sertoli in 1865, when he had just graduated in Medicine at the University of Pavia, Italy (Griswold, 1998). On his original work, Enrico Sertoli named his newly identified cells as "branched cells" due to their peculiar appearance, with many cytoplasmic projections surrounding germ cells (Sertoli, 1865). Sertoli was able to notice with a regular optic microscope and a limited number of samples (check original again) several other histological details and cellular ultrastructure, such as the large nucleus (Sertoli, 1865). SCs would only get their current designation 22 years later, by the Austrian histologist Victor von Ebner (Ebner, 1887). In spite of the immediate recognition of SC research potential, bibliography about them is scarce until the mid-1950s, when electronic microscopy and new biochemistry techniques, such as immunohistochemistry and immunocytochemistry, became available and after the discovery of DNA and RNA structure (França et al., 2016). Since then, the number of journal articles has been steadily increasing, to the present 400 articles per year.

This chapter focuses on the molecular mechanisms and the signaling pathways responsible for SC metabolic regulation and, thus, regulation of the environment for proper germ cell development and differentiation.

A.1.2 Relevance of Sertoli Cells to Spermatogenesis

There is still some debate concerning the specific roles for SCs, but they are usually reported as the main supporters of spermatogenesis (Griswold, 1998; P. F. Oliveira & Alves, 2015a;

Rato, Alves, Socorro, Duarte, et al., 2012). This evidence is supported by three experimental premises. Firstly, SCs are always present in the testis, whereas there is the SC-only condition where the testis does not present any germ cells (Rebourcet et al., 2014). When SC population suffers significant loss by any cause, germ cells are quickly lost, too (Rebourcet et al., 2014). This observation is further confirmed *in vitro*, where germ cells struggle to develop and thrive in the absence of SCs, and spermatogenesis has not been fully achieved in culture, while the closest attempts to mimic it are always based on co-cultures with SCs (França et al., 2016; Griswold, 1998; Nagano et al., 1998; Xie et al., 2015; Zanganeh et al., 2013).

Secondly, there is evidence that the number of germ cells supported by a single SC is limited and species-dependent; hence, the number of germ cells is directly proportional to the number of SCs (França et al., 2016; Griswold, 1998; L. Johnson et al., 2008; Schlatt & Ehmcke, 2014). Experimental evidence comes from studies where the number of SCs was controlled and the spermatogenic output quantified (Griswold, 1998; L. Johnson et al., 2008; Orth et al., 1988; Orth et al., 1984; Simorangkir et al., 1995). When SC numbers were restricted by inhibition of proliferation during testicular development, either by hormonal or chemical action, spermatogenic output and testicular volume decreased, also illustrating that the number of SCs significantly contributes to a normal testicular volume (Orth et al., 1988). When the procedure was hemicastration (ablation of a testis) during the development phase, the remaining testis had bigger volume and spermatogenic output (Orth et al., 1984; Simorangkir et al., 1995). However, in both experimental approaches, the SC-spermatid ratio remains constant (Griswold, 1998). The direct relation between spermatogenic output and SC number is further supported by evidence that, in some species, adult SC proliferation occurs at the breeding season to increase their spermatogenic output, but, in humans, SCs have limited mitotic capacity *in vivo* (L. Johnson et al., 2008).

Lastly, it is observed that spermatogenesis is highly dependent upon hormonal regulation from testosterone and FSH, and those hormones only exert their action directly over SCs, not germ cells (França et al., 2016; Griswold, 1998). Experimental evidence relies in several *in vitro* and *in vivo* models. FSH and thyroid hormones mediate SC proliferation during testis development, thus affecting testis size, weight, and spermatogenic output (Schlatt & Ehmcke, 2014). In addition, there were several gene mutations identified in the subunit of FSHR of SCs, which resulted in dysfunctional protein and infertility in men (Layman et al., 2002; Schlatt & Ehmcke, 2014). The affected individuals presented small testicular size and azoospermia (Layman et al., 2002; Schlatt & Ehmcke, 2014). It is evidenced *in vivo* that the expansion of germ cell reserve is FSH-dependent rather than androgen-dependent but

always primarily mediated by SC action (Arslan et al., 1981; Griswold, 1998). Furthermore, differentiation from germ cells to round spermatids by FSH stimulation is possible, but its yield is only about 5 % of the normal, SC-mediated, yield (Schlatt & Ehmcke, 2014).

The advances on SC research now elicit that their critical role on spermatogenesis is related, but not limited, to germ cell physical support, environmental control, BTB maintenance and modulation, tubular fluid dynamics, endocrine and paracrine regulation, biochemical stimulation, and germ cell nutritional support (Alves, Martins, Rato, et al., 2013; Dimitriadis et al., 2015; Griswold, 1998; P. F. Oliveira & Alves, 2015a; P. F. Oliveira et al., 2015; Rato, Alves, Socorro, Duarte, et al., 2012; Rato et al., 2016).

SCs ensure the physical support to male germline cells due to a very distinguishable feature: long and thin cytoplasmic arms, similar to branches (as observed by Enrico Sertoli) (Sertoli, 1865). These elongations create a cuplike form to hold germ cells, dramatically increasing the membrane surface area and creating stage-specific microenvironments for germ cells throughout their differentiation (França et al., 2016). However, germ cells are not only stabilized by it but also gain protection and other important features. Another significant and singular characteristic is the apical ectoplasmic specialization, a cell-cell adherent junction based on actin filaments, responsible for the germ cell movement and docking along the SC's membrane (França et al., 2016). In a larger extent, SCs are the very nature of the seminiferous tubule epithelium, since their polarization and side-by-side placement create a tubular structure and two distinct mediums (Wong & Cheng, 2005).

SCs are responsible for the maintenance of the two-compartment system found in the testis, which is a critical condition for spermatogenesis (P. F. Oliveira & Alves, 2015a). The BTB separates the interstitial fluid, which surrounds peripheral cells, and the tubular fluid, which fills the lumen of the seminiferous tubules (P. F. Oliveira & Alves, 2015a). *In vitro* studies have demonstrated that human stem cells are able to differentiate into spermatogonia in a SC-conditioned culture medium (Geens et al., 2011; Xie et al., 2015), a strong evidence of the environment importance for spermatogenesis but also of a significant contribution from SCs, rather than from germ cells, in this process. The SCs maintain tubular fluid homeostasis by guaranteeing the integrity of BTB and regulating its composition (Bernardino et al., 2016; Bernardino et al., 2013; Martins et al., 2014; Rato, Alves, Socorro, Duarte, et al., 2012). Due to the fact that SC is highly polarized, SCs are the most important structure of the BTB, since its basal membrane is directed toward the interstitial fluid, while its apical membrane is directed toward the seminiferous tubule lumen (Janecki & Steinberger, 1986). Tubulobulbar complexes are another SC peculiar feature. They are another anchoring system between cells

at the BTB that works similarly to a puzzle: cytoplasmic elongations of a SC, ending on a spherical structure containing a cistern-like complex related to the endoplasmic reticulum, fit into corresponding invaginations on the adjacent cell (França et al., 2016; Vogl et al., 2013). This cell junction is further reinforced by the action of other anchoring systems present in these cells (Cheng & Mruk, 2002; Wong & Cheng, 2005). The tubular complexes at the basal region, tight junctions, gap junctions, and desmosome-like junctions, between SCs are responsible for making this blood-tissue barrier even more impermeable (Cheng & Mruk, 2002; Wong & Cheng, 2005). SCs dock themselves through hemidesmosomes to the basal lamina, the physical border of seminiferous tubules consisting in the basement membrane, a collagen network, and a layer of peritubular MCs (P. F. Oliveira & Alves, 2015a; Rato, Alves, Socorro, Duarte, et al., 2012). The apical Tubulobulbar complexes mainly promote interaction with germ cells, in late maturation stages (spermatid), and are thought to be crucial for spermiation in the cytoplasmic reduction and mature spermatozoa release processes (Wong & Cheng, 2005).

The most important endocrine mechanism mediated by SCs is its role on the reproductive axis which is the central regulator for the reproductive function. It starts at the beginning of puberty, with the pulsatile release of GnRH. GnRH neurons secreting GnRH are derived from the nasal placode and migrate into the brain during prenatal development. Once within the brain, GnRH neurons become integral components of the hypothalamic-pituitary-gonadal axis, essential for reproductive maturation and maintenance of reproductive function in adults. GnRH induces adenohypophysis production and release of gonadotropins, FSH, and LH. In male reproductive system, LH stimulates steroidogenesis in LCs, namely, androgens such as testosterone, while FSH stimulates SC activity, metabolism, and endocrine function, as inhibin source. The axis is under negative feedback control; therefore, inhibin and androgens inhibit GnRH and gonadotropin release when their bloodstream concentration overcomes its feedback threshold (Alves, Rato, et al., 2013; Cardoso et al., 2017; Schlatt & Ehmcke, 2014). In males, this cycle lasts for life but can be disrupted by any abnormality in any of the players. When it happens, spermatogenesis might be seriously affected (Alves, Rato, et al., 2013; J. E. Anderson & Thliveris, 1986; Cardoso et al., 2017). Spermatogenesis depends on paracrine regulation from SCs too, as they provide several differentiation factors (Alves, Rato, et al., 2013; Rato et al., 2016). Nevertheless, SC endocrine and paracrine roles on spermatogenesis may also be indirect. LCs, the most important steroidogenic cell of the testis and the main testosterone source, suffer dramatic population losses, and peritubular MCs, BTB is responsible for the immune-privilege, also suffer a significant drop in their

activity in the absence of SCs (Rebourcet et al., 2014).

The critical role of SCs in spermatogenesis begins long before sexual maturation and at the beginning of regular spermatogenesis. Their influence starts as soon as on testicular embryonic development (França et al., 2016; Rebourcet et al., 2014). In addition to their stimulation by FSH to a normal testis development, SCs also secrete the anti-Mullerian hormone, a critical endocrine modulator involved on the correct development of the primary sexual characteristics (Alves, Rato, et al., 2013; Griswold, 1998; P. F. Oliveira & Alves, 2015a). Thus, these cells are involved in virtually all steps required for a normal spermatogenesis.

A.1.3 Germ Cell Dependence of Sertoli Cell Metabolism

Germ cells cross several differentiation stages during spermatogenesis. Their reliance on SCss are not the same at all stages, but it is a pivotal feature at all steps (P. F. Oliveira & Alves, 2015a). The least differentiated cells in this line are the spermatogonia, diploid germ cells that constitute the germline reserve. Within spermatogonia there is a low differentiation degree between those which are committed to mitotic division for germ cell reserve preservation (type A) and those which are committed to meiotic division and undergo spermatogenesis (type B) (Griswold, 1998; P. F. Oliveira & Alves, 2015a; Schlatt & Ehmcke, 2014). The nomenclature for spermatogenesis stages differs from author to author, but here we will consider the following: spermatogonium (2n), primary spermatocyte (4n), secondary spermatocyte (n), round spermatid (n), elongated spermatid (n), and spermatozoon (n).

As mentioned, the two-compartment system, delimitating two distinctive environments, the interstitial fluid and the tubular fluid, is of the utmost importance for germ cell survival and differentiation (Schlatt & Ehmcke, 2014; Wong & Cheng, 2005). SC metabolism controls the metabolic microenvironment needed for each stage of germ cell differentiation. This is evident on the onset of the first meiotic division, when a type B spermatogonium enters phase S on its cell cycle and doubles its genetic charge (4n). Some authors define this phase as two distinct stages, the preleptotene and leptotene spermatocyte (França et al., 2016; Schlatt & Ehmcke, 2014). However, the interstitial environment is not suitable for meiosis, and in order to complete the first meiotic division, the cell crosses the BTB into the adluminal environment, where it readies meiosis (primary spermatocyte stage) and finally completes the first division to originate two secondary spermatocytes. The conversion of type A to type B spermatogonium seems to be modulated by RA, a vitamin A derivate that cannot pass the BTB and is locally secreted by SCs, so it acts as a signal for meiosis initiation and thus spermatogenesis (Whitmore & Ye, 2015).

Overall, testes are oxygen-deprived organs; hence, germ cells, independently from its maturation stage, mainly rely on glycolytic metabolism to obtain energy (Alves, Rato, et al., 2013; Boussouar & Benahmed, 2004). The importance of glycolytic pathways is such that germ cells present, while undergoing spermatogenesis, unique enzyme isoforms related to it (P. F. Oliveira et al., 2015). Glycolytic potential decreases along the differentiation stages of spermatogenesis. Spermatozoon is the illustrious exception. It is even the most glycolytic cell of the male germline family, but has the lowest TCA cycle activity, and relies almost exclusively on hexoses as substrate (Miki, 2006; Rato, Alves, Socorro, Duarte, et al., 2012). Lactate and pyruvate resulting from SC metabolism is an important energy substrate for germ cells (Alves, Martins, Rato, et al., 2013). SCs export both to the tubular fluid via MCT membrane transporter family members, while germ cells also import those substrates via MCTs (Alves, Martins, Rato, et al., 2013; Martins et al., 2013; P. F. Oliveira et al., 2012). Therefore, lactate availability for germ cells depends on its production and release rate from SCs, which in turn is dependent upon MCT protein expression and glycolytic flux (P. F. Oliveira et al., 2015; Rato, Alves, Socorro, Duarte, et al., 2012). Nevertheless, SCs' glycolytic activity depends on substrate availability, *i.e.*, glucose import from the extracellular, interstitial environment, mediated by GLUT family member (P. F. Oliveira et al., 2015; Rato, Alves, Socorro, Duarte, et al., 2012). Summarily, germ cell substrate availability is intimately related to SCs' own substrate availability and metabolic rate. Fortunately for germ cells, albeit oxygen deprivation promotes the glycolytic metabolism rather than more oxidative metabolic pathways, SCs are competent metabolic "factories" able to metabolize a large spectra of substrates, including fatty acids and amino acids, in order to sustain the production of lactate and pyruvate (P. F. Oliveira & Alves, 2015a; Rato, Alves, Socorro, Duarte, et al., 2012). SCs direct three quarters of all their own pyruvate production to germ cells, either as lactate or pyruvate, whereas β-oxidation is suggested to be used only to satisfy their own energy needs (Grootegoed et al., 1986). Interestingly, SC metabolism resembles a cancer cell metabolism, heavily relying on glycolytic metabolism and glutaminolysis; however, contrarily to cancer cells which direct such energy for cell proliferation, SCs seem to direct it for production of substrates for developing germ cells (Alves, Rato, et al., 2013; P. F. Oliveira et al., 2015).

Besides being the preferred energy substrate, a novel evidence for a role of lactate as a signaling molecule in the seminiferous tubule has been provided, and lactate might be considered a paracrine factor secreted by SCs. In this context, lactate, taken up by germ cells, becomes oxidized to pyruvate with the resultant increase in ROS which act as second mes-

sengers regulating signal transduction pathways and gene expression (Galardo et al., 2014). In addition, lactate has also anti-apoptotic properties on germ cells and promotes RNA and protein synthesis on spermatids, by promoting NADPH oxidation for PPP and ATP production for protein synthesis (Erkkilä et al., 2002). On the other hand, SCs are relevant for germ cell apoptosis through other mechanisms (Murphy & Richburg, 2014). The division rate of spermatogonia is higher than the spermatozoa release rate. In mammals, germ cell death is conspicuous during spermatogenesis and occurs spontaneously at various phases of germ cell development such that seminiferous epithelium yields fewer spermatozoa than might be anticipated from spermatogonial proliferations. Although most of the apoptotic cells are type B spermatogonia and primary spermatocytes, programmed cell death can occur at any differentiation stage (Murphy & Richburg, 2014). SCs quickly absorb their apoptotic bodies by phagocytosis, because they express SR-BI, a receptor sensitive to the apoptosis-marker phospholipid phosphatidylserine (Murphy & Richburg, 2014). Thus, apoptotic cells are rarely spotted on histological preparations (Murphy & Richburg, 2014). Notably, SCs are reported to even use the apoptotic bodies as energy source (Xiong et al., 2009) illustrating their high metabolic capacity to metabolize substrates and convert them into relevant metabolites for spermatogenesis.

SCs are responsible for ionic balance and pH control in tubular fluid, due to their metabolic and secretory activity (Bernardino et al., 2016; Bernardino et al., 2013; Martins et al., 2014). The hydrocarbonate ion plays a decisive role on pH balance, and its concentration depends on SC protein expression of CFTR (Bernardino et al., 2016; Bernardino et al., 2013). Another SC-related pH regulation mechanism is the SLC9 Na^+/H^+ exchangers (Martins et al., 2014). AQPs expressed by SCs are another important transporter family for tubular fluid homeostasis and are reported to be co-expressed with CFTR (Jesus et al., 2014a; 2014b). Germ cells heavily rely on this regulation, as they are very fragile against environmental aggression. Indeed, oxidative stress is a major external aggression for germ cells, as they lack antioxidant mechanisms. Oxidative stress induces germ cell apoptosis due to lipid peroxidation of the germ cell membrane's lipids (Rato, Alves, Socorro, Duarte, et al., 2012). The physical supportive role of SCs is a key factor for germ cell survival as shown before the end of spermiation when a premature germ cell detachment causes apoptosis on detached germ cells (Cheng & Mruk, 2002). In the occurrence of SC death, the previously nursed germ cells, which became freely suspended in the lumen, rapidly engage programmed cell death (Cheng & Mruk, 2002). This might be caused by the germ cell stage-specific environmental needs, only maintainable at the microenvironments created by the SC's membrane invagina-

tions and by the necessity of growth factors synthesized by these cells (Cheng & Mruk, 2002; Vogl et al., 2013). Figure A.1 summarily illustrates the most important roles of SCs in the testicular tissue.

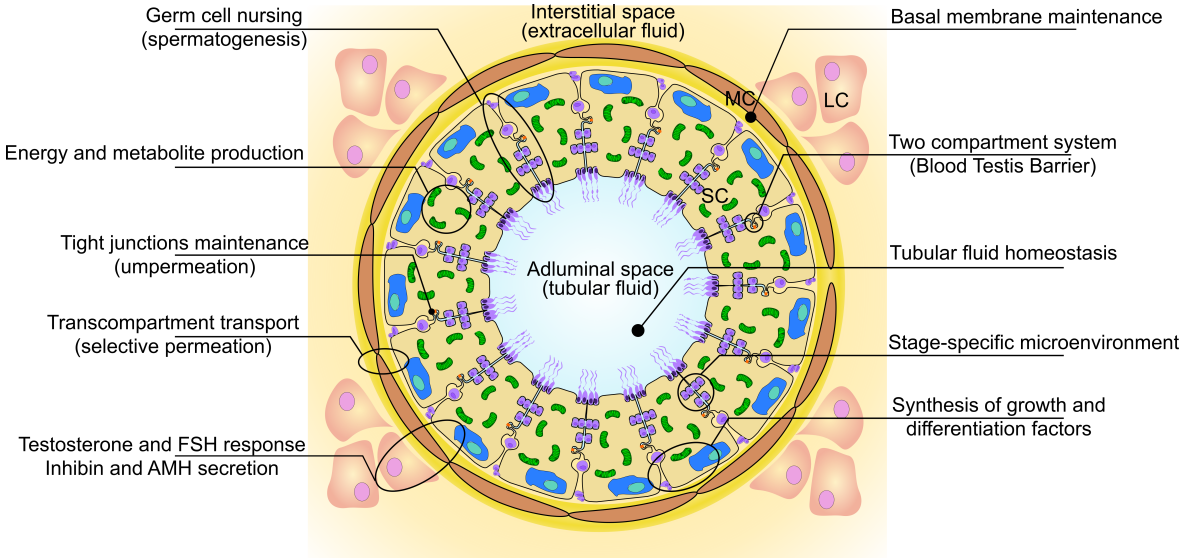


Figure A.1: The central role of Sertoli cells in testicular tissue environment and organization. Sertoli cells play several roles in testicular tissue's physiology. Besides their critical role on spermatogenesis, granting physical and nutritional support to developing germ cells, they also show paracrine and endocrine properties and are the main component of the blood-testis barrier, one of the tightest blood-tissue barriers of the human body.

Within paracrine mediators and growth factors in the cross talk between SCs and germ cells, perhaps the most described players are bFGF and IL-1. bFGF is expressed by Sertoli and germ cells, but only SCs express its receptor, where it acts as a major metabolic modulator. It modulates transferrin, E_2 and lactate secretion, glucose uptake, and FSHR expression (Alves, Rato, et al., 2013). Transferrin is an iron-binding plasmatic glycoprotein that, due to BTB impermeability, cannot transfer their ferric ions to the adluminal compartment where it is needed by the differentiating germ cells. Once again, SCs mediate the ferric ion transport from the interstitial fluid to the tubular fluid. Many proteins have been demonstrated to be implicated in iron transport to germ cells. Iron import to the seminiferous tubule by transferrin was demonstrated, and it was hypothesized that SC could import iron basolaterally, transport it across the BTB, and supply iron to the developing male germ cells. In this way, the entry of iron to the germ cells is thought to be mediated by transferrin secreted by SC (Griswold, 1998). Furthermore, ferroportin transcript levels were detected in SC and can contribute with this transport. In addition to transferrin and TfR1, other proteins involved in iron transport, such as DMT1 and glycosyl phosphatidylinositol-anchored ceruloplasmin, and iron storage, such as cytosolic and mitochondrial ferritin, were found in the testis. IL-1 is a multifunctional cytokine, having two isoforms: IL-1α and IL-1β. In the testis, IL-1α is

produced by SCs and germ cells, while IL-1β is produced by LCs and interstitial macrophages (Rato et al., 2016). Surprisingly, IL-1α causes antispermatogenic effects over SCs, while IL-1β promotes pro-spermatogenic responses from them (Alves, Rato, et al., 2013). On the other hand, IL-1 is specially involved in meiosis initiation, promoting DNA synthesis and differentiation of type B spermatogonium into preleptotene spermatocytes (Alves, Rato, et al., 2013). Nevertheless, we have to take into account that the majority of the studies of paracrine and growth factor regulation of spermatogenesis were performed *in vitro*; hence, differences in *in vivo* behavior must be considered (Alves, Rato, et al., 2013).

A.1.4 Maintaining Seminiferous Tubule Energetic Homeostasis: Role of the AMP-Activated Protein Kinase (AMPK)

It is widely known that SCs assume the responsibility for maintaining energy balance in germ cells situated beyond BTB. Thus, it is expectable that a signaling pathway might be activated by any energy deprivation signal arising from the seminiferous tubules.

AMPK cascade is a key regulator of cellular energy homeostasis (Hardie, 2003). It is a heterotrimeric enzyme consisting of a catalytic α-subunit and regulatory β and γ subunits. In vertebrates, each subunit exists as multiple isoforms encoded by distinct genes. In mammals, Prkaa1 and Prkaa2 encode for α1AMPK and α2AMPK subunits, Prkab1 and Prkab2 encode for β1AMPK and β2AMPK isoforms, and Prkag1, Prkag2, and Prkag3 encode for γ1AMPK, γ2AMPK, and γ3AMPK subunits, respectively (Cheung et al., 2000; Stapleton et al., 1996; Thornton et al., 1998). These seven subunit isoforms could potentially give rise to 12 heterotrimeric combinations. The question of whether these distinct heterotrimeric combinations have different functions has not received much attention, although interesting new evidence is beginning to emerge.

AMPK is activated by an increase in the AMP:ATP ratio, and its activation involves several mechanisms:

1. The AMPK ability to sensitively respond to subtle changes in the AMP:ATP ratio is given by two binding sites for AMP, known as Bateman domains, in γ subunit. When AMP levels are increased, the nucleotide competitively displaces the ATP bound to the enzyme; and now, the bound AMP induces a conformational change that makes the protein a better substrate for the phosphorylation of the α-subunit at a specific threonine residue - Thr 172 - by AMPK upstream kinases (AMPKKs).

2. Phosphorylation by AMPKK constitutes the primary mechanism for the stimulation of AMPK activity.

3. The conformational modification of the protein induced by AMP binding also provokes a decrease in dephosphorylation by phosphatases and contributes to a sustained activation of the enzyme (Hardie, 2004).

Altogether, these mechanisms make the AMPK system ultrasensitive to energy changes, an important fact for the ability of the enzyme to maintain the energy status of the cell within narrow limits.

Several reviews have dealt with the downstream targets and processes regulated by AMPK (Hardie, 2005; Hardie & Ashford, 2014; Hardie & Pan, 2002). In general, activation of AMPK downregulates biosynthetic pathways such as fatty acid and cholesterol biosynthesis yet switches on catabolic pathways that generate ATP, such as fatty acid oxidation, glucose uptake, and glucose oxidation. AMPK regulates the above mentioned phenomena not only through direct phosphorylation of metabolic enzymes but also through effects on the expression of genes that are important to these metabolic pathways. Although protein expression, as a whole, is downregulated following AMPK activation, specific proteins, which are essential for metabolic adaptation, will bypass the condition of energy saving associated with the activation of this kinase and will increase their expression (Hardie & Hawley, 2001).

The expression of AMPK in the testis was demonstrated by Cheung and collaborators in 2000 (Cheung et al., 2000). These authors have also shown that the 75 unit% of AMPK activity in the whole testis is attributed to α1AMPK and the remaining 25 % to α2AMPK. However, it was not until 2007 that the role of AMPK in SC nutritional function gained increasing relevance.

AMPK activation leads to metabolic changes responsible for obtaining a maximum energy yield of glucose oxidation with a reduction in lactate production (Ceddia & Sweeney, 2004; Putman et al., 2003; A. C. Smith et al., 2005). In this context, Faubert et al. (2013) have recently demonstrated that AMPK is a negative regulator of Warburg effect in cancer cells. Surprisingly, the activation of AMPK in SCs leads to an increase in lactate production (Galardo et al., 2007). The latter piece of evidence suggested that under those conditions when there is an increase in the AMP:ATP ratio, indicative of low energy levels, SCs privilege lactate secretion in order to maintain the energy supply to germ cells. This asseveration was supported by the findings of Riera et al. (2009). The researchers have demonstrated that SCs adapt to conditions of glucose deprivation by AMPK activation to ensure an adequate lactate concentration in the microenvironment where germ cell development occurs. It is worth mentioning that α1AMPK-deficient male mice present decreased fertility and altered sperm morphology; meanwhile knockout mice for α2AMPK do not show any anomaly in fertility or

sperm morphology (Tartarin et al., 2012). Further research has shown that specific deletion of the α1AMPK gene in mouse SCs results in a 25 % reduction in male fertility associated with a deregulation in energy metabolism in SCs and thin-shaped sperm heads (Bertoldo et al., 2016). In agreement with the results obtained by Galardo et al. (2007), these authors also suggest that AMPK is a positive regulator of Warburg effect in SCs. Considering that AMPK activation leads to an increase in aerobic glycolytic pathway, it is tempting to speculate that one of the mechanisms underlying low fertility rate in the above mentioned animal model may be related to the inability to produce sufficient amounts of lactate to maintain germ cell development.

As previously mentioned, several biochemical mechanisms may contribute to an increase in SC lactate secretion. The rise in lactate by AMPK activation in SCs is accompanied by an increase in glucose uptake (explained by GLUT1 expression augmentation), a downregulation of MCT1 mRNA levels, and a rise in MCT4 expression (Galardo et al., 2007; Riera et al., 2009). The manner in which MCT1, specialized in lactate import, and MCT4, specialized in lactate export, are regulated in SCs by AMPK activation may reflect a situation of increased lactate export and decreased lactate recapture from the extracellular milieu leading to adequate lactate levels at the disposal of germ cells.

Taking into account that lactate is the main energy source for spermatocytes and spermatids and its production is tightly regulated (Boussouar & Benahmed, 1999; Meroni et al., 2002; Nehar et al., 1998; Rato, Alves, Socorro, Carvalho, et al., 2012; Regueira et al., 2015; Riera et al., 2007; Rocha et al., 2014), it is quite probable that hormones are not the only regulators of lactate production in SCs. Factors of nonprotein nature secreted by germ cells, which indicate a disbalance in energy levels in this cell type, should be considered. Regarding the signaling pathways that might be triggered in SCs by the latter factors released by germ cells, AMPK was postulated as the main candidate involved in restoring ATP levels in seminiferous tubule.

Many years ago, Monaco and Conti (1986) postulated that adenosine resulting from ATP dephosphorylation in germ cells adequates SC function in order to promote energy balance reestablishment in germ cells. In support of the hypothesis that adenosine is secreted by germ cells, it is worth mentioning that Gelain et al. (2003) showed that germ cells in culture release measurable adenosine levels to the extracellular milieu. Even more, the cellular production of adenosine is triggered by an increase in energy consumption or by hypoxia (Newby, 1984).

Most of the adenosine actions demonstrated in a variety of tissues have been studied by treatment with non-metabolizable nucleoside derivatives which bind to purinergic receptors

coupled to specific signal transduction pathways (Bjelobaba et al., 2015). The studies carried out in SCs incubated with nucleoside derivatives enabled to understand that the observed effects were mediated by type A1 purinergic receptor coupled to Gi protein, which activation leads to a decrease in cAMP levels (Conti et al., 1989; Conti et al., 1988; Meroni et al., 1998; Monaco et al., 1984). In addition to binding to purinergic receptors, adenosine can traverse cell membranes through concentrative nucleoside transporters (CNTs) and equilibrative nucleoside transporters (ENTs) which have been described in testicular cells (Kato et al., 2005).

It was shown that in SCs, adenosine increases lactate production regulating the expression of GLUT1, LDHA, and MCT4. Even more, the adenosine effects differ from those by N6-cyclohexyladenosine, a type A1 purinergic receptor agonist fully resistant to cellular uptake. These authors have also demonstrated that adenosine, after entering to SC and being converted into AMP by adenosine kinase, activates AMPK and that the augmentation in lactate production, induced by the nucleoside, depends on AMPK activation (Galardo et al., 2010). Figure A.2 depicts the molecular mechanism involved in the regulation of lactate production regulated by adenosine in the seminiferous tubule.

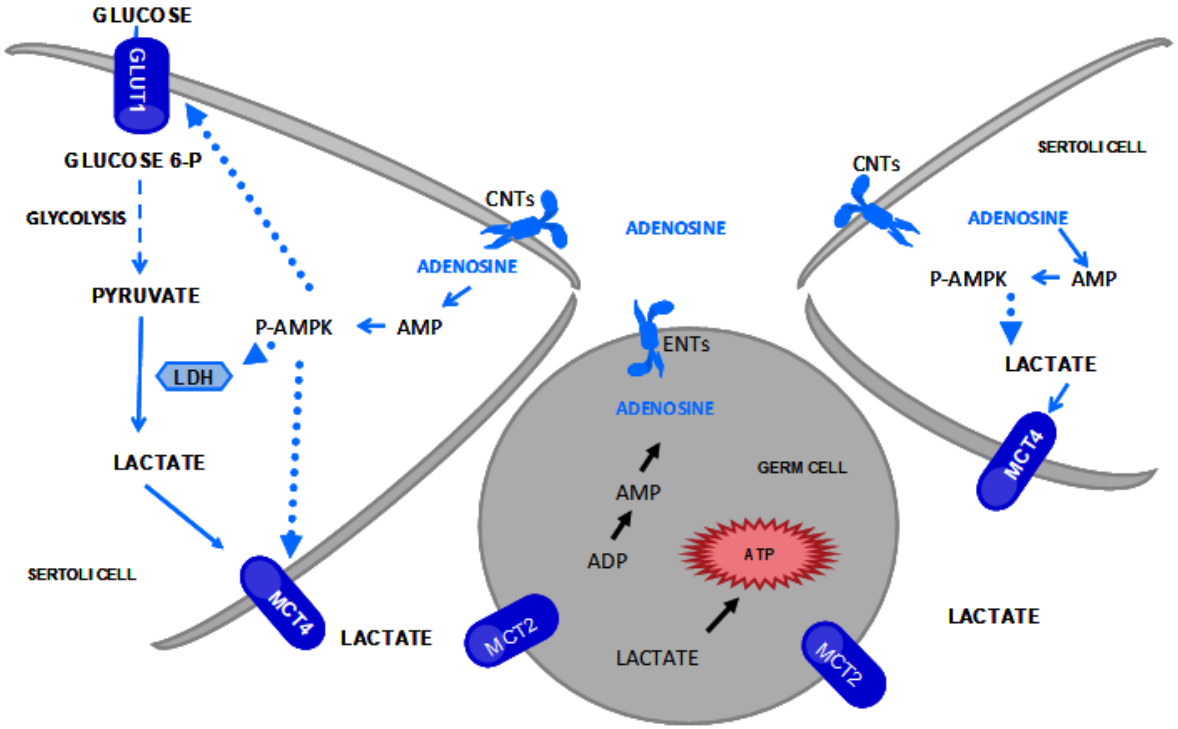


Figure A.2: Molecular mechanism involved in the regulation of lactate production by adenosine in the seminiferous tubule. Molecular mechanism involved in the regulation of lactate production by adenosine in the seminiferous tubule. Adenosine acts as a paracrine regulator between germ cells and Sertoli cells. Adenosine is secreted by germ cell through equilibrative nucleoside transporters (ENTs) and is later imported by Sertoli cells through concentrative nucleoside transporters (CNTs). There, it promotes GLUT1, LDHA and MCT4 expression and increases glucose uptake and LDH activity, resulting in lactate production augmentation. This lactate is exported to germ cells, which use it as nutritional source. Resulting ATP is used by germ cells, inevitably leading to ADP, later converted to AMP, and, finally, adenosine, reinitiating this cycle.

A.1.5 Involvement of Hypoxia-Inducible Factors (HIFs) in Sertoli Cell Nutritional Function

The hypoxia-inducible factor (HIF) plays a central role in the regulation of mammalian metabolism. Active HIF is a heterodimer consisting of an oxygen-dependent HIFα subunit and a constitutively expressed HIFβ subunit. Under normoxic conditions, HIFα subunit is rapidly degraded via a mechanism that involves its hydroxylation by oxygen-dependent prolyl-hydroxylases. Prolyl-hydroxylase inhibition by low oxygen tension or transition metals like cobalt^{2+} (Co^{2+}) (Biswas et al., 2013; Chan et al., 2002) leads to HIFα subunit stabilization, which enables HIFα and HIFβ subunits to associate and form active HIF. Active HIF interacts with a consensus hypoxia response element (HRE) in the promoter region of target genes in order to stimulate transcription. Three isoforms of HIFα subunit (HIF1α HIF2α and HIF3α) have been characterized (Kietzmann et al., 2001). The association of each HIFα subunit with HIFβ subunit constitutes three active transcription factors: HIF1, HIF2, and HIF3 (Kietzmann et al., 2001).

Classically HIF transcriptional activity has been associated with hypoxic conditions. However, some years ago, regulation by hormones of HIF transcriptional activity under normoxic conditions was demonstrated (Fukuda et al., 2002; Laughner et al., 2001). The physiological roles of HIFs are multifarious. While HIF1 and HIF2 regulate the expression of many genes in common, HIF1 preferentially induces genes involved in the glycolytic pathway (Hu et al., 2007; Hu et al., 2003; V. Wang et al., 2005), and HIF2 induces genes important for cell cycle progression like c-Myc (Gordan et al., 2007) and cyclin D1 (Raval et al., 2005).

The expression of HIF1α and HIF2α in SCs was observed (Gruber et al., 2010; Guven et al., 2014), and in rather elegant experiments, a role of HIF2 in BTB integrity was demonstrated (Zimmermann et al., 2015).

Regarding SC nutritional function, the participation of HIFs in the regulation of lactate production was recently evidenced under normoxic conditions (Galardo et al., 2017). Authors have also shown that HIF transcriptional activity is modulated by FSH through the increase of HIF1α and HIF2α expression. Even more, FSH-stimulated lactate production depends on HIF transcriptional activity. The experimental strategy utilized by these authors to evaluate HIF participation in the regulation of lactate production by FSH does not enable to discern which hypoxia-inducible factor, HIF1 and/or HIF2, is involved. Considering that HIF1 is the master regulator of glycolysis, HIF1 may be predicted as the hypoxia-inducible factor that mostly contributes to the regulation of lactate production by FSH. The genes induced by FSH in a HIF-dependent manner, which explain the increase in lactate production, are

GLUT1, PKM2, and LDHA (Firth et al., 1995). Precisely, the latter genes present HRE sequences in their promoters (Hayashi et al., 2004; W. Luo et al., 2011).

The mechanisms that regulate SC metabolism are essential for the maintenance of spermatogenesis and male fertility and deserve deep study. The relevant role of HIFs in modulating SC nutritional function has been elucidated. In addition, HIFs are key components of the intricate pathways utilized by FSH to regulate the provision of lactate for germ cells. Considering that FSH is the master endocrine regulator of SCs, it is not surprising that this hormone may employ several regulatory mechanisms to fulfill the nourishing functions of this cell type and to create the microenvironment where germ cell development takes place.

A.1.6 Regulation of Sertoli Cell Energy Metabolism: Participation of Peroxisome Proliferator-Activated Receptors (PPARs)

As described in previous paragraphs, there are a vast number of studies sought to clarify the molecular mechanisms involved in the maintenance of energy homeostasis of germ cells through SC function modulation. However, the molecular mechanisms that participate in the regulation of ATP levels in SCs have been neglected for many years.

As mentioned before, SC metabolism presents unique characteristics. Glucose is not an important source of energy for SCs; actually it has been shown that this cell type can survive in culture for at least 48 h in the absence of glucose (Riera et al., 2009). Glucose is metabolized to lactate since germ cells situated beyond the blood-testis barrier rely on SC production of this hydroxy acid to obtain energy. In this metabolic scenario, it was observed that palmitate could be oxidized to CO_2, and it was suggested that the oxidation of FAs might yield much of the energy required by SC (Jutte et al., 1985). More recently, it has been demonstrated that FA must be readily available to sustain SC energy status (Xiong et al., 2009). The presence of numerous lipid droplets (LDs) is characteristic in SC, and it may be assumed that these LDs constitute the storage of FAs. The possible regulation of FA utilization and storage, which would ensure FA availability for SC energetic requirements, by receptors that sense lipid status can be imagined.

Peroxisome proliferator-activated receptors (PPARs), α (NR1C1), β/δ (NR1C2), and γ (NR1C3), are ligand-activated transcription factors that are members of the nuclear-hormone receptor superfamily (Issemann & Green, 1990; Michalik et al., 2006). All PPARs function as sensors of FAs and FA derivatives and thus control metabolic pathways involved in lipid and energy metabolism (Green & Wahli, 1994; Krey et al., 1997). It has been well documented that PPARγ mainly mediates lipid anabolism, while PPARα and PPARβ/δ are regulators

of lipid catabolism (Burkart et al., 2007; Chawla et al., 1994; Jump et al., 2005; Tontonoz et al., 1994; Y.-X. Wang et al., 2003). The expression of PPARs varies in different tissues (Kliewer et al., 2001). In adult SCs, PPARα, PPARβ, and PPARγ seem to be expressed at variable degrees (Braissant et al., 1996). Additionally, a developmental pattern of PPAR gene expression in SCs during spermatogenesis, probably reflecting an important physiological role of these nuclear receptors in germ cell development, has been demonstrated (Thomas et al., 2010). Considering the importance of FA utilization and storage for SC metabolic activity, a central role of PPARs in the regulation of SC energetic metabolism could be envisaged. In this context, the role of catabolic PPARs was highlighted by a study carried out by Regueira et al. (2014). These authors have shown that pharmacological PPARα and PPARβ/δ activation regulates the expression of genes involved in FA catabolism such as Fat/CD36, carnitine palmitoyltransferase 1 (CPT1), and long- and medium-chain 3-hydroxyacyl-CoA dehydrogenases (LCAD and MCAD) in SC (Regueira et al., 2014). They have also observed that PPARβ/δ activation can simultaneously regulate FA oxidation and lactate production, and these results have been interpreted as a coordinated mechanism that will ensure the concurrent provision of energy to SC and germ cells.

Regarding PPARγ role in SC metabolism, it is tempting to speculate that activation of this anabolic PPAR exerts effects related with lipid synthesis and storage. Recent evidence shows the participation of PPARγ in the regulation of triacylglyceride (TAG) synthesis and lipid droplet formation in SCs (Gorga et al., 2017). The authors show that the increase in TAG and lipid droplet levels was accompanied by a rise in the expression of genes that are involved in fatty acid storage such as the fatty acid transporter FAT/CD36, the glycerol-3-phosphate-acyltransferase 1 (GPAT1), and perilipins 1, 2, and 3, proteins that participate in lipid droplet formation and stabilization. On the other hand, PPARγ activation increased lactate production. This increase was accompanied by an augmentation in glucose uptake and *Glut2* expression. Altogether, the results suggest that PPARγ activation in SCs participates in the regulation of lipid storage and lactate production ensuring simultaneously the energetic metabolism for Sertoli and germ cells.

In summary, PPAR activation in SC plays a central role in lipid and lactate utilization. On the one hand, PPARα and PPARβ/δ (PPARs with catabolic functions) regulate the expression of genes involved in fatty acid transport and catabolism, and on the other hand, PPARγ regulates anabolic lipid metabolism, increasing TAG and lipid droplet levels. Both PPARβ/δ and PPARγ also participate in the regulation of lactate production to provide energetic substrates for germ cell and positioning these PPARs as main players in the reg-

ulation of seminiferous tubule metabolism. Figure A.3 shows how different PPAR isoform combinations shift SC metabolism toward specific pathways.

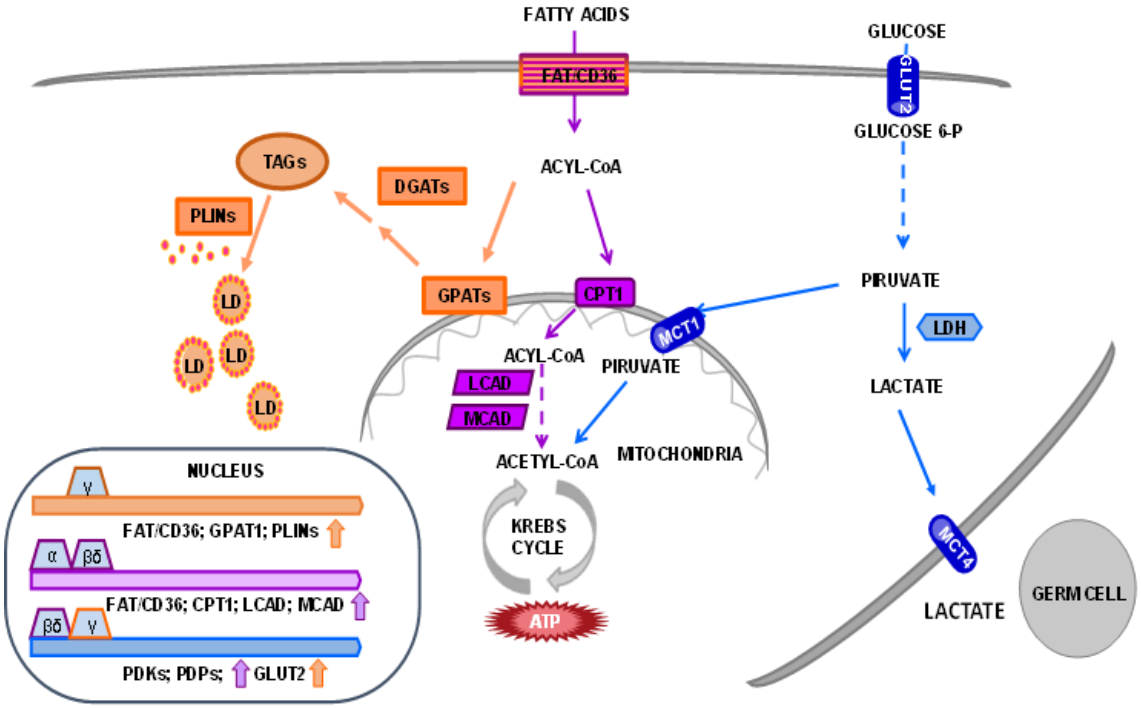


Figure A.3: Role of PPARs in the regulation of fatty acid metabolism and lactate production in Sertoli cells. Each isoform of peroxisome proliferator-activated receptor (PPAR) promotes different metabolic pathways in Sertoli cells. When PPARγis activated, the expression of FAT/CD36, GPAT1 and PLINs is promoted, causing a shift toward lipogenesis. On the other hand, when PPARαand PPARβ/δare activated, Sertoli cell metabolism moves toward full free fatty acid oxidation, due to an increase in FAT/CD36, CPT1, LCAD, and MCAD expression resulting in ATP augmentation. Simultaneously, activation of PPARγand PPARβ/δstimulates the glycolytic pathway, through the increase in GLUT2, PDKs and PDPs expression. In this scenario, lactate production and export to germ cells is also promoted, while a smaller portion of pyruvate is used to obtain ATP.

A.1.7 Impact of Whole-Body Metabolic Dysfunction in Sertoli Cell Metabolism and Spermatogenesis

In the last decades, we have been witnessing a steady increase in male fertility dysfunction and a decrease on fertility rates worldwide but particularly significant on high HDI countries (Hamilton & Ventura, 2006). Simultaneously, the incidence of metabolic disorders, particularly obesity and T2D, significantly increased worldwide and again with special impact in developed countries (WHO, 2000). The overlapping nature of both premises led to the hypothesis that a relation between metabolic dysfunction and male infertility may occur (**Crisóstomo**, Sousa, et al., 2017). In fact, clinical studies seem to confirm this association: obese and overweight men have poorer fertility outcomes than lean men, and thus, couples with a man obese or overweight will have problems to conceive (**Crisóstomo**, Sousa, et al., 2017). Although some causes for those fertility problems were not difficult to associate to

excess of weight, the same is not true for other metabolic disorders such as T2D. Obese and overweight men often have their fertility compromised by mechanic factors, such as testicular overheat due to adipose tissue accumulation or even coitus difficulties and erectile dysfunction linked to cardiovascular complications (Chambers & Richard, 2015). Diabetic men also present fertility dysfunction linked to microvascular disease, reflected as lower tubule diameter, abnormal testicular capillary and lymphatic endothelia, SC-only tubules, and ultrastructural damage in SC apical cytoplasm (Alves, Martins, Cavaco, et al., 2013). It is also reported that microvascular disease increases vascular density and cellular matrix thickening, simultaneously promoting germ cell apoptosis due to increased scrotal temperature and hypoxiainduced cellular damage (Alves, Martins, Cavaco, et al., 2013; J. E. Anderson & Thliveris, 1986; Cai et al., 2000). Moreover, T2D patients often present ejaculatory defects, such as erectile dysfunction and retrograde ejaculation (Alves, Martins, Cavaco, et al., 2013). But those factors do not reflect most of infertility's etiology.

One of the first molecular mechanisms explaining male infertility due to obesity was based on the steroidogenic potential of adipocytes (Belanger et al., 2002). Obese men present higher E_2 levels than the average male population, because they have more fat cells, and those cells present p450 aromatase, an enzyme that converts testosterone to E_2 (Roumaud & Martin, 2015). Estrogens in men have similar effects over the pituitary as in women, exerting a negative feedback over LH and FSH release (Cardoso et al., 2017) and a consequent decrease of testosterone secretion and SC activity. Ultimately, the reproductive axis disruption leads to spermatogenesis defects or even arrest (Alves, Rato, et al., 2013; Cardoso et al., 2017; Rato, Alves, Duarte, et al., 2015). Furthermore, E_2 impairs ionic transport via CFTR on SCs, resulting in less ionic exchanges between lumen and intracellular environment, cytoplasmic acidification, and possibly spermatogenic defects (Bernardino et al., 2016; Bernardino et al., 2013). On the opposite side, fasting suppresses GnRH pulses, thus perturbing reproductive axis, first by inhibiting LH release, which in turn inhibits the release of gonadotropins, and lastly by decreasing androgen secretion by LCs and SC activity (P. F. Oliveira, Sousa, et al., 2017; Rato, Alves, Socorro, Duarte, et al., 2012).

The SC is the central target of environmental toxicants responsible to male reproductive defects, as evidenced by *in vitro* and *in vivo* models (Reis et al., 2015). The first compromising action of those toxicants is the negative effect on junction between SCs and Sertoli-germ cells (Reis et al., 2015). Chemotherapeutic drugs (W. H. B. Wallace et al., 1989), gossypol (Monsees et al., 2000), glycerol (Wiebe et al., 2000), alkylphenols (Raychoudhury et al., 1999), pesticides (Alves, Neuhaus-Oliveira, et al., 2013), and heavy metals (Bizarro et al., 2003) are

just examples of compounds known for interfering with cells' adhesion. Furthermore, some pesticides, phthalates, and obesogens can also work as endocrine disruptors, since their chemical structure is either similar to steroid hormones or competitively inhibits gonadotropin's receptors in SCs (Cardoso et al., 2017; Reis et al., 2015). Those compounds are known as endocrine disruptors because their action causes imbalances on the tightly balanced reproductive axis, disrupting the expected negative feedback mechanisms between the hypothalamus and hypophysis complex and the gonads (Cardoso et al., 2017; Reis et al., 2015). Obesogens aggravate their disrupting effect by promoting adipose tissue accumulation, which causes (1) increasing storage of environmental toxicants, since most of them are fat-soluble, and (2) a self-endocrine disruptor, since adipocytes present significant activity levels of aromatase, an enzyme that converts testosterone to E_2 (Cardoso et al., 2017).

However, the nefarious effects of metabolic disorders are not restricted to systemic defects; they are also reflected *in loco*. Metabolic hormones affect whole-body cell metabolism, and testes are no exception (Alves et al., 2016; Martins et al., 2015; Martins et al., 2016; P. F. Oliveira et al., 2012; P. F. Oliveira, Sousa, et al., 2017; Rato, Alves, Socorro, Duarte, et al., 2012). One of the main metabolites secreted by SC is acetate, which is suggested to be involved in germ cell membrane remodeling. Interestingly, in the absence of insulin, SCs totally inhibit acetate production (Alves, Martins, Rato, et al., 2013), illustrating that insulin dysfunction may cause serious defects on the metabolic support of spermatogenesis by SCs. The close metabolic cooperation between Sertoli and germ cells is based on their proximity, maintained by the numerous cell junctions, which are threatened by changes on environment composition, and metabolic by-products capable to disrupt those junctions (Reis et al., 2015). Glycerol is a suitable example. Its antispermatogenic properties when directly injected in testes, at high concentrations, are caused by BTB disassembly due to tight junctions' cleavage and overall SeT disorganization (Wiebe & Barr, 1984; Wiebe et al., 2000). There are several examples of hormones linked to whole-body metabolic status that also exert direct and indirect effects on SC metabolism (Alves et al., 2016; Martins et al., 2015; P. F. Oliveira et al., 2012; P. F. Oliveira, Sousa, et al., 2017; Rato, Alves, Socorro, Duarte, et al., 2012; Rato et al., 2016).

Leptin increases fat mobilization from adipocytes so it can be used as fuel by cells. Liver is the main destiny of adipocyte-derived triacylglycerols, where they suffer lipolysis. The resulting fatty acids are used for energy production, mainly through β-oxidation (Rodríguez et al., 2011). Resulting glycerol is phosphorylated into G3P and enters the G3P shuttle, a cyclic reaction group that links lipidic and carbohydrate metabolism, TCA cycle, and electron ETC

(Lin, 1977). Obese and diabetic men present higher serum glycerol concentrations resulting from triacylglycerol breakdown (Hagström-Toft et al., 1997). The combination of insulin and leptin also promotes lipid mobilization in SCs for energy production, and they express several AQGPs, a AQP subfamily capable of transporting small solutes such as glycerol, in addition to water (Jesus et al., 2014b; Rodríguez et al., 2011; Yeung et al., 2010). However, glycerol has antispermatogenic properties, since it destabilizes the actin filaments that allow the BTB and the germ cell attachment to SCs (Wiebe et al., 2000). Leptin also promotes GLUT expression and LDH activity in SCs but decreases acetate production (Martins et al., 2015).

Ghrelin is another metabolic hormone whose receptor is present in SCs. Its whole-body metabolic effect is glucose blood and interstitial concentration decrease, due to increased insulin sensitivity of the cells (Vestergaard et al., 2013). Ghrelin concentration is inversely proportional to nutritional state, so obese individuals show the lowest blood concentration (and higher insulin resistance), while undernourished present the highest blood concentration (and lowest insulin resistance) (Martins et al., 2016). In cultured SCs, ghrelin media concentration seems to be inversely proportional to lactate production (Martins et al., 2016), which indicates that fasting may be worse than overeating in what concerns the effects of ghrelin on the nutritional support of spermatogenesisby SCs. However, the ghrelin concentration corresponding to the average in lean man increases the efficiency of glycolytic metabolism, *i.e.*, redirects the cell metabolism to obtain a better lactate-to-glucose ratio, increasing LDH activity while decreasing glucose consumption (Martins et al., 2016). Since reproductive function requires a proper nutritional state, and according to ghrelin concentration variation in function of the nutritional state of the individual, it is legitimate to declare that ghrelin acts as a nutritional state sensor (Lorenzi et al., 2009). Ghrelin inhibits LH release by pituitary in dose-dependent manner. This is especially important for puberty onset, as it needs proper energy store, although this has only been observed in males (Lorenzi et al., 2009). Yet, this effect is valid for adulthood as well; thus, ghrelin may impair the reproductive axis due to LH release inhibition and consequent decrease in testosterone (Lorenzi et al., 2009). In Figure A.4, all the hormonal mechanisms which influence SC function mentioned in this chapter are depicted.

A.1.8 Conclusions

The importance of SCs in spermatogenesis is undeniable. They are extremely specialized cells and had developed unique characteristics to accomplish their function effectively. We must

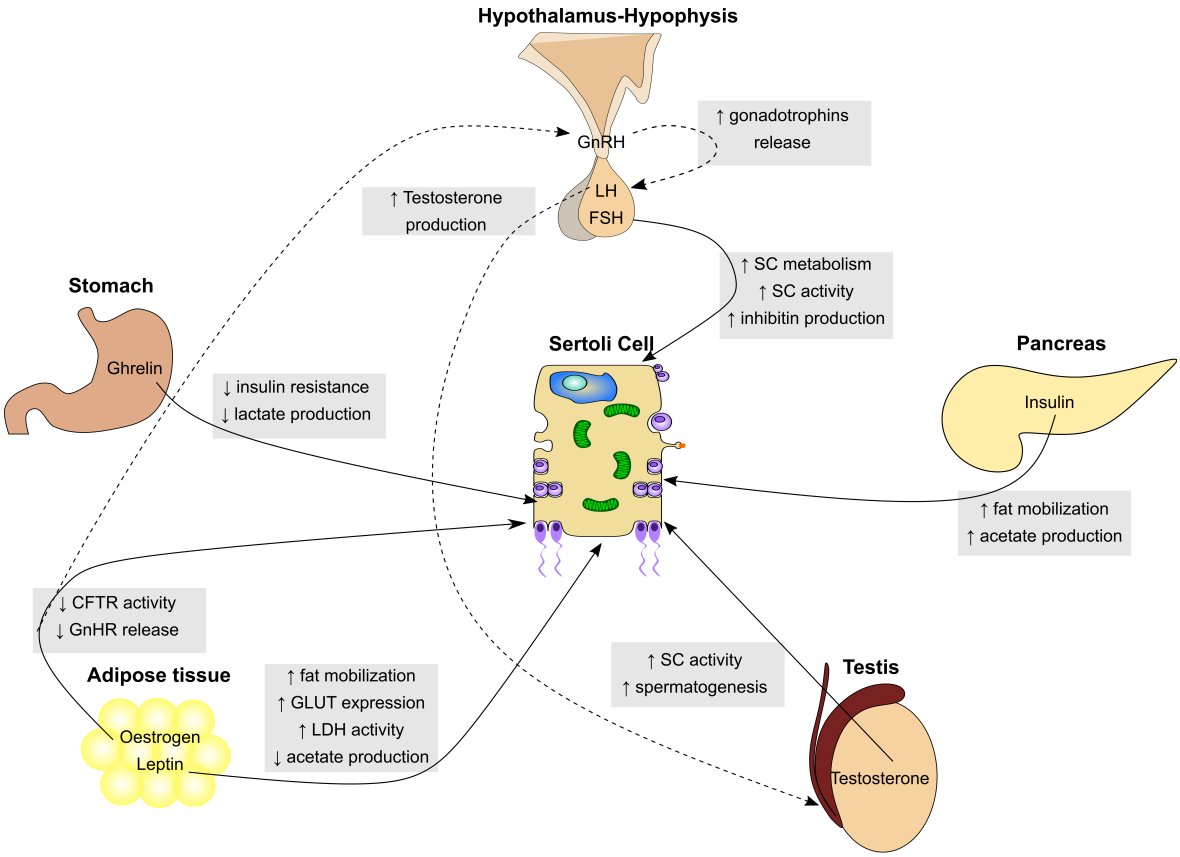


Figure A.4: Hormonal regulation of Sertoli cells. Sertoli cells are directly targeted and react to several hormones. Due to their central role on testicular physiology, those responses potentially affect spermatogenesis outcomes, either positively or negatively. Moreover, the tightly regulated hormonal circuits mean that there are hormones that can indirectly affect Sertoli cell function.

not forget that germ cells are also unique cells in the human body, thus requiring unparalleled conditions. SCs reflect their unique needs and are responsible for creating the suitable environment for germ cell differentiation and survival throughout spermatogenesis, acting as an interface between them and the extracellular environment. Knowing this principle, it becomes easier to understand all the actions promoted by SCs to achieve a successful spermatogenesis. They create the BTB, by the tight junctions, hemidesmosomes and tubulobulbar complexes between SCs, creating the two-compartment system. This barrier impermeates the adluminal space, where germ cells differentiate, while granting it immunological immunity; otherwise, germ cells would be regarded as targets of the immune system. However, this insulation makes difficult the diffusion of substrates for germ cells. Once more, SCs mediate their diffusion, importing the substrates from the extracellular medium and then providing them to the germ cells or exporting them to the tubular fluid while maintaining the right pH and ionic balance of this fluid.

The regulation of the abovementioned functions is complex. SCs must respond to tubular fluid and extracellular medium composition, to the cooperation of the neighboring cells, and

to hormonal regulation. In this chapter, we had shed light over the molecular mechanisms which regulate SC response to the various stimuli. Understanding those processes is pivotal on male fertility research. Any alteration of the metabolites, hormones, and messengers on these processes can deeply impact male fertility. Although there are already plenty bibliographies on the subject, many questions arise from the actual knowledge. The molecular mechanisms which regulate SC is a fertile research ground. Advances on the research on this subject may lead us to new therapeutical approaches against the ever-increasing number of male infertility cases.

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B.2 A switch from high-fat to normal diet does not restore sperm quality but prevents metabolic syndrome

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Author: Pereira, Sara C; Crisóstomo, Luís

Publication: Environmental Epigenetics

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B.4 Diet during early life defines testicular lipid content and sperm quality in adulthood

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Diet during early life defines testicular lipid content and sperm quality in adulthood

Author: Luís Crisóstomo, Romeu A. Videira, Ivana Jarak, et al
Publication: Am J Physiol-Endocrinology and Metabolism
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B.5 Sperm, metabolic memory and echoes from Lamarck

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B.6 Inheritable testicular metabolic memory of high-fat diet causes transgenerational sperm defects in mice

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B.7 Inherited Metabolic Memory of High-Fat Diet Impairs Testicular Fatty Acid Content and Sperm Parameters

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B.8 Testicular "inherited metabolic memory" of ancestral high-fat diet is associated with sperm sncRNA content

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Links and Notes

- Open access citation impact advantage: http://en.wikipedia.org/wiki/Open_access#Authors_and_researchers. For example, a standard research paper "Shutalev, A.D.; Kishko, E.A.; Sivova, N.V.; Kuznetsov, A.Y. *Molecules* **1998**, *3*, 100-106" has been cited 51 times, the highest number among all the papers published so far by the same author.
- Lin, S.-K. *Editorial:* Non-Open Access and Its Adverse Impact on Molecules. *Molecules* **2007**, *12*, 1436-1437 (PDF format 16 K, HTML format).
- Recently a research paper of 30 pages has been published: *Molecules* **2008**, *13*(5), 1081-1110.
- Some well written papers have been peer reviewed and published in less than two weeks from manuscript submission, see e.g.: *Molecules* **2006**, *11*(4), 212-218.

Appendix C

Scripts

C.1 Inheritable testicular metabolic memory of high-fat diet causes transgenerational sperm defects in mice

This annex contains scripts used in the analysis of the published work:

Crisóstomo, **L.**, Jarak, I., Rato, L. P., Raposo, J. F., Batterham, R. L., Oliveira, P. F., & Alves, M. G. (2021). Inheritable testicular metabolic memory of high-fat diet causes transgenerational sperm defects in mice. *Scientific Reports*, *11*(1), 9444. https://doi.org/10.1038/s41598-021-88981-3

These scripts and the datasets are available online at https://doi.org/10.5281/zenodo.4770647.

```
#check package requirements
list.of.packages <- c("dplyr", "curl", "vegan", "RVAideMemoire")
new.packages <- list.of.packages[!(list.of.packages %in% installed.
    packages()[,"Package"])]
if(length(new.packages)) install.packages(new.packages, ask = F,
    dependencies = T)

#import PCA factors file
library(curl)

pca_data <- read.csv(curl("https://raw.githubusercontent.com/luis-
    crisostomo/transgenerational_metabolomics/main/pca_data.csv"))
pca_data <- pca_data[c(2:12)]

#Create Generation-specific dataframes (numeric)
library("dplyr")

gen0 <- mutate_all((subset.data.frame(pca_data, Generation == 0, c
    (3:5))), function(x) as.numeric(as.character(x)))

gen1 <- mutate_all((subset.data.frame(pca_data, Generation == 1, c
    (3,6:7))), function(x) as.numeric(as.character(x)))

gen2 <- mutate_all((subset.data.frame(pca_data, Generation == 2, c
    (3,8:9))), function(x) as.numeric(as.character(x)))

genall <- mutate_all((subset.data.frame(pca_data, select = c
    (2:3,10:11))), function(x) as.numeric(as.character(x)))

#PermANOVA with pairwise comparisons (Vegan + RVAideMemoire package
    ) for forced extraction PCA with 2 principal components
library("vegan")
library("RVAideMemoire")
```

```
#Generation F0 - Global permANOVA result (vegan package)
gen0.stat1 <- adonis2(subset.data.frame(gen0, select = c(2:3)) ~ as
    .factor(gen0$Group), data = gen0, method = "euclidean", by = "
    terms", parallel = getOption("mc.cores"))

#Generation F0 - Pairwise permANOVA results (RVAideMemoire package)
n <- dist(subset.data.frame(gen0, select = c(2:3)), "euclidean")
gen0.stat2raw <- pairwise.perm.manova(n, gen0$Group, nperm = 999,
    progress = FALSE, p.method = "BH", F = TRUE, R2 = TRUE)

vec <- c(c(gen0.stat2raw[["F.value"]]), c(gen0.stat2raw[["R2.value"
    ]]), c(gen0.stat2raw[["p.value"]]))
vec <- vec[!is.na(vec)]
gen0.stat2 <- as.data.frame(matrix(vec, c(3, 3)))
rownames(gen0.stat2) <- c("CTRL vs. HFD", "CTRL vs. HFDt", "HFD vs.
     HFDt")
colnames(gen0.stat2) <- c("F.value", "R2.value", "p.value")

#Generation F0 - write output to .csv
write.table(gen0.stat1, "gen0_global_permanova.csv", sep=";", col.
    names = NA)
write.table(gen0.stat2, "gen0_pairwise_permanova.csv", sep=";", col
    .names = NA)


#Generation F1 - Global permANOVA result (vegan package)
gen1.stat1 <- adonis2(subset.data.frame(gen1, select = c(2:3)) ~ as
    .factor(gen1$Group), data = gen1, method = "euclidean", by = "
    terms", parallel = getOption("mc.cores"))

#Generation F1 - Pairwise permANOVA results (RVAideMemoire package)
n <- dist(subset.data.frame(gen1, select = c(2:3)), "euclidean")
gen1.stat2raw <- pairwise.perm.manova(n, gen1$Group, nperm = 999,
```

```
    progress = FALSE, p.method = "BH", F = TRUE, R2 = TRUE)

vec <- c(c(gen1.stat2raw[["F.value"]]), c(gen1.stat2raw[["R2.value"
    ]]), c(gen1.stat2raw[["p.value"]]))
vec <- vec[!is.na(vec)]
gen1.stat2 <- as.data.frame(matrix(vec, c(3, 3)))
rownames(gen1.stat2) <- c("CTRL vs. HFD", "CTRL vs. HFDt", "HFD vs.
     HFDt")
colnames(gen1.stat2) <- c("F.value", "R2.value", "p.value")

#Generation F1 - write output to .csv
write.table(gen1.stat1, "gen1_global_permanova.csv", sep=";", col.
    names = NA)
write.table(gen1.stat2, "gen1_pairwise_permanova.csv", sep=";", col
    .names = NA)



#Generation F2 - Global permANOVA result (vegan package)
gen2.stat1 <- adonis2(subset.data.frame(gen2, select = c(2:3)) ~ as
    .factor(gen2$Group), data = gen2, method = "euclidean", by = "
    terms", parallel = getOption("mc.cores"))

#Generation F2 - Pairwise permANOVA results (RVAideMemoire package)
n <- dist(subset.data.frame(gen2, select = c(2:3)), "euclidean")
gen2.stat2raw <- pairwise.perm.manova(n, gen2$Group, nperm = 999,
    progress = FALSE, p.method = "BH", F = TRUE, R2 = TRUE)

vec <- c(c(gen2.stat2raw[["F.value"]]), c(gen2.stat2raw[["R2.value"
    ]]), c(gen2.stat2raw[["p.value"]]))
vec <- vec[!is.na(vec)]
gen2.stat2 <- as.data.frame(matrix(vec, c(3, 3)))
rownames(gen2.stat2) <- c("CTRL vs. HFD", "CTRL vs. HFDt", "HFD vs.
     HFDt")
colnames(gen2.stat2) <- c("F.value", "R2.value", "p.value")
```

```
#Generation F2 - write output to .csv
write.table(gen2.stat1, "gen2_global_permanova.csv", sep=";", col.
    names = NA)
write.table(gen2.stat2, "gen2_pairwise_permanova.csv", sep=";", col
    .names = NA)

#All Generations - Global permANOVA result (vegan package)
genall.stat1 <- adonis2(subset.data.frame(genall, select = c(3:4))
    ~ as.factor(genall$Group) + as.factor(genall$Generation) + as.
    factor(genall$Group)*as.factor(genall$Generation), data = genall
    , method = "euclidean", by = "terms", parallel = getOption("mc.
    cores"))

#All Generations - write output to .csv
write.table(genall.stat1, "genall_global_permanova.csv", sep=";",
    col.names = NA)
```

C.2 Inherited metabolic memory of high-fat diet impairs testicular fatty acid content and sperm parameters

This annex contains scripts used in the analysis of the published work:

Crisóstomo, **L.**, Videira, R. A., Jarak, I., Starčević, K., Mašek, T., Rato, L., Raposo, J. F., Batterham, R. L., Oliveira, P. F., & Alves, M. G. (2022). Inherited metabolic memory of high-fat diet impairs testicular fatty acid content and sperm parameters. *Molecular Nutrition and Food Research*, *66*(5), 2100680. https://doi.org/10.1002/mnfr.202100680

These scripts and the datasets are available online at https://doi.org/10.5281/zenodo.4770647.

```
#check package requirements
##CRAN
list.of.packages <- c('dplyr', 'curl', 'vegan', 'mixOmics', 'igraph
    ')
new.packages <- list.of.packages[!(list.of.packages %in% installed.
    packages()[,"Package"])]
if(length(new.packages)) install.packages(new.packages, ask = F,
    dependencies = T)

##Bioconda
if (!requireNamespace('BiocManager', quietly = TRUE))
install.packages('BiocManager')
BiocManager::install()
BiocManager::install('mixOmics')

#import metabolomics and lipidomics files
library(curl)
lipids <- read.csv(curl("https://raw.githubusercontent.com/luis-
    crisostomo/transgenerational_lipidomics/master/Lipidomics.csv"),
     header=TRUE, row.names=1)
metabolites <- read.csv(curl("https://raw.githubusercontent.com/
    luis-crisostomo/transgenerational_lipidomics/master/Metabolomics
    .csv"), header=TRUE, row.names=1)

#Generation F0

##sorting data for statistical methods
lipids_f0 <- subset(lipids, generation == 'F0', 5:19) #select
    lipidomics F0
lipids_f0[] <- lapply(lipids_f0, as.numeric) #change column type to
     numeric (essential for operations)

metabolites_f0 <- subset(metabolites, generation == 'F0', 5:28) #
```

```
    select metabolites F0
metabolites_f0[] <- lapply(metabolites_f0, as.numeric)

lip_met <- c("Glycerol","Choline","Acetate","Phosphocholine","
    Ethanolamine","Phosphoethanolamine","myo.Inositol","triOHbut") #
    vector for metabolite selector
metlip_f0 <- metabolites_f0[lip_met] #dataframe with metabolites
    selected/assigned as lipid precursors

design_f0 <- subset(lipids, generation == 'F0', c('sample', 'group'
    )) #sample info
f0_factors <- as.factor(design_f0$group) #phenotype selectors for
    PLS-DA (exp. groups)

##remove missing values and adapt factors
purge_f0 <- c("c14_0","c16_0","c16_1n7","c18_0","c18_1n9","c18_1n7"
    ,"c18_2n6","c20_3n6","c20_4n6","c22_5n6","c22_6n3") #drop
    columns without data
cleanlipids_f0 <- na.omit(lipids_f0[purge_f0]) #remove rows with
    missing values
cleanfactor_f0 <- as.factor(rep(c("CTRL","HFD","HFDt"), times = c
    (5,3,5))) #remaining samples
clean.metlip_f0 <- metlip_f0[!(rownames(metlip_f0) %in% c("F0_HFD1"
    ,"F0_HFD3","F0_HFD6")),] #equalize row number in the other
    dimension for 2-omics
clean.metabolites_f0 <- metabolites_f0[!(rownames(metabolites_f0) %
    in% c("F0_HFD1","F0_HFD3","F0_HFD6")), !(colnames(metabolites_f0
    ) %in% lip_met)]
labels_f0 <- c("CTRL1","CTRL2","CTRL3","CTRL4","CTRL5","HFD2","HFD4
    ","HFD5","HFDt1","HFDt2","HFDt3","HFDt4","HFDt5")

##run PLS-DA and sPLS-DA
library('mixOmics')
library('igraph')
```

```
###obtain variable loading factors by PLS-DA (2 components) and
    write the output in .csv
result.plsda_f0 <- plsda(cleanlipids_f0, cleanfactor_f0, ncomp = 2)
write.table(result.plsda_f0[["loadings"]][["X"]], "PLS-DA Gen0 -
    Component load.csv", sep=";", col.names = NA)

###sPLS-DA, two components, selecting the number of variables with
    PLS-DA component load > |0.2| for components 1 and 2
result.splsda_f0 <- splsda(cleanlipids_f0, cleanfactor_f0, ncomp =
    2, keepX = c(10, 5))

###Save sPLS-DA plot
tiff("sPLS-DA - Generation F0.tiff", width=600, height=600, units="
    px", res=100)
plotIndiv(result.splsda_f0, ind.names = FALSE, legend=TRUE, ellipse
    = TRUE, title="sPLS-DA - Generation F0")
dev.off()

###Save sPLS-DA plot with background
tiff("sPLS-DA background - Generation F0.tiff", width=600, height
    =600, units="px", res=100)
plotIndiv(result.splsda_f0, comp = 1:2, group = cleanfactor_f0, ind
    .names = labels_f0, title = "Generation F0: sPLS-DA",
legend = FALSE, centroid = TRUE, col.per.group = c("dodgerblue","
    red3","darkgreen"), abline = TRUE, background = background)
dev.off()

##use PERMANOVA to find p-values for sPLS-DA and two-omics sPLS (
    vegan package)
library(vegan)
result.permanova.splsda_f0 <- adonis2(result.splsda_f0[["variates"
    ]][["X"]] ~ cleanfactor_f0, data = as.data.frame(result.splsda_
    f0[["variates"]][["X"]]),
```

```
method = "euclidean", by = "terms", permutations = 999, parallel =
    getOption("mc.cores"))
write.table(result.permanova.splsda_f0, "sPLS-DA Gen0 - PERMANOVA.
    csv", sep=";", col.names = NA)

##run two-omics PLS and sPLS
###obtain variable loading factors by PLS (2 blocks, 2 components)
    and write the output in .csv
result.2omics.pls_f0 <- pls(cleanlipids_f0, clean.metlip_f0)
write.table(result.2omics.pls_f0[["loadings"]][["X"]], "PLS Gen0 -
    Fatty acids.csv", sep=";", col.names = NA)
write.table(result.2omics.pls_f0[["loadings"]][["Y"]], "sPLS Gen0 -
     Lipid-related metabolites.csv", sep=";", col.names = NA)

##two-omics sPLS
###two-omics sPLS, two blocks (fatty acids vs. lipid-related
    metabolites) and two components, selecting the number of
    variables with PLS component load > |0.2| for components 1 and 2
result.2omics.spls_f0 <- spls(cleanlipids_f0, clean.metlip_f0,
    ncomp = 2, keepX = c(9, 5), keepY = c(5,6))

###save 2-Omics sPLS plot
tiff("2-omics sPLS - Generation F0.tiff", width=600, height=600,
    units="px", res=100)
plotIndiv(result.2omics.spls_f0, group = cleanfactor_f0, col.per.
    group = c("dodgerblue","red3","darkgreen"),
rep.space = "XY-variate", centroid = TRUE, ellipse = TRUE, abline =
     TRUE, size.xlabel = 12, size.ylabel = 12, ind.names = labels_f0
    ,
xlim = c(-5,5), ylim = c(-5,5), title = 'Generation F0: 2-omics
    sPLS')
dev.off()

###save 2-Omics sPLS network plot
```

```
svg("2-omics sPLS network - Generation F0.svg")
network(result.2omics.spls_f0, comp = c(1,2), color.node = c('
    orchid', 'brown'),
row.names = c(" C14:0 "," C16:0 ", "C16:1n7", " C18:0 ", "C18:1n9",
     "C18:1n7", "C18:2n6", "C20:3n6", "C20:4n6", "C22:5n6", "C22:6n3
    "),
col.names = c("Glycerol","Choline","Acetate","P-Choline","EtNH2","P
    -EtNH2","m-Inositol","3OH-But"),
cex.node.name = 10, lty.edge = c("solid", "dashed"), lwd.edge = c
    (2,2), cutoff = 0.49)
dev.off()

##use PERMANOVA to find p-values for two-omics sPLS (vegan package)
     and write to .csv
average.dist_f0 <- (result.2omics.spls[["variates"]][["X"]]+result
    .2omics.spls[["variates"]][["Y"]])/2
result.permanova.2omicsspls_f0 <- adonis2(average.dist_f0 ~
    cleanfactor_f0, data = as.data.frame(average.dist_f0), method =
    "euclidean", by = "terms",
permutations = 999, parallel = getOption("mc.cores"))
write.table(result.permanova.2omicsspls_f0, "2omics sPLS Gen0 -
    PERMANOVA.csv", sep=";", col.names = NA)


#Generation F1

##sorting data for statistical methods
lipids_f1 <- subset(lipids, generation == 'F1', 5:19)
lipids_f1[] <- lapply(lipids_f1, as.numeric)

metabolites_f1 <- subset(metabolites, generation == 'F1', 5:28)
metabolites_f1[] <- lapply(metabolites_f1, as.numeric)

lip_met_f1 <- c("Glycerol","Choline","Acetate","Phosphocholine","
```

```
    Ethanolamine","Phosphoethanolamine","myo.Inositol","triOHbut") #
    vector for metabolite selector
metlip_f1 <- metabolites_f1[lip_met_f1]

design_f1 <- subset(lipids, generation == 'F1', c('sample', 'group'
    ))
f1_factors <- as.factor(design_f1$group)

##remove missing values and adapt factors
cleanlipids_f1 <- na.omit(lipids_f1[!(rownames(lipids_f1) %in% c("
    F1_HFDt3"))),])
clean.metlip_f1 <- metlip_f1[rownames(cleanlipids_f1),]
clean.metabolites_f1 <- metabolites_f1[rownames(cleanlipids_f1), !(
    colnames(metabolites_f1) %in% lip_met_f1)]
all(rownames(cleanlipids_f1) == rownames(clean.metlip_f1))
all(rownames(cleanlipids_f1) == rownames(clean.metabolites_f1))
cleanfactor_f1 <- as.factor(rep(c("CTRL","HFD","HFDt"), times = c
    (5,6,5)))
labels_f1 <- c("CTRL1","CTRL3","CTRL4","CTRL5","CTRL6","HFD1","HFD2
    ","HFD3","HFD4","HFD5","HFD6","HFDt1","HFDt2","HFDt4","HFDt5","
    HFDt6")

##run PLS-DA and sPLS-DA

###obtain variable loading factors by PLS-DA (2 components) and
    write the output in .csv
result.plsda_f1 <- plsda(cleanlipids_f1, cleanfactor_f1, ncomp = 2)
write.table(result.plsda_f1[["loadings"]][["X"]], "PLS-DA gen1 -
    Component load.csv", sep=";", col.names = NA)

###sPLS-DA, two components, selecting the number of variables with
    PLS-DA component load > |0.2| for components 1 and 2
result.splsda_f1 <- splsda(cleanlipids_f1, cleanfactor_f1, ncomp =
    2, keepX = c(10, 5))
```

```
###Save sPLS-DA plot
tiff("sPLS-DA - Generation f1.tiff", width=600, height=600, units="
    px", res=100)
plotIndiv(result.splsda_f1, ind.names = FALSE, legend=TRUE, ellipse
    = TRUE, title="sPLS-DA - Generation f1")
dev.off()

###Save sPLS-DA plot with background
tiff("sPLS-DA background - Generation f1.tiff", width=600, height
    =600, units="px", res=100)
plotIndiv(result.splsda_f1, comp = 1:2, group = cleanfactor_f1, ind
    .names = labels_f1, title = "Generation f1: sPLS-DA",
legend = FALSE, centroid = TRUE, col.per.group = c("dodgerblue","
    red3","darkgreen"), abline = TRUE, background = background)
dev.off()

##use PERMANOVA to find p-values for sPLS-DA (vegan package)
result.permanova.splsda_f1 <- adonis2(result.splsda_f1[["variates"
    ]][["X"]] ~ cleanfactor_f1, data = as.data.frame(result.splsda_
    f1[["variates"]][["X"]]),
method = "euclidean", by = "terms", permutations = 999, parallel =
    getOption("mc.cores"))
write.table(result.permanova.splsda_f1, "sPLS-DA gen1 - PERMANOVA.
    csv", sep=";", col.names = NA)

##run two-omics PLS and sPLS
###obtain variable loading factors by PLS (2 blocks, 2 components)
    and write the output in .csv
result.2omics.pls_f1 <- pls(cleanlipids_f1, clean.metlip_f1)
write.table(result.2omics.pls_f1[["loadings"]][["X"]], "PLS gen1 -
    Fatty acids.csv", sep=";", col.names = NA)
write.table(result.2omics.pls_f1[["loadings"]][["Y"]], "sPLS gen1 -
     Lipid-related metabolites.csv", sep=";", col.names = NA)
```

```
##two-omics sPLS
###two-omics sPLS, two blocks (fatty acids vs. lipid-related
    metabolites) and two components, selecting the number of
    variables with PLS component load > |0.2| for components 1 and 2
result.2omics.spls_f1 <- spls(cleanlipids_f1, clean.metlip_f1,
    ncomp = 2, keepX = c(9, 5), keepY = c(5,6))

###save 2-Omics sPLS plot
tiff("2-omics sPLS - Generation f1.tiff", width=600, height=600,
    units="px", res=100)
plotIndiv(result.2omics.spls_f1, group = cleanfactor_f1, col.per.
    group = c("dodgerblue","red3","darkgreen"),
rep.space = "XY-variate", centroid = TRUE, ellipse = TRUE, abline =
     TRUE, size.xlabel = 12, size.ylabel = 12, ind.names = labels_f1
    ,
xlim = c(-5,5), ylim = c(-5,5), title = 'Generation f1: 2-omics
    sPLS')
dev.off()

###save 2-Omics sPLS network plot
svg("2-omics sPLS network - Generation f1.svg")
network(result.2omics.spls_f1, comp = c(1,2), color.node = c('
    orchid', 'brown'),
row.names = c(" C14:0 "," C16:0 ", "C16:1n7", " C18:0 ", "C18:1n9",
     "C18:1n7", "C18:2n6", "C20:3n6", "C20:4n6", "C22:5n6", "C22:6n3
    "),
col.names = c("Glycerol","Choline","Acetate","P-Choline","EtNH2","P
    -EtNH2","m-Inositol","3OH-But"),
cex.node.name = 10, lty.edge = c("solid", "dashed"), lwd.edge = c
    (2,2), cutoff = 0.49)
dev.off()

##use PERMANOVA to find p-values for two-omics sPLS (vegan package)
```

```
    and write to .csv
average.dist_f1 <- (result.2omics.spls[["variates"]][["X"]]+result
    .2omics.spls[["variates"]][["Y"]])/2
result.permanova.2omicsspls_f1 <- adonis2(average.dist_f1 ~
    cleanfactor_f1, data = as.data.frame(average.dist_f1), method =
    "euclidean", by = "terms",
permutations = 999, parallel = getOption("mc.cores"))
write.table(result.permanova.2omicsspls_f1, "2omics sPLS gen1 -
    PERMANOVA.csv", sep=";", col.names = NA)


#Generation F2

##sorting data for statistical methods

lipids_f2 <- subset(lipids, generation == 'F2', 5:19)
lipids_f2[] <- lapply(lipids_f2, as.numeric)

metabolites_f2 <- subset(metabolites, generation == 'F2', 5:28)
metabolites_f2[] <- lapply(metabolites_f2, as.numeric)

lip_met_f2 <- c("Glycerol","Choline","Acetate","Phosphocholine","
    Ethanolamine","Phosphoethanolamine","myo.Inositol","triOHbut")
metlip_f2 <- metabolites_f2[lip_met_f2]

design_f2 <- subset(lipids, generation == 'F2', c('sample', 'group'
    ))
f2_factors <- as.factor(design_f2$group)

##remove missing values and adapt factors
###for sPLS-DA only
cleanlipids_1_f2 <- lipids_f2[!(rownames(lipids_f2) %in% "F2_CTRL1"
    ),] #remove rows with missing values
cleanfactor_1_f2 <- as.factor(rep(c("CTRL","HFD","HFDt"), times = c
```

```
    (5,5,6))) #remaining samples
labels_1_f2 <- c("CTRL2","CTRL3","CTRL4","CTRL5","CTRL6","HFD1","
    HFD2","HFD3","HFD5","HFD6","HFDt1","HFDt2","HFDt3","HFDt4","
    HFDt5","HFDt6")

cleanlipids_f2 <- na.omit(lipids_f2[!(rownames(lipids_f2) %in% c("
    F2_CTRL1","F2_HFD2","F2_HFDt5")),])
clean.metlip_f2 <- metlip_f2[rownames(cleanlipids_f2),] #remove
    samples with missing value in lipids
clean.metabolites_f2 <- metabolites_f2[rownames(cleanlipids_f2), !(
    colnames(metabolites_f2) %in% lip_met_f2)]
all(rownames(cleanlipids_f2) == rownames(clean.metlip_f2))
all(rownames(cleanlipids_f2) == rownames(clean.metabolites_f2)) #
    verify consistency between tables
cleanfactor_f2 <- as.factor(rep(c("CTRL","HFD","HFDt"), times = c
    (5,4,5))) #remaining samples
labels_f2 <- c("CTRL2","CTRL3","CTRL4","CTRL5","CTRL6","HFD1","HFD3
    ","HFD5","HFD6","HFDt1","HFDt2","HFDt3","HFDt4","HFDt6")

##run PLS-DA and sPLS-DA

###obtain variable loading factors by PLS-DA (2 components) and
    write the output in .csv
result.plsda_f2 <- plsda(cleanlipids_1_f2, cleanfactor_1_f2, ncomp
    = 2)
write.table(result.plsda_f2[["loadings"]][["X"]], "PLS-DA gen2 -
    Component load.csv", sep=";", col.names = NA)

###sPLS-DA, two components, selecting the number of variables with
    PLS-DA component load > |0.2| for components 1 and 2
result.splsda_f2 <- splsda(cleanlipids_1_f2, cleanfactor_1_f2,
    ncomp = 2, keepX = c(10, 5))

###Save sPLS-DA plot
```

```
tiff("sPLS-DA - Generation f2.tiff", width=600, height=600, units="
    px", res=100)
plotIndiv(result.splsda_f2, ind.names = FALSE, legend=TRUE, ellipse
    = TRUE, title="sPLS-DA - Generation f2")
dev.off()

###Save sPLS-DA plot with background
tiff("sPLS-DA background - Generation f2.tiff", width=600, height
    =600, units="px", res=100)
plotIndiv(result.splsda_f2, comp = 1:2, group = cleanfactor_1_f2,
    ind.names = labels_1_f2, title = "Generation f2: sPLS-DA",
legend = FALSE, centroid = TRUE, col.per.group = c("dodgerblue","
    red3","darkgreen"), abline = TRUE, background = background)
dev.off()

##use PERMANOVA to find p-values for sPLS-DA (vegan package)
library(vegan)
result.permanova.splsda_f2 <- adonis2(result.splsda_f2[["variates"
    ]][["X"]] ~ cleanfactor_1_f2, data = as.data.frame(result.splsda
    _f2[["variates"]][["X"]]),
method = "euclidean", by = "terms", permutations = 999, parallel =
    getOption("mc.cores"))
write.table(result.permanova.splsda_f2, "sPLS-DA gen2 - PERMANOVA.
    csv", sep=";", col.names = NA)

##run two-omics PLS and sPLS
###obtain variable loading factors by PLS (2 blocks, 2 components)
    and write the output in .csv
result.2omics.pls_f2 <- pls(cleanlipids_f2, clean.metlip_f2)
write.table(result.2omics.pls_f2[["loadings"]][["X"]], "PLS gen2 -
    Fatty acids.csv", sep=";", col.names = NA)
write.table(result.2omics.pls_f2[["loadings"]][["Y"]], "sPLS gen2 -
     Lipid-related metabolites.csv", sep=";", col.names = NA)
```

```
##two-omics sPLS
###two-omics sPLS, two blocks (fatty acids vs. lipid-related
    metabolites) and two components, selecting the number of
    variables with PLS component load > |0.2| for components 1 and 2
result.2omics.spls_f2 <- spls(cleanlipids_f2, clean.metlip_f2,
    ncomp = 2, keepX = c(9, 5), keepY = c(5,6))

###save 2-Omics sPLS plot
tiff("2-omics sPLS - Generation f2.tiff", width=600, height=600,
    units="px", res=100)
plotIndiv(result.2omics.spls_f2, group = cleanfactor_f2, col.per.
    group = c("dodgerblue","red3","darkgreen"),
rep.space = "XY-variate", centroid = TRUE, ellipse = TRUE, abline =
     TRUE, size.xlabel = 12, size.ylabel = 12, ind.names = labels_f2
    ,
xlim = c(-5,5), ylim = c(-5,5), title = 'Generation f2: 2-omics
    sPLS')
dev.off()

###save 2-Omics sPLS network plot
svg("2-omics sPLS network - Generation f2.svg")
network(result.2omics.spls_f2, comp = c(1,2), color.node = c('
    orchid', 'brown'),
row.names = c(" C14:0 "," C16:0 ", "C16:1n7", " C18:0 ", "C18:1n9",
     "C18:1n7", "C18:2n6", "C20:3n6", "C20:4n6", "C22:5n6", "C22:6n3
    "),
col.names = c("Glycerol","Choline","Acetate","P-Choline","EtNH2","P
    -EtNH2","m-Inositol","3OH-But"),
cex.node.name = 10, lty.edge = c("solid", "dashed"), lwd.edge = c
    (2,2), cutoff = 0.49)
dev.off()

##use PERMANOVA to find p-values for two-omics sPLS (vegan package)
     and write to .csv
```

```
average.dist_f2 <- (result.2omics.spls[["variates"]][["X"]]+result
    .2omics.spls[["variates"]][["Y"]])/2
result.permanova.2omicsspls_f2 <- adonis2(average.dist_f2 ~
    cleanfactor_f2, data = as.data.frame(average.dist_f2), method =
    "euclidean", by = "terms",
permutations = 999, parallel = getOption("mc.cores"))
write.table(result.permanova.2omicsspls_f2, "2omics sPLS gen2 -
    PERMANOVA.csv", sep=";", col.names = NA)
```

C.3 Testicular "inherited metabolic memory" of ancestral high-fat diet is associated with sperm sncRNA content

This annex contains scripts used in the analysis of the published work:

Crisóstomo, **L.**, Bourgery, M., Rato, L., Raposo, J. F., Batterham, R. L., Kotaja, N., & Alves, M. G. (2022). Testicular *"inherited metabolic memory"* of ancestral high-fat diet is associated with sperm sncrna content. *Biomedicines*, *10*(4), 909. https://doi.org/10.3390/biomedicines10040909

These scripts and the datasets are available online at https://doi.org/10.5281/zenodo.6001903.

Sequence annotation

```
Access the databases:
-g ~/Documents/Databases/Mus_musculus_db/UCSC/mm10/Sequence/
    BowtieIndex/genome
-m ~/Documents/Databases/Mus_musculus_db/miRBase_22/miRBase_22-mmu
-r ~/Documents/Databases/Mus_musculus_db/rRNAdb/mouse_rRNA
-t ~/Documents/Databases/Mus_musculus_db/GtRNAdb/mm10-tRNAs
-w ~/Documents/Databases/Mus_musculus_db/piRBase/piR_mouse

Access the file directory:
/home/luis_crisostomo/SncRNA_analysis/SMALL_RNA_rawdata

tar -zxvf */F0_CTRL7_S3_R1_001.fastq.gz

gunzip -dk */F0_CTRL7_S3_R1_001.fastq.gz

Run cutadapt, adapted for Illumina sequencing system (unrestricted
    number of bases per sequence)
cutadapt -j 0 -a TGGAATTCTCGGGTGCCAAGG --nextseq-trim=20 --discard-
    untrimmed ~/Documents/sncRNA_analysis/SMALL_RNA_rawdata/
    F0_CTRL7_S3_R1_001.fastq | cutadapt -j 0 -u 4 -u -4 - > ~/
    Documents/sncRNA_analysis/Trimmed_samples/
    F0_CTRL7_S3_R1_001_trim.fastq
cutadapt -j 0 -a TGGAATTCTCGGGTGCCAAGG --nextseq-trim=20 --discard-
    untrimmed ~/Documents/sncRNA_analysis/SMALL_RNA_rawdata/
    F0_CTRL8_S4_R1_001.fastq | cutadapt -j 0 -u 4 -u -4 - > ~/
    Documents/sncRNA_analysis/Trimmed_samples/
    F0_CTRL8_S4_R1_001_trim.fastq
cutadapt -j 0 -a TGGAATTCTCGGGTGCCAAGG --nextseq-trim=20 --discard-
    untrimmed ~/Documents/sncRNA_analysis/SMALL_RNA_rawdata/
    F0_CTRL9_S5_R1_001.fastq | cutadapt -j 0 -u 4 -u -4 - > ~/
    Documents/sncRNA_analysis/Trimmed_samples/
    F0_CTRL9_S5_R1_001_trim.fastq
```

```
cutadapt -j 0 -a TGGAATTCTCGGGTGCCAAGG --nextseq-trim=20 --discard-
    untrimmed ~/Documents/sncRNA_analysis/SMALL_RNA_rawdata/
    F0_HFD4_S6_R1_001.fastq | cutadapt -j 0 -u 4 -u -4 - > ~/
    Documents/sncRNA_analysis/Trimmed_samples/F0_HFD4_S6_R1_001_trim
    .fastq
cutadapt -j 0 -a TGGAATTCTCGGGTGCCAAGG --nextseq-trim=20 --discard-
    untrimmed ~/Documents/sncRNA_analysis/SMALL_RNA_rawdata/
    F0_HFD7_S7_R1_001.fastq | cutadapt -j 0 -u 4 -u -4 - > ~/
    Documents/sncRNA_analysis/Trimmed_samples/F0_HFD7_S7_R1_001_trim
    .fastq
cutadapt -j 0 -a TGGAATTCTCGGGTGCCAAGG --nextseq-trim=20 --discard-
    untrimmed ~/Documents/sncRNA_analysis/SMALL_RNA_rawdata/
    F0_HFD8_S8_R1_001.fastq | cutadapt -j 0 -u 4 -u -4 - > ~/
    Documents/sncRNA_analysis/Trimmed_samples/F0_HFD8_S8_R1_001_trim
    .fastq
cutadapt -j 0 -a TGGAATTCTCGGGTGCCAAGG --nextseq-trim=20 --discard-
    untrimmed ~/Documents/sncRNA_analysis/SMALL_RNA_rawdata/
    F0_HFDT1_S9_R1_001.fastq | cutadapt -j 0 -u 4 -u -4 - > ~/
    Documents/sncRNA_analysis/Trimmed_samples/
    F0_HFDT1_S9_R1_001_trim.fastq
cutadapt -j 0 -a TGGAATTCTCGGGTGCCAAGG --nextseq-trim=20 --discard-
    untrimmed ~/Documents/sncRNA_analysis/SMALL_RNA_rawdata/
    F0_HFDT5_S11_R1_001.fastq | cutadapt -j 0 -u 4 -u -4 - > ~/
    Documents/sncRNA_analysis/Trimmed_samples/
    F0_HFDT5_S11_R1_001_trim.fastq
cutadapt -j 0 -a TGGAATTCTCGGGTGCCAAGG --nextseq-trim=20 --discard-
    untrimmed ~/Documents/sncRNA_analysis/SMALL_RNA_rawdata/
    F0_HFDT8_S10_R1_001.fastq | cutadapt -j 0 -u 4 -u -4 - > ~/
    Documents/sncRNA_analysis/Trimmed_samples/
    F0_HFDT8_S10_R1_001_trim.fastq
cutadapt -j 0 -a TGGAATTCTCGGGTGCCAAGG --nextseq-trim=20 --discard-
    untrimmed ~/Documents/sncRNA_analysis/SMALL_RNA_rawdata/
    F1_CTRL3_S12_R1_001.fastq | cutadapt -j 0 -u 4 -u -4 - > ~/
    Documents/sncRNA_analysis/Trimmed_samples/
```

```
    F1_CTRL3_S12_R1_001_trim.fastq
cutadapt -j 0 -a TGGAATTCTCGGGTGCCAAGG --nextseq-trim=20 --discard-
    untrimmed ~/Documents/sncRNA_analysis/SMALL_RNA_rawdata/
    F1_CTRL6_S13_R1_001.fastq | cutadapt -j 0 -u 4 -u -4 - > ~/
    Documents/sncRNA_analysis/Trimmed_samples/
    F1_CTRL6_S13_R1_001_trim.fastq
cutadapt -j 0 -a TGGAATTCTCGGGTGCCAAGG --nextseq-trim=20 --discard-
    untrimmed ~/Documents/sncRNA_analysis/SMALL_RNA_rawdata/
    F1_HFD5_S14_R1_001.fastq | cutadapt -j 0 -u 4 -u -4 - > ~/
    Documents/sncRNA_analysis/Trimmed_samples/
    F1_HFD5_S14_R1_001_trim.fastq
cutadapt -j 0 -a TGGAATTCTCGGGTGCCAAGG --nextseq-trim=20 --discard-
    untrimmed ~/Documents/sncRNA_analysis/SMALL_RNA_rawdata/
    F1_HFD8_S15_R1_001.fastq | cutadapt -j 0 -u 4 -u -4 - > ~/
    Documents/sncRNA_analysis/Trimmed_samples/
    F1_HFD8_S15_R1_001_trim.fastq
cutadapt -j 0 -a TGGAATTCTCGGGTGCCAAGG --nextseq-trim=20 --discard-
    untrimmed ~/Documents/sncRNA_analysis/SMALL_RNA_rawdata/
    F1_HFDT1_S2_R1_001.fastq | cutadapt -j 0 -u 4 -u -4 - > ~/
    Documents/sncRNA_analysis/Trimmed_samples/
    F1_HFDT1_S2_R1_001_trim.fastq
cutadapt -j 0 -a TGGAATTCTCGGGTGCCAAGG --nextseq-trim=20 --discard-
    untrimmed ~/Documents/sncRNA_analysis/SMALL_RNA_rawdata/
    F1_HFDT2_S16_R1_001.fastq | cutadapt -j 0 -u 4 -u -4 - > ~/
    Documents/sncRNA_analysis/Trimmed_samples/
    F1_HFDT2_S16_R1_001_trim.fastq
cutadapt -j 0 -a TGGAATTCTCGGGTGCCAAGG --nextseq-trim=20 --discard-
    untrimmed ~/Documents/sncRNA_analysis/SMALL_RNA_rawdata/
    F2_CTRL5_S17_R1_001.fastq | cutadapt -j 0 -u 4 -u -4 - > ~/
    Documents/sncRNA_analysis/Trimmed_samples/
    F2_CTRL5_S17_R1_001_trim.fastq
cutadapt -j 0 -a TGGAATTCTCGGGTGCCAAGG --nextseq-trim=20 --discard-
    untrimmed ~/Documents/sncRNA_analysis/SMALL_RNA_rawdata/
    F2_CTRL7_S18_R1_001.fastq | cutadapt -j 0 -u 4 -u -4 - > ~/
```

```
    Documents/sncRNA_analysis/Trimmed_samples/
    F2_CTRL7_S18_R1_001_trim.fastq
cutadapt -j 0 -a TGGAATTCTCGGGTGCCAAGG --nextseq-trim=20 --discard-
    untrimmed ~/Documents/sncRNA_analysis/SMALL_RNA_rawdata/
    F2_CTRL9_S19_R1_001.fastq | cutadapt -j 0 -u 4 -u -4 - > ~/
    Documents/sncRNA_analysis/Trimmed_samples/
    F2_CTRL9_S19_R1_001_trim.fastq
cutadapt -j 0 -a TGGAATTCTCGGGTGCCAAGG --nextseq-trim=20 --discard-
    untrimmed ~/Documents/sncRNA_analysis/SMALL_RNA_rawdata/
    F2_HFD5_S1_R1_001.fastq | cutadapt -j 0 -u 4 -u -4 - > ~/
    Documents/sncRNA_analysis/Trimmed_samples/F2_HFD5_S1_R1_001_trim
    .fastq
cutadapt -j 0 -a TGGAATTCTCGGGTGCCAAGG --nextseq-trim=20 --discard-
    untrimmed ~/Documents/sncRNA_analysis/SMALL_RNA_rawdata/
    F2_HFD6_S20_R1_001.fastq | cutadapt -j 0 -u 4 -u -4 - > ~/
    Documents/sncRNA_analysis/Trimmed_samples/
    F2_HFD6_S20_R1_001_trim.fastq
cutadapt -j 0 -a TGGAATTCTCGGGTGCCAAGG --nextseq-trim=20 --discard-
    untrimmed ~/Documents/sncRNA_analysis/SMALL_RNA_rawdata/
    F2_HFD11_S21_R1_001.fastq | cutadapt -j 0 -u 4 -u -4 - > ~/
    Documents/sncRNA_analysis/Trimmed_samples/
    F2_HFD11_S21_R1_001_trim.fastq
cutadapt -j 0 -a TGGAATTCTCGGGTGCCAAGG --nextseq-trim=20 --discard-
    untrimmed ~/Documents/sncRNA_analysis/SMALL_RNA_rawdata/
    F2_HFDT4_S24_R1_001.fastq | cutadapt -j 0 -u 4 -u -4 - > ~/
    Documents/sncRNA_analysis/Trimmed_samples/
    F2_HFDT4_S24_R1_001_trim.fastq
cutadapt -j 0 -a TGGAATTCTCGGGTGCCAAGG --nextseq-trim=20 --discard-
    untrimmed ~/Documents/sncRNA_analysis/SMALL_RNA_rawdata/
    F2_HFDT5_S22_R1_001.fastq | cutadapt -j 0 -u 4 -u -4 - > ~/
    Documents/sncRNA_analysis/Trimmed_samples/
    F2_HFDT5_S22_R1_001_trim.fastq
cutadapt -j 0 -a TGGAATTCTCGGGTGCCAAGG --nextseq-trim=20 --discard-
    untrimmed ~/Documents/sncRNA_analysis/SMALL_RNA_rawdata/
```

```
    F2_HFDT8_S23_R1_001.fastq | cutadapt -j 0 -u 4 -u -4 - > ~/
    Documents/sncRNA_analysis/Trimmed_samples/
    F2_HFDT8_S23_R1_001_trim.fastq

Annotation using SPORTS1.1, untrimming sequences:
sports.pl -i ~/Documents/sncRNA_analysis/Trimmed_samples/ -p 8 -g
    ~/Documents/Databases/Mus_musculus_db/UCSC/mm10/Genome/genome -m
     ~/Documents/Databases/Mus_musculus_db/miRBase_22/miRBase_22-mmu
     -r ~/Documents/Databases/Mus_musculus_db/rRNAdb/mouse_rRNA -t
    ~/Documents/Databases/Mus_musculus_db/GtRNAdb/mm10/mm10-tRNAs -w
     ~/Documents/Databases/Mus_musculus_db/piRBase/piR_mouse -o ~/
    Documents/sncRNA_analysis/Output_SPORTS/ -k


Pipeline for piRNA clusters and repRNA using HISAT2/Samtools/
    featureCounts

-Codes for HISAT2
hisat2 -p 4 -x ~/Documents/Mus_musculus_db/hisat2-mm10/genome -q ~/
    Documents/sncRNA_analysis/Trimmed_samples/
    F0_CTRL7_S3_R1_001_trim.fastq -S ~/Documents/Output_HISAT2/
    F0_CTRL7.sam

-Samtools
samtools sort -@ 4 -o ~/Documents/Output_HISAT2/F0_CTRL7.bam ~/
    Documents/Output_HISAT2/F0_CTRL7.sam
samtools index ~/Documents/Output_HISAT2/F0_CTRL7.bam ~/Documents/
    Output_HISAT2/F0_CTRL7.bam.bai

-FeatureCounts (pachytene piRNA clusters):
featureCounts -t piRNA -g ID -M --fraction -a ~/Documents/
    Mus_musculus_db/merge_mm10_piRNAcluster.bed -o ~/Documents/
    Output_FC/counts_piRNAs_clusters.txt ~/Documents/Output_HISAT2/
    F0_CTRL7.sam
```

```
-FeatureCounts (repeats):
featureCounts -t piRNA -g ID -M --fraction -a ~/Documents/
    Mus_musculus_db/rmsk1_notRNA_norRNA.bed -o ~/Documents/Output_FC
    /counts_repeat_elements.txt ~/Documents/Output_HISAT2/F0_CTRL7.
    sam
```

Differential expression: sncRNA biotypes

```
#read input file
library(readxl)
library(dplyr)
countData <- read_excel("C:/Users/luisd/Dropbox/UP/Doutoramento em
    Ciências Biomédicas/Resultados/RNAseq/Results/sncRNA_
    distribution_results/cts.xlsx", col_types = c("text", "numeric",
     "numeric", "numeric", "numeric", "numeric", "numeric", "numeric
    ", "numeric", "numeric", "numeric", "numeric", "numeric", "
    numeric", "numeric", "numeric", "numeric", "numeric", "numeric",
     "numeric", "numeric", "numeric", "numeric", "numeric", "numeric
    "))

u <- pull(countData [, 1])
countData <- subset.data.frame(countData,,c(2:25))
rownames(countData) <- u

#read sample info file
colData <- read_excel("C:/Users/luisd/Dropbox/UP/Doutoramento em
    Ciências Biomédicas/Resultados/RNAseq/Results/sncRNA_
    distribution_results/coldata.xlsx")
colData <- mutate_if(colData, is.character, as.factor)
u <- pull(colData [, 1])
rownames(colData) <- u
colData <- subset.data.frame(colData,,c(2:4))
rownames(colData) <- u
```

```
remove(u)
all(rownames(colData) == colnames(countData)) #verify consistency
    between tables



library(DESeq2)
#normalization of all samples seting CTRL_F0 as reference
#dds_total <- DESeqDataSetFromMatrix(countData = countData, colData
    = colData, design = ~ Gen + Group + Gen:Group) #create
    DESeqDataSet object
dds_total <- DESeqDataSetFromMatrix(countData = countData,colData =
     colData, design = ~ GenGroup)
dds$GenGroup <- relevel(dds$GenGroup, ref = "CTRL_F0") #set
    reference
dds_total2 <- DESeq(dds_total)

##obtain the F0 comparisons
res_total1 <- results(dds_total2, contrast=c("GenGroup","CTRL_F0","
    HFD_F0"))
res_total2 <- results(dds_total2, contrast=c("GenGroup","CTRL_F0","
    HFDt_F0"))
res_total3 <- results(dds_total2, contrast=c("GenGroup","HFD_F0","
    HFDt_F0"))
write.table(as.data.frame(res_total1), file="CTRL_F0 vs. HFD_F0.csv
    ", sep=";", col.names = NA)
write.table(as.data.frame(res_total2), file="CTRL_F0 vs. HFDt_F0.
    csv", sep=";", col.names = NA)
write.table(as.data.frame(res_total3), file="HFD_F0 vs. HFDt_F0.csv
    ", sep=";", col.names = NA)

##obtain the F1 comparisons
res_total1 <- results(dds_total2, contrast=c("GenGroup","CTRL_F1","
    HFD_F1"))
res_total2 <- results(dds_total2, contrast=c("GenGroup","CTRL_F1","
```

```
    HFDt_F1"))
res_total3 <- results(dds_total2, contrast=c("GenGroup","HFD_F1","
    HFDt_F1"))
write.table(as.data.frame(res_total1), file="CTRL_F1 vs. HFD_F1.csv
    ", sep=";", col.names = NA)
write.table(as.data.frame(res_total2), file="CTRL_F1 vs. HFDt_F1.
    csv", sep=";", col.names = NA)
write.table(as.data.frame(res_total3), file="HFD_F1 vs. HFDt_F1.csv
    ", sep=";", col.names = NA)

##obtain the F2 comparisons
res_total1 <- results(dds_total2, contrast=c("GenGroup","CTRL_F2","
    HFD_F2"))
res_total2 <- results(dds_total2, contrast=c("GenGroup","CTRL_F2","
    HFDt_F2"))
res_total3 <- results(dds_total2, contrast=c("GenGroup","HFD_F2","
    HFDt_F2"))
write.table(as.data.frame(res_total1), file="CTRL_F2 vs. HFD_F2.csv
    ", sep=";", col.names = NA)
write.table(as.data.frame(res_total2), file="CTRL_F2 vs. HFDt_F2.
    csv", sep=";", col.names = NA)
write.table(as.data.frame(res_total3), file="HFD_F2 vs. HFDt_F2.csv
    ", sep=";", col.names = NA)

##obtain CTRL transgenerational comparisons
res_total1 <- results(dds_total2, contrast=c("GenGroup","CTRL_F0","
    CTRL_F1"))
res_total2 <- results(dds_total2, contrast=c("GenGroup","CTRL_F0","
    CTRL_F2"))
res_total3 <- results(dds_total2, contrast=c("GenGroup","CTRL_F1","
    CTRL_F2"))
write.table(as.data.frame(res_total1), file="CTRL_F0 vs. CTRL_F1.
    csv", sep=";", col.names = NA)
write.table(as.data.frame(res_total2), file="CTRL_F0 vs. CTRL_F2.
```

```
    csv", sep=";", col.names = NA)
write.table(as.data.frame(res_total3), file="CTRL_F1 vs. CTRL_F2.
    csv", sep=";", col.names = NA)

##obtain HFD transgenerational comparisons
res_total1 <- results(dds_total2, contrast=c("GenGroup","HFD_F0","
    HFD_F1"))
res_total2 <- results(dds_total2, contrast=c("GenGroup","HFD_F0","
    HFD_F2"))
res_total3 <- results(dds_total2, contrast=c("GenGroup","HFD_F1","
    HFD_F2"))
write.table(as.data.frame(res_total1), file="HFD_F0 vs. HFD_F1.csv"
    , sep=";", col.names = NA)
write.table(as.data.frame(res_total2), file="HFD_F0 vs. HFD_F2.csv"
    , sep=";", col.names = NA)
write.table(as.data.frame(res_total3), file="HFD_F1 vs. HFD_F2.csv"
    , sep=";", col.names = NA)

##obtain HFDt transgenerational comparisons
res_total1 <- results(dds_total2, contrast=c("GenGroup","HFDt_F0","
    HFDt_F1"))
res_total2 <- results(dds_total2, contrast=c("GenGroup","HFDt_F0","
    HFDt_F2"))
res_total3 <- results(dds_total2, contrast=c("GenGroup","HFDt_F1","
    HFDt_F2"))
write.table(as.data.frame(res_total1), file="HFDt_F0 vs. HFDt_F1.
    csv", sep=";", col.names = NA)
write.table(as.data.frame(res_total2), file="HFDt_F0 vs. HFDt_F2.
    csv", sep=";", col.names = NA)
write.table(as.data.frame(res_total3), file="HFDt_F1 vs. HFDt_F2.
    csv", sep=";", col.names = NA)

#obtain normalized counts
counts(estimateSizeFactors(dds_f0), normalized=TRUE)
```

```
counts(estimateSizeFactors(dds_f1), normalized=TRUE)
counts(estimateSizeFactors(dds_f2), normalized=TRUE)
counts(estimateSizeFactors(dds_total), normalized=TRUE)
write.table(counts(estimateSizeFactors(dds_total), normalized=TRUE)
    , "normalized_counts.csv", sep=";", col.names = NA)

#obtain base means (normalizing factor)
rowMeans(counts(estimateSizeFactors(dds_f0), normalized=TRUE))
rowMeans(counts(estimateSizeFactors(dds_f1), normalized=TRUE))
rowMeans(counts(estimateSizeFactors(dds_f2), normalized=TRUE))

write.csv(as.data.frame(counts(estimateSizeFactors(dds_f0),
    normalized=TRUE)), file="Bar_data_F0.csv")
write.csv(as.data.frame(counts(estimateSizeFactors(dds_f1),
    normalized=TRUE)), file="Bar_data_F1.csv")
write.csv(as.data.frame(counts(estimateSizeFactors(dds_f2),
    normalized=TRUE)), file="Bar_data_F2.csv")
write.table(as.data.frame(counts(estimateSizeFactors(dds_total),
    normalized=TRUE)), "normalized_counts.csv", sep=";", col.names =
     NA)

#bar graphs
library(ggplot2)
require(scales)
library(readxl)
bar_plot <- read_excel("bar_chart_data.xlsx", col_types = c("text",
     "text", "text", "skip", "skip", "skip", "skip", "skip", "skip",
     "numeric", "numeric"))
bar_plot$group <- factor(bar_plot$group, levels = c("CTRL", "HFD",
    "HFDt"))
bar_plot$gen <- factor(bar_plot$gen, levels = c("F0", "F1", "F2"))

bar_plot_big <- subset(bar_plot, gene_grp == "miRNA" | gene_grp ==
    "tsRNA" | gene_grp == "piRNA" | gene_grp == "rsRNA")
```

```
bar_plot_small <- subset(bar_plot, gene_grp == "mtRNA" | gene_grp
    == "Y RNA")

tiff("Figure 1a - sncRNA categories.tiff", width=2160, height=1000,
     units="px", res = 300)
ggplot(data=bar_plot_big, aes(y=mean, x=gene_grp, fill=group)) +
geom_bar(stat="identity", position=position_dodge(preserve = '
    single')) +
geom_errorbar(aes(ymin=mean-sd, ymax=mean+sd), width=.5,position=
    position_dodge(.9)) +
facet_grid(~bar_plot_big$gen, labeller = label_parsed) +
scale_y_continuous(breaks = pretty(bar_plot_big$mean)) +
scale_fill_brewer(palette="Set1") +
labs(x = "RNA biotype", y = "Normalized counts", fill = "Group") +
theme(axis.title = element_text(size = 12, face="bold")) +
theme(legend.title = element_text(size = 12, face="bold")) +
theme(strip.text.x = element_text(size = 14, face="bold"))
dev.off()

tiff("Figure 1b - sncRNA categories.tiff", width=1400, height=1000,
     units="px", res = 300)
ggplot(data=bar_plot_small, aes(y=mean, x=gene_grp, fill=group)) +
geom_bar(stat="identity", position=position_dodge(preserve = '
    single')) +
geom_errorbar(aes(ymin=mean-sd, ymax=mean+sd), width=.5,position=
    position_dodge(.9)) +
facet_grid(~bar_plot_small$gen, labeller = label_parsed) +
scale_y_continuous(breaks = pretty(bar_plot_small$mean)) +
scale_fill_brewer(palette="Set1") +
labs(x = "RNA biotype", y = "Normalized counts", fill = "Group") +
theme(axis.title = element_text(size = 12, face="bold")) +
theme(legend.title = element_text(size = 12, face="bold")) +
theme(strip.text.x = element_text(size = 14, face="bold"))
dev.off()
```

Differential expression: miRNA

```
#Help
help(package="DESeq2",help="html")
vignette("DESeq2")

#libraries
library("DESeq2")
library("vsn")
library("pheatmap")
library("ggplot2")
library(ggrepel)
library(tidyverse)
library("genefilter")
library("sqldf")
library(RColorBrewer)
library(data.table)
library(ggpubr)

cts <- read.table("merge_miRNAs.txt", header = T, sep = "", row.
    names="Annotation")
cts$row.names <- NULL
cts[is.na(cts)] = 0
coldata <- read.csv(file = "sample_annotation.csv", header = T, row
    .names = 1)
head(cts,2)
coldata
all(rownames(coldata) == colnames(cts))

dds <- DESeqDataSetFromMatrix(countData = cts, colData = coldata,
    design = ~ Condition)
dds
nrow(dds)
dds <- dds[ rowSums(counts(dds)) > 10, ]
```

```
nrow(dds)

#Data vizualization
lambda <- 10^seq(from = -1, to = 2, length = 1000)
cts <- matrix(rpois(1000*100, lambda), ncol = 100)
meanSdPlot(cts, ranks = FALSE)
log.cts.one <- log2(cts + 1)
meanSdPlot(log.cts.one, ranks = FALSE)

#Data transformation
vsd <- varianceStabilizingTransformation(dds)

#Heatmap plot 1000 * 600
sampleDists <- dist(t(assay(vsd)))
sampleDistMatrix <- as.matrix( sampleDists )
rownames(sampleDistMatrix) <- paste( vsd$name, vsd$Condition, sep =
    " - " )
colnames(sampleDistMatrix) <- NULL
colors <- colorRampPalette( rev(brewer.pal(9, "Blues")) )(255)
pheatmap(sampleDistMatrix,
clustering_distance_rows = sampleDists,
clustering_method="ward.D2",
clustering_distance_cols = sampleDists,
col = colors, fontsize = 15)

#PCA plot 1500 * 900
pcaData <- plotPCA(vsd, intgroup = c( "name", "Condition"),
   returnData = TRUE)
percentVar <- round(100 * attr(pcaData, "percentVar"))
ggplot(pcaData, aes(x= PC1, y= PC2, color=Condition, shape=
   Condition))+
scale_shape_manual(values=c(0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11))+
xlab(paste0("PC1: ", percentVar[1], "% variance")) +
ylab(paste0("PC2: ", percentVar[2], "% variance")) +
```

```
theme_bw(base_size = 35) +
geom_point(size = 5)
geom_text_repel(aes(label=Condition), size=6, vjust=0.6, hjust=0.5)


#Making sure to set up which control samples to use
dds$Condition <- relevel(dds$Condition, ref = "HFD_F0")
as.data.frame(colData(dds))
dds <- DESeq(dds)
res <- results(dds)
res
resultsNames(dds)
summary(res)

#Running the analysis
res <- results( dds, contrast = c("Condition", "HFDT_F0", "HFD_F0")
    )
res
resOrdered <- res[order(res$padj),]
head(resOrdered)
mcols(res, use.names = TRUE)
summary(res)
sum( res$padj < 0.1, na.rm = TRUE)
table( is.na(res$pvalue))
sum( res$padj < 0.1, na.rm = TRUE)
resSig <- res[ which(res$padj < 0.1 & abs(res$log2FoldChange) >
   1.5), ]
head( resSig)
tail( resSig )
write.csv( as.data.frame(res), file="results_miRNAs_HFDT_F0vsHFD_F0
   .csv" )

df <- read.csv("results_miRNAs_HFDT_F0vsHFD_F0.csv", header = T)
setnames(df, "X", "Gene_id")
```

```
write.csv( as.data.frame(df), file="results_miRNAs_HFDT_F0vsHFD_F0.
    csv", row.names = FALSE )

#MA plot of the results 1000 * 600
ggmaplot(res, fdr = 0.1, fc = 2.828427, size = 1,
palette = c("#B31B21", "#1465AC", "darkgray"),
legend="top", top = 10, font.label = c("bold", 10),
main = "HFDT_F0 vs HFD_F0 miRNAs",
label.rectangle = TRUE, font.legend = c("bold",12),font.main = "
    bold")



resdata <- merge(as.data.frame(res), as.data.frame(counts(dds,
    normalized=TRUE)), by="row.names", sort=FALSE)
names(resdata)[1] <- "Gene"
#resdatafilt <- filter(resdata, baseMean >= 100)
#resdatafilt <- dplyr::filter(resdatafilt, grepl("p", Gene))
resdata$Gene <- gsub("mature-tRNA-", "", resdata$Gene)
resdata$Gene <- gsub("mature-", "", resdata$Gene)

resdata$Significant = as.factor(abs(resdata$log2FoldChange) > 1.5 &
     resdata$padj < 0.1)
ggplot(resdata, aes(x=log2FoldChange, y=-log10(padj))) +
geom_point(aes(color=Significant), size =6) +
scale_color_manual(values = c("grey", "green")) +
theme_bw(base_size = 35) + theme(legend.position = "none") +
xlim(c(-6, 6)) + ylim(c(0, 3)) +
labs(title = "Volcano plot LF...Met vs LF...LF diet") +
geom_vline(xintercept = 1.5, col = "red", linetype = "dotted", size
    = 2) +
geom_vline(xintercept = -1.5, col = "red", linetype = "dotted",
    size = 2) +
geom_hline(yintercept = -log10(0.1), col = "red", linetype = "
    dotted", size = 2) +
```

```
geom_text_repel(
data = subset(resdata, resdata$padj < 0.1 & abs(resdata$
    log2FoldChange) > 1.5),
aes(label = Gene),
size=6
)


#create heatmap from sample variances
topVarGenes <- head(order(rowVars(assay(rld)), decreasing = TRUE),
    200)
mat  <- assay(rld)[ topVarGenes, ]
mat  <- mat - rowMeans(mat)
mat <- as.data.frame(mat, header=T, sep="")
write.csv(as.data.frame(mat), file="test.csv")
matfilt <- read.csv(file = "test.csv", header = T, sep=",")
write.csv( as.data.frame(resSig), file="results_tRNAs_HFHFvsChCh_
    Pvalue.csv" )
res <- read.csv("results_tRNAs_HFHFvsChCh_Pvalue.csv")
matfilt2 <- sqldf("select * from matfilt f1 inner join res f2 on (
    f1.X == f2.X) ")
matfilt2$X <- NULL
matfilt2$baseMean <- NULL
matfilt2$log2FoldChange <- NULL
matfilt2$lfcSE <- NULL
matfilt2$stat <- NULL
matfilt2$pvalue <- NULL
matfilt2$padj <- NULL
matfilt3 <- matfilt2[,c
    (19,1,2,3,4,5,6,7,8,9,10,11,12,13,14,15,16,17,18)]
write.csv(as.data.frame(matfilt3), file="test.csv")
matfilt <- read.csv(file = "test.csv", header = T, row.names = "X")
matfilt$X.1 <- NULL
```

```
anno <- as.data.frame(colData(rld)[, c("Condition","Type")])
anno$Type <- NULL
levels(anno$Condition)
anno$Condition <- factor(anno$Condition, levels = unique(anno$
    Condition))
levels(anno$Condition)
pheatmap(matfilt, annotation_col = anno, annotation_names_col = F,
    show_colnames = FALSE, fontsize = 24, cellwidth = 22, cellheight
     = 22, cluster_rows = FALSE, main = "HFHFvsChCh")

#For Luis data
dds <- dds[ , dds$time == "48h" ]
dds$time <- droplevels( dds$time )
dds$treatment <- relevel( dds$treatment, "Control" )
as.data.frame( colData(dds) )

#Count outliers
assays (dds) [["cooks"]]
par(mar=c(8,5,2,2))
boxplot(log10(assays(dds)[["cooks"]]), range = 0, las = 2)
plotDispEsts(dds)

#plot counts

#HFvsCh
plotCounts(dds, gene="mature-mt_tRNA-Glu-TTC_3_end", intgroup= "
    Condition")

#HFHFvsChCh
par(mfrow=c(6,3), cex = 1.1, cex.main=1.2, cex.lab=1.2)
plotCounts(dds, gene="mature-tRNA-Gln-CTG", intgroup= "Condition")
plotCounts(dds, gene="mature-mt_tRNA-Leu-TAG_5_end", intgroup= "
    Condition")
plotCounts(dds, gene="mature-mt_tRNA-Tyr-GTA_5_end", intgroup= "
```

```
    Condition")
plotCounts(dds, gene="mature-tRNA-Ala-AGC_3_end", intgroup= "
    Condition")
plotCounts(dds, gene="mature-tRNA-Gly-TCC_5_end", intgroup= "
    Condition")
plotCounts(dds, gene="mature-tRNA-iMet-CAT_5_end", intgroup= "
    Condition")
plotCounts(dds, gene="mature-tRNA-Ser-AGA_3_end", intgroup= "
    Condition")
plotCounts(dds, gene="mature-tRNA-Ser-CGA_3_end", intgroup= "
    Condition")

#HFRunvsHFHF
plotCounts(dds, gene="mature-tRNA-Glu-CTC_CCA_end", intgroup= "
    Condition")
```

Differential expression: tsRNA

```
#Help
help(package="DESeq2",help="html")
vignette("DESeq2")

#libraries
library("DESeq2")
library("vsn")
library("pheatmap")
library("ggplot2")
library(ggrepel)
library(tidyverse)
library("genefilter")
library("sqldf")
library(RColorBrewer)
library(data.table)
library(ggpubr)
```

```
#Datasets preparation

cts <- read.table("merge_tsRNAs.txt", header = T, sep = "", row.
    names = "Annotation")
cts <- cts[, c(2:25)]
cts[is.na(cts)] = 0

coldata <- read.csv("sample_annotation.csv", header = T, row.names
    = 1)

all(rownames(coldata) == colnames(cts))
dds <- DESeqDataSetFromMatrix(countData = cts, colData = coldata,
    design = ~ Condition)
dds
nrow(dds)
dds <- dds[ rowSums(counts(dds)) > 10, ]
nrow(dds)

#Data vizualization
lambda <- 10^seq(from = -1, to = 2, length = 1000)
cts <- matrix(rpois(1000*100, lambda), ncol = 100)
meanSdPlot(cts, ranks = FALSE)
log.cts.one <- log2(cts + 1)
meanSdPlot(log.cts.one, ranks = FALSE)

#Data transformation
vsd <- varianceStabilizingTransformation(dds)

#Heatmap plot 1000 * 600
sampleDists <- dist(t(assay(vsd)))
sampleDistMatrix <- as.matrix( sampleDists )
rownames(sampleDistMatrix) <- paste( vsd$name, vsd$Condition, sep =
     " - " )
```

```
colnames(sampleDistMatrix) <- NULL
colors <- colorRampPalette( rev(brewer.pal(9, "Blues")) )(255)
pheatmap(sampleDistMatrix,
clustering_distance_rows = sampleDists,
clustering_method="ward.D2",
clustering_distance_cols = sampleDists,
col = colors, fontsize = 15)

#PCA plot 1500 * 900
pcaData <- plotPCA(vsd, intgroup = c( "name", "Condition"),
    returnData = TRUE)
percentVar <- round(100 * attr(pcaData, "percentVar"))
ggplot(pcaData, aes(x= PC1, y= PC2, color=Condition, shape=
    Condition))+
scale_shape_manual(values=c(0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11))+
xlab(paste0("PC1: ", percentVar[1], "% variance")) +
ylab(paste0("PC2: ", percentVar[2], "% variance")) +
theme_bw(base_size = 35) +
geom_point(size = 5)
geom_text_repel(aes(label=Condition), size=6, vjust=0.6, hjust=0.5)

#Making sure to set up which control samples to use
dds$Condition <- relevel(dds$Condition, ref = "HFD_F0")
as.data.frame(colData(dds))
dds <- DESeq(dds)
res <- results(dds)
res
resultsNames(dds)
summary(res)

#Running the analysis
res <- results( dds, contrast = c("Condition", "HFDT_F0", "HFD_F0")
    )
res
```

```
resOrdered <- res[order(res$padj),]
head(resOrdered)
mcols(res, use.names = TRUE)
summary(res)
sum( res$padj < 0.1, na.rm = TRUE)
table( is.na(res$pvalue))
sum( res$padj < 0.1, na.rm = TRUE)
resSig <- res[ which(res$padj < 0.1 & abs(res$log2FoldChange) >
    1.5), ]
head( resSig)
tail( resSig )
write.csv( as.data.frame(res), file="results_tsRNAs_HFDT_F0vsHFD_F0
    .csv" )

df <- read.csv("results_tsRNAs_HFDT_F0vsHFD_F0.csv", header = T)
setnames(df, "X", "Gene_id")
write.csv( as.data.frame(df), file="results_tsRNAs_HFDT_F0vsHFD_F0.
    csv", row.names = FALSE )

#MA plot of the results 1000 * 600
ggmaplot(res, fdr = 0.1, fc = 2.828427, size = 1,
palette = c("#B31B21", "#1465AC", "darkgray"),
legend="top", top = 10, font.label = c("bold", 10),
main = "HFDT_F0 vs HFD_F0 tsRNAs",
label.rectangle = TRUE, font.legend = c("bold",12),font.main = "
    bold",
max.overlaps = Inf)
```

Differential expression: tiRNA

```
df <- read.csv("results_tiRNAs_HFHFvsChCh_without_outliers.csv",
    header = T )
setnames(df, "X", "Gene_id")
```

```
#Help
help(package="DESeq2",help="html")
vignette("DESeq2")

#libraries
library("DESeq2")
library("vsn")
library("pheatmap")
library("ggplot2")
library(ggrepel)
library(tidyverse)
library("genefilter")
library("sqldf")
library(RColorBrewer)
library(data.table)
library(ggpubr)

#Datasets preparation

cts <- read.table("merge_tiRNAs.txt", header = T, sep = "", row.
    names = "Annotation")
cts <- cts[, c(2:25)]
cts[is.na(cts)] = 0

coldata <- read.csv("sample_annotation.csv", header = T, row.names
    = 1)

all(rownames(coldata) == colnames(cts))
dds <- DESeqDataSetFromMatrix(countData = cts, colData = coldata,
    design = ~ Condition)
dds
nrow(dds)
dds <- dds[ rowSums(counts(dds)) > 10, ]
nrow(dds)
```

```
#Data vizualization
lambda <- 10^seq(from = -1, to = 2, length = 1000)
cts <- matrix(rpois(1000*100, lambda), ncol = 100)
meanSdPlot(cts, ranks = FALSE)
log.cts.one <- log2(cts + 1)
meanSdPlot(log.cts.one, ranks = FALSE)

#Data transformation
vsd <- varianceStabilizingTransformation(dds)

#Heatmap plot 1000 * 600
sampleDists <- dist(t(assay(vsd)))
sampleDistMatrix <- as.matrix( sampleDists )
rownames(sampleDistMatrix) <- paste( vsd$name, vsd$Condition, sep =
    " - " )
colnames(sampleDistMatrix) <- NULL
colors <- colorRampPalette( rev(brewer.pal(9, "Blues")) )(255)
pheatmap(sampleDistMatrix,
clustering_distance_rows = sampleDists,
clustering_method="ward.D2",
clustering_distance_cols = sampleDists,
col = colors, fontsize = 15)

#PCA plot 1500 * 900
pcaData <- plotPCA(vsd, intgroup = c( "name", "Condition"),
   returnData = TRUE)
percentVar <- round(100 * attr(pcaData, "percentVar"))
ggplot(pcaData, aes(x= PC1, y= PC2, color=Condition, shape=
   Condition))+
scale_shape_manual(values=c(0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11))+
xlab(paste0("PC1: ", percentVar[1], "% variance")) +
ylab(paste0("PC2: ", percentVar[2], "% variance")) +
theme_bw(base_size = 35) +
```

```
geom_point(size = 5)
geom_text_repel(aes(label=Condition), size=6, vjust=0.6, hjust=0.5)

#Making sure to set up which control samples to use
dds$Condition <- relevel(dds$Condition, ref = "HFD_F0")
as.data.frame(colData(dds))
dds <- DESeq(dds)
res <- results(dds)
res
resultsNames(dds)
summary(res)

#Running the analysis
res <- results( dds, contrast = c("Condition", "HFDT_F0", "HFD_F0")
    )
res
resOrdered <- res[order(res$padj),]
head(resOrdered)
mcols(res, use.names = TRUE)
summary(res)
sum( res$padj < 0.1, na.rm = TRUE)
table( is.na(res$pvalue))
sum( res$padj < 0.1, na.rm = TRUE)
resSig <- res[ which(res$padj < 0.1 & abs(res$log2FoldChange) >
   1.5), ]
head( resSig)
tail( resSig )
write.csv( as.data.frame(res), file="results_tiRNAs_HFDT_F0vsHFD_F0
   .csv" )

df <- read.csv("results_tiRNAs_HFDT_F0vsHFD_F0.csv", header = T)
setnames(df, "X", "Gene_id")
write.csv( as.data.frame(df), file="results_tiRNAs_HFDT_F0vsHFD_F0.
   csv", row.names = FALSE )
```

```
#MA plot of the results 1000 * 600
ggmaplot(res, fdr = 0.1, fc = 2.828427, size = 1,
palette = c("#B31B21", "#1465AC", "darkgray"),
legend="top", top = 15, font.label = c("bold", 10),
main = "HFDT_F0 vs HFD_F0 tiRNAs",
label.rectangle = TRUE, font.legend = c("bold",12),font.main = "
    bold")
```

Differential expression: tRF

```
#Help
help(package="DESeq2",help="html")
vignette("DESeq2")

#libraries
library("DESeq2")
library("vsn")
library("pheatmap")
library("ggplot2")
library(ggrepel)
library(tidyverse)
library("genefilter")
library("sqldf")
library(RColorBrewer)
library(data.table)
library(ggpubr)

#Datasets preparation

cts <- read.table("merge_tRFs.txt", header = T, sep = "", row.names
    = "Annotation")
cts <- cts[, c(2:25)]
cts[is.na(cts)] = 0
```

```
coldata <- read.csv("sample_annotation.csv", header = T, row.names
    = 1)

all(rownames(coldata) == colnames(cts))
dds <- DESeqDataSetFromMatrix(countData = cts, colData = coldata,
    design = ~ Condition)
dds
nrow(dds)
dds <- dds[ rowSums(counts(dds)) > 10, ]
nrow(dds)

#Data vizualization
lambda <- 10^seq(from = -1, to = 2, length = 1000)
cts <- matrix(rpois(1000*100, lambda), ncol = 100)
meanSdPlot(cts, ranks = FALSE)
log.cts.one <- log2(cts + 1)
meanSdPlot(log.cts.one, ranks = FALSE)

#Data transformation
vsd <- varianceStabilizingTransformation(dds)

#Heatmap plot 1000 * 600
sampleDists <- dist(t(assay(vsd)))
sampleDistMatrix <- as.matrix( sampleDists )
rownames(sampleDistMatrix) <- paste( vsd$name, vsd$Condition, sep =
     " - " )
colnames(sampleDistMatrix) <- NULL
colors <- colorRampPalette( rev(brewer.pal(9, "Blues")) )(255)
pheatmap(sampleDistMatrix,
clustering_distance_rows = sampleDists,
clustering_method="ward.D2",
clustering_distance_cols = sampleDists,
col = colors, fontsize = 15)
```

```
#PCA plot 1500 * 900
pcaData <- plotPCA(vsd, intgroup = c( "name", "Condition"),
    returnData = TRUE)
percentVar <- round(100 * attr(pcaData, "percentVar"))
ggplot(pcaData, aes(x= PC1, y= PC2, color=Condition, shape=
    Condition))+
scale_shape_manual(values=c(0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11))+
xlab(paste0("PC1: ", percentVar[1], "% variance")) +
ylab(paste0("PC2: ", percentVar[2], "% variance")) +
theme_bw(base_size = 35) +
geom_point(size = 5)
geom_text_repel(aes(label=Condition), size=6, vjust=0.6, hjust=0.5)

#Making sure to set up which control samples to use
dds$Condition <- relevel(dds$Condition, ref = "HFD_F1")
as.data.frame(colData(dds))
dds <- DESeq(dds)
res <- results(dds)
res
resultsNames(dds)
summary(res)

#Running the analysis
res <- results( dds, contrast = c("Condition", "HFDT_F1", "HFD_F1")
    )
res
resOrdered <- res[order(res$padj),]
head(resOrdered)
mcols(res, use.names = TRUE)
summary(res)
sum( res$padj < 0.1, na.rm = TRUE)
table( is.na(res$pvalue))
sum( res$padj < 0.1, na.rm = TRUE)
```

```
resSig <- res[ which(res$padj < 0.1 & abs(res$log2FoldChange) >
    1.5), ]
head( resSig)
tail( resSig )
write.csv( as.data.frame(res), file="results_tRFs_HFDT_F1vsHFD_F1.
    csv" )

df <- read.csv("results_tRFs_HFDT_F1vsHFD_F1.csv", header = T)
setnames(df, "X", "Gene_id")
write.csv( as.data.frame(df), file="results_tRFs_HFDT_F1vsHFD_F1.
    csv", row.names = FALSE )

#MA plot of the results 1000 * 600
ggmaplot(res, fdr = 0.1, fc = 2.828427, size = 1,
palette = c("#B31B21", "#1465AC", "darkgray"),
legend="top", top = 10, font.label = c("bold", 10),
main = "HFDT_F1 vs HFD_F1 tRFs",
label.rectangle = TRUE, font.legend = c("bold",12),font.main = "
    bold")
```

Differential expression: piRNA cluster

```
#Help
help(package="DESeq2",help="html")
vignette("DESeq2")

#libraries
library("DESeq2")
library("vsn")
library("pheatmap")
library("ggplot2")
library(ggrepel)
library(tidyverse)
library("genefilter")
```

```
library("sqldf")
library(RColorBrewer)
library(data.table)
library(ggpubr)

cts <- read.table("counts_piRNA_cluster_s0.txt", header = T, sep =
    "", row.names="Geneid")
cts <- cts[, c(6:29)]
names(cts) <- gsub(".sam", "", names(cts))
names(cts) <- gsub("X", "Reads.", names(cts))
cts[is.na(cts)] = 0
cts <- round(cts, digits = 0)

coldata <- read.csv(file = "sample_annotation.csv", header = T, row
    .names = 1)
all(rownames(coldata) == colnames(cts))

dds <- DESeqDataSetFromMatrix(countData = cts, colData = coldata,
    design = ~ Condition)
dds
nrow(dds)
dds <- dds[ rowSums(counts(dds)) > 10, ]
nrow(dds)

#Data vizualization
lambda <- 10^seq(from = -1, to = 2, length = 1000)
cts <- matrix(rpois(1000*100, lambda), ncol = 100)
meanSdPlot(cts, ranks = FALSE)
log.cts.one <- log2(cts + 1)
meanSdPlot(log.cts.one, ranks = FALSE)

#Data transformation
vsd <- varianceStabilizingTransformation(dds)
```

```
#Heatmap plot 1000 * 600
sampleDists <- dist(t(assay(vsd)))
sampleDistMatrix <- as.matrix( sampleDists )
rownames(sampleDistMatrix) <- paste( vsd$name, vsd$Condition, sep =
    " - " )
colnames(sampleDistMatrix) <- NULL
colors <- colorRampPalette( rev(brewer.pal(9, "Blues")) )(255)
pheatmap(sampleDistMatrix,
clustering_distance_rows = sampleDists,
clustering_method="ward.D2",
clustering_distance_cols = sampleDists,
col = colors, fontsize = 15)

#PCA plot 1500 * 900
pcaData <- plotPCA(vsd, intgroup = c( "name", "Condition"),
   returnData = TRUE)
percentVar <- round(100 * attr(pcaData, "percentVar"))
ggplot(pcaData, aes(x= PC1, y= PC2, color=Condition, shape=
   Condition))+
scale_shape_manual(values=c(0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11))+
xlab(paste0("PC1: ", percentVar[1], "% variance")) +
ylab(paste0("PC2: ", percentVar[2], "% variance")) +
theme_bw(base_size = 35) +
geom_point(size = 5)
geom_text_repel(aes(label=Condition), size=6, vjust=0.6, hjust=0.5)


#Making sure to set up which control samples to use
dds$Condition <- relevel(dds$Condition, ref = "HFD_F0")
as.data.frame(colData(dds))
dds <- DESeq(dds)
res <- results(dds)
res
resultsNames(dds)
```

```
summary(res)

#Running the analysis
res <- results( dds, contrast = c("Condition", "HFDT_F0", "HFD_F0")
    )
res
resOrdered <- res[order(res$padj),]
head(resOrdered)
mcols(res, use.names = TRUE)
summary(res)
sum( res$padj < 0.1, na.rm = TRUE)
table( is.na(res$pvalue))
sum( res$padj < 0.1, na.rm = TRUE)
resSig <- res[ which(res$padj < 0.1 & abs(res$log2FoldChange) >
   1.5), ]
head( resSig)
tail( resSig )
write.csv( as.data.frame(res), file="results_piRNAcluster_HFDT_
   F0vsHFD_F0.csv" )

df <- read.csv("results_piRNAcluster_HFDT_F0vsHFD_F0.csv", header =
    T)
setnames(df, "X", "Cluster_id")
write.csv( as.data.frame(df), file="results_piRNAcluster_HFDT_
   F0vsHFD_F0.csv", row.names = FALSE )

#MA plot of the results 1000 * 600
ggmaplot(res, fdr = 0.1, fc = 2.828427, size = 1,
palette = c("#B31B21", "#1465AC", "darkgray"),
legend="top", top = 10, font.label = c("bold", 10),
main = "HFDT_F0 vs HFD_F0 piRNA clusters",
label.rectangle = TRUE, font.legend = c("bold",12),font.main = "
   bold")
```

```
resdata <- merge(as.data.frame(res), as.data.frame(counts(dds,
    normalized=TRUE)), by="row.names", sort=FALSE)
names(resdata)[1] <- "Gene"
#resdatafilt <- filter(resdata, baseMean >= 100)
#resdatafilt <- dplyr::filter(resdatafilt, grepl("p", Gene))
resdata$Gene <- gsub("mature-tRNA-", "", resdata$Gene)
resdata$Gene <- gsub("mature-", "", resdata$Gene)

resdata$Significant = as.factor(abs(resdata$log2FoldChange) > 1.5 &
     resdata$padj < 0.1)
ggplot(resdata, aes(x=log2FoldChange, y=-log10(padj))) +
geom_point(aes(color=Significant), size =6) +
scale_color_manual(values = c("grey", "green")) +
theme_bw(base_size = 35) + theme(legend.position = "none") +
xlim(c(-6, 6)) + ylim(c(0, 3)) +
labs(title = "Volcano plot LF...Met vs LF...LF diet") +
geom_vline(xintercept = 1.5, col = "red", linetype = "dotted", size
    = 2) +
geom_vline(xintercept = -1.5, col = "red", linetype = "dotted",
    size = 2) +
geom_hline(yintercept = -log10(0.1), col = "red", linetype = "
    dotted", size = 2) +
geom_text_repel(
data = subset(resdata, resdata$padj < 0.1 & abs(resdata$
    log2FoldChange) > 1.5),
aes(label = Gene),
size=6
)


#create heatmap from sample variances
topVarGenes <- head(order(rowVars(assay(rld)), decreasing = TRUE),
    200)
```

```
mat  <- assay(rld)[ topVarGenes, ]
mat  <- mat - rowMeans(mat)
mat <- as.data.frame(mat, header=T, sep="")
write.csv(as.data.frame(mat), file="test.csv")
matfilt <- read.csv(file = "test.csv", header = T, sep=",")
write.csv( as.data.frame(resSig), file="results_tRNAs_HFHFvsChCh_
    Pvalue.csv" )
res <- read.csv("results_tRNAs_HFHFvsChCh_Pvalue.csv")
matfilt2 <- sqldf("select * from matfilt f1 inner join res f2 on (
    f1.X == f2.X) ")
matfilt2$X <- NULL
matfilt2$baseMean <- NULL
matfilt2$log2FoldChange <- NULL
matfilt2$lfcSE <- NULL
matfilt2$stat <- NULL
matfilt2$pvalue <- NULL
matfilt2$padj <- NULL
matfilt3 <- matfilt2[,c
    (19,1,2,3,4,5,6,7,8,9,10,11,12,13,14,15,16,17,18)]
write.csv(as.data.frame(matfilt3), file="test.csv")
matfilt <- read.csv(file = "test.csv", header = T, row.names = "X")
matfilt$X.1 <- NULL

anno <- as.data.frame(colData(rld)[, c("Condition","Type")])
anno$Type <- NULL
levels(anno$Condition)
anno$Condition <- factor(anno$Condition, levels = unique(anno$
    Condition))
levels(anno$Condition)
pheatmap(matfilt, annotation_col = anno, annotation_names_col = F,
    show_colnames = FALSE, fontsize = 24, cellwidth = 22, cellheight
     = 22, cluster_rows = FALSE, main = "HFHFvsChCh")

#For Luis data
```

```
dds <- dds[ , dds$time == "48h" ]
dds$time <- droplevels( dds$time )
dds$treatment <- relevel( dds$treatment, "Control" )
as.data.frame( colData(dds) )

#Count outliers
assays (dds) [["cooks"]]
par(mar=c(8,5,2,2))
boxplot(log10(assays(dds)[["cooks"]]), range = 0, las = 2)
plotDispEsts(dds)

#plot counts

#HFvsCh
plotCounts(dds, gene="mature-mt_tRNA-Glu-TTC_3_end", intgroup= "
    Condition")

#HFHFvsChCh
par(mfrow=c(6,3), cex = 1.1, cex.main=1.2, cex.lab=1.2)
plotCounts(dds, gene="mature-tRNA-Gln-CTG", intgroup= "Condition")
plotCounts(dds, gene="mature-mt_tRNA-Leu-TAG_5_end", intgroup= "
    Condition")
plotCounts(dds, gene="mature-mt_tRNA-Tyr-GTA_5_end", intgroup= "
    Condition")
plotCounts(dds, gene="mature-tRNA-Ala-AGC_3_end", intgroup= "
    Condition")
plotCounts(dds, gene="mature-tRNA-Gly-TCC_5_end", intgroup= "
    Condition")
plotCounts(dds, gene="mature-tRNA-iMet-CAT_5_end", intgroup= "
    Condition")
plotCounts(dds, gene="mature-tRNA-Ser-AGA_3_end", intgroup= "
    Condition")
plotCounts(dds, gene="mature-tRNA-Ser-CGA_3_end", intgroup= "
    Condition")
```

```
#HFRunvsHFHF
plotCounts(dds, gene="mature-tRNA-Glu-CTC_CCA_end", intgroup= "
    Condition")

df <- read.csv("results_tiRNAs_HFHFvsChCh_without_outliers.csv",
    header = T )
setnames(df, "X", "Gene_id")
```

Differential expression: repRNA

```
#Help
help(package="DESeq2",help="html")
vignette("DESeq2")

#libraries
library("DESeq2")
library("vsn")
library("pheatmap")
library("ggplot2")
library(ggrepel)
library(tidyverse)
library("genefilter")
library("sqldf")
library(RColorBrewer)
library(data.table)
library(ggpubr)

dat <- fread("counts_piRNA_rmsk_s0.txt", select = c(1,7:30))
write.table( as.data.frame(dat), file="counts_piRNA_rmsk.txt", row.
    names = FALSE )

cts <- read.table("counts_piRNA_rmsk.txt", header = T, sep = "",
    row.names="Geneid")
```

```
names(cts) <- gsub(".sam", "", names(cts))
names(cts) <- gsub("X", "Reads.", names(cts))
cts[is.na(cts)] = 0
cts <- round(cts, digits = 0)

coldata <- read.csv(file = "sample_annotation.csv", header = T, row
    .names = 1)
all(rownames(coldata) == colnames(cts))

dds <- DESeqDataSetFromMatrix(countData = cts, colData = coldata,
    design = ~ Condition)
dds
nrow(dds)
dds <- dds[ rowSums(counts(dds)) > 10, ]
nrow(dds)

#Data vizualization
lambda <- 10^seq(from = -1, to = 2, length = 1000)
cts <- matrix(rpois(1000*100, lambda), ncol = 100)
meanSdPlot(cts, ranks = FALSE)
log.cts.one <- log2(cts + 1)
meanSdPlot(log.cts.one, ranks = FALSE)

#Data transformation
vsd <- varianceStabilizingTransformation(dds)

#Heatmap plot 1000 * 600
sampleDists <- dist(t(assay(vsd)))
sampleDistMatrix <- as.matrix( sampleDists )
rownames(sampleDistMatrix) <- paste( vsd$name, vsd$Condition, sep =
    " - " )
colnames(sampleDistMatrix) <- NULL
colors <- colorRampPalette( rev(brewer.pal(9, "Blues")) )(255)
pheatmap(sampleDistMatrix,
```

```
clustering_distance_rows = sampleDists,
clustering_method="ward.D2",
clustering_distance_cols = sampleDists,
col = colors, fontsize = 15)

#PCA plot 1500 * 900
pcaData <- plotPCA(vsd, intgroup = c( "name", "Condition"),
    returnData = TRUE)
percentVar <- round(100 * attr(pcaData, "percentVar"))
ggplot(pcaData, aes(x= PC1, y= PC2, color=Condition, shape=
    Condition))+
scale_shape_manual(values=c(0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11))+
xlab(paste0("PC1: ", percentVar[1], "% variance")) +
ylab(paste0("PC2: ", percentVar[2], "% variance")) +
theme_bw(base_size = 35) +
geom_point(size = 5)
geom_text_repel(aes(label=Condition), size=6, vjust=0.6, hjust=0.5)


#Making sure to set up which control samples to use
dds$Condition <- relevel(dds$Condition, ref = "HFD_F0")
as.data.frame(colData(dds))
dds <- DESeq(dds)
res <- results(dds)
res
resultsNames(dds)
summary(res)

#Running the analysis
res <- results( dds, contrast = c("Condition", "HFDT_F0", "HFD_F0")
    )
res
resOrdered <- res[order(res$padj),]
head(resOrdered)
```

```
mcols(res, use.names = TRUE)
summary(res)
sum( res$padj < 0.1, na.rm = TRUE)
table( is.na(res$pvalue))
sum( res$padj < 0.1, na.rm = TRUE)
resSig <- res[ which(res$padj < 0.1 & abs(res$log2FoldChange) >
    1.5), ]
head( resSig)
tail( resSig )
write.csv( as.data.frame(res), file="results_rmsk_HFDT_F0vsHFD_F0.
    csv" )

df <- read.csv("results_rmsk_HFDT_F0vsHFD_F0.csv", header = T)
setnames(df, "X", "Repeat_id")
write.csv( as.data.frame(df), file="results_rmsk_HFDT_F0vsHFD_F0.
    csv", row.names = FALSE )

#MA plot of the results 1000 * 600
ggmaplot(res, fdr = 0.1, fc = 2.828427, size = 1,
palette = c("#B31B21", "#1465AC", "darkgray"),
legend="top", top = 10, font.label = c("bold", 10),
main = "HFDT_F0 vs HFD_F0 rmsk",
label.rectangle = TRUE, font.legend = c("bold",12),font.main = "
    bold")

#anno <- read.table("rmsk1_notRNA_norRNA.bed", header = F, sep = "\
    t")
#anno <- anno %>%
#separate(V9, c("Gene_id", "Repeat_Alias", "Repeat_Name"), ";")
#anno$Gene_id <- gsub(".*=", "", anno$Gene_id)
#anno$Repeat_Alias <- gsub(".*=", "", anno$Repeat_Alias)
#anno$Repeat_Name <- gsub(".*=", "", anno$Repeat_Name)
#anno <- anno[,c(9:11)]
#anno <- anno[!duplicated(anno$Gene_id), ]
```

```
total_anno <- sqldf("select * from df f1 left outer join anno f2 on
    (f1.Repeat_id == f2.Gene_id) ")
write.csv( as.data.frame(df), file="results_rmsk_HFDT_F0vsHFD_F0.
    csv", row.names = FALSE )
```

GO annotation and analysis

```
#Annotate sncRNA targets using GO terms

#will clear all objects includes hidden objects.
rm(list = ls(all.names = TRUE))

#Load packages
library(readr)
library(tidyr)
library(topGO) #annotation tool
library(Mus.musculus) #mouse annotation
#library(org.Mm.eg.db) #loaded by the previous

# custom filter function
count.filter <- structure(function (allScore)
{
    return(allScore > 1)
}, source = c("function(allScore) {", "  return(allScore > 1)",
    "}"))

#Generation F0: CTRL vs. HFDt
##load targets of DEGs
F0_CTRL_HFDt_Targets <- read_csv("F0_CTRL_vs_HFDt - sRNAtools
    Targets.csv")
F0_CTRL_HFDt_Targets <- F0_CTRL_HFDt_Targets %>% separate_rows(
    Targets)
```

```
##data structure
test.genes_F0_CTRL_HFDt <- F0_CTRL_HFDt_Targets[4]
test.genes_F0_CTRL_HFDt$Targets <- gsub("\\..*","",test.genes_F0_
    CTRL_HFDt$Targets)
test.genes_F0_CTRL_HFDt <- as.data.frame(table(test.genes_F0_CTRL_
    HFDt$Targets))
colnames(test.genes_F0_CTRL_HFDt) <- c("Targets","counts")

genecounts_F0_CTRL_HFDt <- test.genes_F0_CTRL_HFDt[[2]]
names(genecounts_F0_CTRL_HFDt) <- test.genes_F0_CTRL_HFDt[[1]]

##prepare topGO dataobjects
topGO.BP_F0_CTRL_vs_HFDt <- new("topGOdata", ontology = "BP",
    allGenes = genecounts_F0_CTRL_HFDt, geneSel = count.filter,
description = "GO analysis BP: CTRL_F0 vs. HFDt_F0", nodeSize = 10,
     annotationFun = annFUN.org, mapping = "org.Mm.eg.db", ID = "
    ensembl")

topGO.MF_F0_CTRL_vs_HFDt <- new("topGOdata", ontology = "MF",
    allGenes = genecounts_F0_CTRL_HFDt, geneSel = count.filter,
description = "GO analysis MF: CTRL_F0 vs. HFDt_F0", nodeSize = 10,
     annotationFun = annFUN.org, mapping = "org.Mm.eg.db", ID = "
    ensembl")

topGO.CC_F0_CTRL_vs_HFDt <- new("topGOdata", ontology = "CC",
    allGenes = genecounts_F0_CTRL_HFDt, geneSel = count.filter,
description = "GO analysis CC: CTRL_F0 vs. HFDt_F0", nodeSize = 10,
     annotationFun = annFUN.org, mapping = "org.Mm.eg.db", ID = "
    ensembl")

##perform GO test - weight01 & Fisher
runGO.BP_F0_CTRL_vs_HFDt <- runTest(topGO.BP_F0_CTRL_vs_HFDt,
    algorithm = "weight01", statistic = "fisher")#, topNodes = 5)
runGO.MF_F0_CTRL_vs_HFDt <- runTest(topGO.MF_F0_CTRL_vs_HFDt,
```

```
    algorithm = "weight01", statistic = "fisher")
runGO.CC_F0_CTRL_vs_HFDt <- runTest(topGO.CC_F0_CTRL_vs_HFDt,
    algorithm = "weight01", statistic = "fisher")

##generate result tables
GO.BP_F0_CTRL_vs_HFDt <- GenTable(topGO.BP_F0_CTRL_vs_HFDt,
    Weight01 = runGO.BP_F0_CTRL_vs_HFDt, orderBy = "Weight01",
    topNodes = 30)
GO.MF_F0_CTRL_vs_HFDt <- GenTable(topGO.MF_F0_CTRL_vs_HFDt,
    Weight01 = runGO.MF_F0_CTRL_vs_HFDt, orderBy = "Weight01",
    topNodes = 30)
GO.CC_F0_CTRL_vs_HFDt <- GenTable(topGO.CC_F0_CTRL_vs_HFDt,
    Weight01 = runGO.CC_F0_CTRL_vs_HFDt, orderBy = "Weight01",
    topNodes = 30)

##Write results tables
write.csv2(GO.BP_F0_CTRL_vs_HFDt,"GO Biological Process CTRL-F0 vs
    HFDt-F0.csv", row.names = FALSE)
write.csv2(GO.MF_F0_CTRL_vs_HFDt,"GO Molecular Function CTRL-F0 vs
    HFDt-F0.csv", row.names = FALSE)
write.csv2(GO.CC_F0_CTRL_vs_HFDt,"GO Cellular Component CTRL-F0 vs
    HFDt-F0.csv", row.names = FALSE)



#Generation F1: CTRL vs. HFD
##load targets of DEGs
F1_CTRL_HFD_Targets <- read_csv("F1_CTRL_vs_HFD - sRNAtools
    Targets.csv")
F1_CTRL_HFD_Targets <- F1_CTRL_HFD_Targets %>% separate_rows(
    Targets)

##data structure
test.genes_F1_CTRL_HFD <- F1_CTRL_HFD_Targets[4]
test.genes_F1_CTRL_HFD$Targets <- gsub("\\..*","",test.genes_F1_
```

```
    CTRL_HFD$Targets)
test.genes_F1_CTRL_HFD <- as.data.frame(table(test.genes_F1_CTRL_
    HFD$Targets))
colnames(test.genes_F1_CTRL_HFD) <- c("Targets","counts")

genecounts_F1_CTRL_HFD <- test.genes_F1_CTRL_HFD[[2]]
names(genecounts_F1_CTRL_HFD) <- test.genes_F1_CTRL_HFD[[1]]

##prepare topGO dataobjects
topGO.BP_F1_CTRL_vs_HFD <- new("topGOdata", ontology = "BP",
    allGenes = genecounts_F1_CTRL_HFD, geneSel = count.filter,
description = "GO analysis BP: CTRL_F1 vs. HFD_F1", nodeSize = 10,
    annotationFun = annFUN.org, mapping = "org.Mm.eg.db", ID = "
    ensembl")

topGO.MF_F1_CTRL_vs_HFD <- new("topGOdata", ontology = "MF",
    allGenes = genecounts_F1_CTRL_HFD, geneSel = count.filter,
description = "GO analysis MF: CTRL_F1 vs. HFD_F1", nodeSize = 10,
    annotationFun = annFUN.org, mapping = "org.Mm.eg.db", ID = "
    ensembl")

topGO.CC_F1_CTRL_vs_HFD <- new("topGOdata", ontology = "CC",
    allGenes = genecounts_F1_CTRL_HFD, geneSel = count.filter,
description = "GO analysis CC: CTRL_F1 vs. HFD_F1", nodeSize = 10,
    annotationFun = annFUN.org, mapping = "org.Mm.eg.db", ID = "
    ensembl")

##perform GO test - weight01 & Fisher
runGO.BP_F1_CTRL_vs_HFD <- runTest(topGO.BP_F1_CTRL_vs_HFD,
    algorithm = "weight01", statistic = "fisher")#, topNodes = 5)
runGO.MF_F1_CTRL_vs_HFD <- runTest(topGO.MF_F1_CTRL_vs_HFD,
    algorithm = "weight01", statistic = "fisher")
runGO.CC_F1_CTRL_vs_HFD <- runTest(topGO.CC_F1_CTRL_vs_HFD,
    algorithm = "weight01", statistic = "fisher")
```

```
#Generation F1: CTRL vs. HFDt
##load targets of DEGs
F1_CTRL_HFDt_Targets <- read_csv("F1_CTRL_vs_HFDt - sRNAtools
    Targets.csv")
F1_CTRL_HFDt_Targets <- F1_CTRL_HFDt_Targets %>% separate_rows(
    Targets)

##data structure
test.genes_F1_CTRL_HFDt <- F1_CTRL_HFDt_Targets[4]
test.genes_F1_CTRL_HFDt$Targets <- gsub("\\..*","",test.genes_F1_
    CTRL_HFDt$Targets)
test.genes_F1_CTRL_HFDt <- as.data.frame(table(test.genes_F1_CTRL_
    HFDt$Targets))
colnames(test.genes_F1_CTRL_HFDt) <- c("Targets","counts")

genecounts_F1_CTRL_HFDt <- test.genes_F1_CTRL_HFDt[[2]]
names(genecounts_F1_CTRL_HFDt) <- test.genes_F1_CTRL_HFDt[[1]]

##prepare topGO dataobjects
topGO.BP_F1_CTRL_vs_HFDt <- new("topGOdata", ontology = "BP",
    allGenes = genecounts_F1_CTRL_HFDt, geneSel = count.filter,
description = "GO analysis BP: CTRL_F1 vs. HFDt_F1", nodeSize = 10,
     annotationFun = annFUN.org, mapping = "org.Mm.eg.db", ID = "
    ensembl")

topGO.MF_F1_CTRL_vs_HFDt <- new("topGOdata", ontology = "MF",
    allGenes = genecounts_F1_CTRL_HFDt, geneSel = count.filter,
description = "GO analysis MF: CTRL_F1 vs. HFDt_F1", nodeSize = 10,
     annotationFun = annFUN.org, mapping = "org.Mm.eg.db", ID = "
    ensembl")

topGO.CC_F1_CTRL_vs_HFDt <- new("topGOdata", ontology = "CC",
    allGenes = genecounts_F1_CTRL_HFDt, geneSel = count.filter,
```

```
description = "GO analysis CC: CTRL_F1 vs. HFDt_F1", nodeSize = 10,
    annotationFun = annFUN.org, mapping = "org.Mm.eg.db", ID = "
    ensembl")

##perform GO test - weight01 & Fisher
runGO.BP_F1_CTRL_vs_HFDt <- runTest(topGO.BP_F1_CTRL_vs_HFDt,
    algorithm = "weight01", statistic = "fisher")#, topNodes = 5)
runGO.MF_F1_CTRL_vs_HFDt <- runTest(topGO.MF_F1_CTRL_vs_HFDt,
    algorithm = "weight01", statistic = "fisher")
runGO.CC_F1_CTRL_vs_HFDt <- runTest(topGO.CC_F1_CTRL_vs_HFDt,
    algorithm = "weight01", statistic = "fisher")

##generate result tables
GO.BP_F1_CTRL_vs_HFDt <- GenTable(topGO.BP_F1_CTRL_vs_HFDt,
    Weight01 = runGO.BP_F1_CTRL_vs_HFDt, orderBy = "Weight01",
    topNodes = 30)
GO.MF_F1_CTRL_vs_HFDt <- GenTable(topGO.MF_F1_CTRL_vs_HFDt,
    Weight01 = runGO.MF_F1_CTRL_vs_HFDt, orderBy = "Weight01",
    topNodes = 30)
GO.CC_F1_CTRL_vs_HFDt <- GenTable(topGO.CC_F1_CTRL_vs_HFDt,
    Weight01 = runGO.CC_F1_CTRL_vs_HFDt, orderBy = "Weight01",
    topNodes = 30)

##Write results tables
write.csv2(GO.BP_F1_CTRL_vs_HFDt,"GO Biological Process CTRL-F1 vs
    HFDt-F1.csv", row.names = FALSE)
write.csv2(GO.MF_F1_CTRL_vs_HFDt,"GO Molecular Function CTRL-F1 vs
    HFDt-F1.csv", row.names = FALSE)
write.csv2(GO.CC_F1_CTRL_vs_HFDt,"GO Cellular Component CTRL-F1 vs
    HFDt-F1.csv", row.names = FALSE)



#Generation F1: HFD vs. HFDt
##load targets of DEGs
```

```
F1_HFD_HFDt_Targets <- read_csv("F1_HFD_vs_HFDt - sRNAtools
    Targets.csv")
F1_HFD_HFDt_Targets <- F1_HFD_HFDt_Targets %>% separate_rows(
    Targets)

##data structure
test.genes_F1_HFD_HFDt <- F1_HFD_HFDt_Targets[4]
test.genes_F1_HFD_HFDt$Targets <- gsub("\\..*","",test.genes_F1_HFD
    _HFDt$Targets)
test.genes_F1_HFD_HFDt <- as.data.frame(table(test.genes_F1_HFD_
    HFDt$Targets))
colnames(test.genes_F1_HFD_HFDt) <- c("Targets","counts")

genecounts_F1_HFD_HFDt <- test.genes_F1_HFD_HFDt[[2]]
names(genecounts_F1_HFD_HFDt) <- test.genes_F1_HFD_HFDt[[1]]

##prepare topGO dataobjects
topGO.BP_F1_HFD_vs_HFDt <- new("topGOdata", ontology = "BP",
    allGenes = genecounts_F1_HFD_HFDt, geneSel = count.filter,
description = "GO analysis BP: HFD_F1 vs. HFDt_F1", nodeSize = 10,
    annotationFun = annFUN.org, mapping = "org.Mm.eg.db", ID = "
    ensembl")

topGO.MF_F1_HFD_vs_HFDt <- new("topGOdata", ontology = "MF",
    allGenes = genecounts_F1_HFD_HFDt, geneSel = count.filter,
description = "GO analysis MF: HFD_F1 vs. HFDt_F1", nodeSize = 10,
    annotationFun = annFUN.org, mapping = "org.Mm.eg.db", ID = "
    ensembl")

topGO.CC_F1_HFD_vs_HFDt <- new("topGOdata", ontology = "CC",
    allGenes = genecounts_F1_HFD_HFDt, geneSel = count.filter,
description = "GO analysis CC: HFD_F1 vs. HFDt_F1", nodeSize = 10,
    annotationFun = annFUN.org, mapping = "org.Mm.eg.db", ID = "
    ensembl")
```

```
##perform GO test - weight01 & Fisher
runGO.BP_F1_HFD_vs_HFDt <- runTest(topGO.BP_F1_HFD_vs_HFDt,
    algorithm = "weight01", statistic = "fisher")#, topNodes = 5)
runGO.MF_F1_HFD_vs_HFDt <- runTest(topGO.MF_F1_HFD_vs_HFDt,
    algorithm = "weight01", statistic = "fisher")
runGO.CC_F1_HFD_vs_HFDt <- runTest(topGO.CC_F1_HFD_vs_HFDt,
    algorithm = "weight01", statistic = "fisher")

##generate result tables
GO.BP_F1_HFD_vs_HFDt <- GenTable(topGO.BP_F1_HFD_vs_HFDt, Weight01
    = runGO.BP_F1_HFD_vs_HFDt, orderBy = "Weight01", topNodes = 30)
GO.MF_F1_HFD_vs_HFDt <- GenTable(topGO.MF_F1_HFD_vs_HFDt, Weight01
    = runGO.MF_F1_HFD_vs_HFDt, orderBy = "Weight01", topNodes = 30)
GO.CC_F1_HFD_vs_HFDt <- GenTable(topGO.CC_F1_HFD_vs_HFDt, Weight01
    = runGO.CC_F1_HFD_vs_HFDt, orderBy = "Weight01", topNodes = 30)

##Write results tables
write.csv2(GO.BP_F1_HFD_vs_HFDt,"GO Biological Process HFD-F1 vs
    HFDt-F1.csv", row.names = FALSE)
write.csv2(GO.MF_F1_HFD_vs_HFDt,"GO Molecular Function HFD-F1 vs
    HFDt-F1.csv", row.names = FALSE)
write.csv2(GO.CC_F1_HFD_vs_HFDt,"GO Cellular Component HFD-F1 vs
    HFDt-F1.csv", row.names = FALSE)



#Generation F2: CTRL vs. HFDt
##load targets of DEGs
F2_CTRL_HFDt_Targets <- read_csv("F2_CTRL_vs_HFDt - sRNAtools
    Targets.csv")
F2_CTRL_HFDt_Targets <- F2_CTRL_HFDt_Targets %>% separate_rows(
    Targets)

##data structure
```

```
test.genes_F2_CTRL_HFDt <- F2_CTRL_HFDt_Targets[4]
test.genes_F2_CTRL_HFDt$Targets <- gsub("\\..*","",test.genes_F2_
    CTRL_HFDt$Targets)
test.genes_F2_CTRL_HFDt <- as.data.frame(table(test.genes_F2_CTRL_
    HFDt$Targets))
colnames(test.genes_F2_CTRL_HFDt) <- c("Targets","counts")

genecounts_F2_CTRL_HFDt <- test.genes_F2_CTRL_HFDt[[2]]
names(genecounts_F2_CTRL_HFDt) <- test.genes_F2_CTRL_HFDt[[1]]

##prepare topGO dataobjects
topGO.BP_F2_CTRL_vs_HFDt <- new("topGOdata", ontology = "BP",
    allGenes = genecounts_F2_CTRL_HFDt, geneSel = count.filter,
description = "GO analysis BP: CTRL_F2 vs. HFDt_F2", nodeSize = 10,
     annotationFun = annFUN.org, mapping = "org.Mm.eg.db", ID = "
    ensembl")

topGO.MF_F2_CTRL_vs_HFDt <- new("topGOdata", ontology = "MF",
    allGenes = genecounts_F2_CTRL_HFDt, geneSel = count.filter,
description = "GO analysis MF: CTRL_F2 vs. HFDt_F2", nodeSize = 10,
     annotationFun = annFUN.org, mapping = "org.Mm.eg.db", ID = "
    ensembl")

topGO.CC_F2_CTRL_vs_HFDt <- new("topGOdata", ontology = "CC",
    allGenes = genecounts_F2_CTRL_HFDt, geneSel = count.filter,
description = "GO analysis CC: CTRL_F2 vs. HFDt_F2", nodeSize = 10,
     annotationFun = annFUN.org, mapping = "org.Mm.eg.db", ID = "
    ensembl")

##perform GO test - weight01 & Fisher
runGO.BP_F2_CTRL_vs_HFDt <- runTest(topGO.BP_F2_CTRL_vs_HFDt,
    algorithm = "weight01", statistic = "fisher")#, topNodes = 5)
runGO.MF_F2_CTRL_vs_HFDt <- runTest(topGO.MF_F2_CTRL_vs_HFDt,
    algorithm = "weight01", statistic = "fisher")
```

```
runGO.CC_F2_CTRL_vs_HFDt <- runTest(topGO.CC_F2_CTRL_vs_HFDt,
    algorithm = "weight01", statistic = "fisher")

##generate result tables
GO.BP_F2_CTRL_vs_HFDt <- GenTable(topGO.BP_F2_CTRL_vs_HFDt,
    Weight01 = runGO.BP_F2_CTRL_vs_HFDt, orderBy = "Weight01",
    topNodes = 30)
GO.MF_F2_CTRL_vs_HFDt <- GenTable(topGO.MF_F2_CTRL_vs_HFDt,
    Weight01 = runGO.MF_F2_CTRL_vs_HFDt, orderBy = "Weight01",
    topNodes = 30)
GO.CC_F2_CTRL_vs_HFDt <- GenTable(topGO.CC_F2_CTRL_vs_HFDt,
    Weight01 = runGO.CC_F2_CTRL_vs_HFDt, orderBy = "Weight01",
    topNodes = 30)

##Write results tables
write.csv2(GO.BP_F2_CTRL_vs_HFDt,"GO Biological Process CTRL-F2 vs
    HFDt-F2.csv", row.names = FALSE)
write.csv2(GO.MF_F2_CTRL_vs_HFDt,"GO Molecular Function CTRL-F2 vs
    HFDt-F2.csv", row.names = FALSE)
write.csv2(GO.CC_F2_CTRL_vs_HFDt,"GO Cellular Component CTRL-F2 vs
    HFDt-F2.csv", row.names = FALSE)



#Generation F2: HFD vs. HFDt
##load targets of DEGs
F2_HFD_HFDt_Targets <- read_csv("F2_HFD_vs_HFDt - sRNAtools
    Targets.csv")
F2_HFD_HFDt_Targets <- F2_HFD_HFDt_Targets %>% separate_rows(
    Targets)

##data structure
test.genes_F2_HFD_HFDt <- F2_HFD_HFDt_Targets[4]
test.genes_F2_HFD_HFDt$Targets <- gsub("\\..*","",test.genes_F2_HFD
    _HFDt$Targets)
```

```
test.genes_F2_HFD_HFDt <- as.data.frame(table(test.genes_F2_HFD_
    HFDt$Targets))
colnames(test.genes_F2_HFD_HFDt) <- c("Targets","counts")

genecounts_F2_HFD_HFDt <- test.genes_F2_HFD_HFDt[[2]]
names(genecounts_F2_HFD_HFDt) <- test.genes_F2_HFD_HFDt[[1]]

##prepare topGO dataobjects
topGO.BP_F2_HFD_vs_HFDt <- new("topGOdata", ontology = "BP",
    allGenes = genecounts_F2_HFD_HFDt, geneSel = count.filter,
description = "GO analysis BP: HFD_F2 vs. HFDt_F2", nodeSize = 10,
    annotationFun = annFUN.org, mapping = "org.Mm.eg.db", ID = "
    ensembl")

topGO.MF_F2_HFD_vs_HFDt <- new("topGOdata", ontology = "MF",
    allGenes = genecounts_F2_HFD_HFDt, geneSel = count.filter,
description = "GO analysis MF: HFD_F2 vs. HFDt_F2", nodeSize = 10,
    annotationFun = annFUN.org, mapping = "org.Mm.eg.db", ID = "
    ensembl")

topGO.CC_F2_HFD_vs_HFDt <- new("topGOdata", ontology = "CC",
    allGenes = genecounts_F2_HFD_HFDt, geneSel = count.filter,
description = "GO analysis CC: HFD_F2 vs. HFDt_F2", nodeSize = 10,
    annotationFun = annFUN.org, mapping = "org.Mm.eg.db", ID = "
    ensembl")

##perform GO test - weight01 & Fisher
runGO.BP_F2_HFD_vs_HFDt <- runTest(topGO.BP_F2_HFD_vs_HFDt,
    algorithm = "weight01", statistic = "fisher")#, topNodes = 5)
runGO.MF_F2_HFD_vs_HFDt <- runTest(topGO.MF_F2_HFD_vs_HFDt,
    algorithm = "weight01", statistic = "fisher")
runGO.CC_F2_HFD_vs_HFDt <- runTest(topGO.CC_F2_HFD_vs_HFDt,
    algorithm = "weight01", statistic = "fisher")
```

```
##generate result tables
GO.BP_F2_HFD_vs_HFDt <- GenTable(topGO.BP_F2_HFD_vs_HFDt, Weight01
    = runGO.BP_F2_HFD_vs_HFDt, orderBy = "Weight01", topNodes = 30)
GO.MF_F2_HFD_vs_HFDt <- GenTable(topGO.MF_F2_HFD_vs_HFDt, Weight01
    = runGO.MF_F2_HFD_vs_HFDt, orderBy = "Weight01", topNodes = 30)
GO.CC_F2_HFD_vs_HFDt <- GenTable(topGO.CC_F2_HFD_vs_HFDt, Weight01
    = runGO.CC_F2_HFD_vs_HFDt, orderBy = "Weight01", topNodes = 30)

##Write results tables
write.csv2(GO.BP_F2_HFD_vs_HFDt,"GO Biological Process HFD-F2 vs
    HFDt-F2.csv", row.names = FALSE)
write.csv2(GO.MF_F2_HFD_vs_HFDt,"GO Molecular Function HFD-F2 vs
    HFDt-F2.csv", row.names = FALSE)
write.csv2(GO.CC_F2_HFD_vs_HFDt,"GO Cellular Component HFD-F2 vs
    HFDt-F2.csv", row.names = FALSE)



#test database
# xx <- annFUN.org("BP", mapping = "org.Mm.eg.db", ID = "ensembl")
# head(xx)

#perform GO tests
# go_test <- runTest(test, algorithm = "weight01", #the more
    balance algorithm. 'classic' (unhierarchical), 'lea' (?), 'elim'
     (more conservative), 'parentchild' (close to weight-9
# statistic = "fisher")



#create charts
#input files were combined and edited in Excel
library(readxl)
library(ggplot2)
library(scales)
```

```
#Biological process - Transgenerational
GO_results_BP <- read_excel("GO_results_Biological process - All
    generations.xlsx", col_types = c("text", "text", "text", "text",
     "numeric", "numeric", "numeric"))
GO_results_BP$group <- factor(GO_results_BP$group, levels = c("CTRL
     vs. HFD", "CTRL vs. HFDt", "HFD vs. HFDt"))
GO_results_BP$Gen <- as.factor(GO_results_BP$Gen)



reverselog_trans <- function(base = exp(1)) {
     trans <- function(x) -log(x, base)
     inv <- function(x) base^(-x)
     trans_new(paste0("reverselog-", format(base)), trans, inv,
     log_breaks(base = base),
     domain = c(1e-100, Inf))
}

tiff("Figure 5 - GO Biological Process.tiff", width=800, height
    =600, units="px")
ggplot(data=GO_results_BP, aes(y=Term, x=p.value, fill=group)) +
geom_bar(stat="identity", position=position_dodge(preserve = '
    single')) +
facet_grid(rows = GO_results_BP$Gen, scales="free_y", space="free")
     +
scale_x_continuous(trans = reverselog_trans(10), breaks = breaks_
    log(n = 7, base = 10)) +
scale_fill_brewer(palette="Set1") +
#labs(x = "-log(p-value)", y = "GO terms", fill = "Group") +
labs(x = "-log(p-adj)", y = "", fill = "Group") +
theme(axis.title = element_text(size = 12, face="bold")) +
theme(legend.title = element_text(size = 12, face="bold")) +
theme(strip.text.y = element_text(size = 12, face="bold"))
dev.off()
```

```
#Molecular function - Transgenerational
GO_results_MF <- read_excel("GO_results_Molecular function - All
    generations.xlsx", col_types = c("text", "text", "text", "text",
     "numeric", "numeric", "numeric"))
GO_results_MF$group <- factor(GO_results_MF$group, levels = c("CTRL
     vs. HFD", "CTRL vs. HFDt", "HFD vs. HFDt"))
GO_results_MF$Gen <- as.factor(GO_results_MF$Gen)

tiff("Figure S1 - GO Molecular Function.tiff", width=800, height
    =550, units="px")
ggplot(data=GO_results_MF, aes(y=Term, x=p.value, fill=group)) +
geom_bar(stat="identity", position=position_dodge(preserve = '
    single')) +
facet_grid(rows = GO_results_MF$Gen, scales="free_y", space="free")
     +
scale_x_continuous(trans = reverselog_trans(10), breaks = breaks_
    log(n = 7, base = 10)) +
scale_fill_brewer(palette="Set1") +
#labs(x = "-log(p-value)", y = "GO terms", fill = "Group") +
labs(x = "-log(p-adj)", y = "", fill = "Group") +
theme(axis.title = element_text(size = 12, face="bold")) +
theme(legend.title = element_text(size = 12, face="bold")) +
theme(strip.text.y = element_text(size = 12, face="bold"))
dev.off()

#Cellular component - Transgenerational
GO_results_CC <- read_excel("GO_results_Cellular component - All
    generations.xlsx", col_types = c("text", "text", "text", "text",
     "numeric", "numeric", "numeric"))
GO_results_CC$group <- factor(GO_results_CC$group, levels = c("CTRL
     vs. HFD", "CTRL vs. HFDt", "HFD vs. HFDt"))
GO_results_CC$Gen <- as.factor(GO_results_CC$Gen)

tiff("Figure S2 - GO Cellular Component.tiff", width=800, height
```

```
    =500, units="px")
ggplot(data=GO_results_CC, aes(y=Term, x=p.value, fill=group)) +
geom_bar(stat="identity", position=position_dodge(preserve = '
    single')) +
facet_grid(rows = GO_results_CC$Gen, scales="free_y", space="free")
    +
scale_x_continuous(trans = reverselog_trans(10), breaks = breaks_
    log(n = 7, base = 10)) +
scale_fill_brewer(palette="Set1") +
#labs(x = "-log(p-value)", y = "GO terms", fill = "Group") +
labs(x = "-log(p-adj)", y = "", fill = "Group") +
theme(axis.title = element_text(size = 12, face="bold")) +
theme(legend.title = element_text(size = 12, face="bold")) +
theme(strip.text.y = element_text(size = 12, face="bold"))
dev.off()


#Euler diagram DEGs accross generations/diets
euler.ult <- c("F0 CTRL vs. HFDt" = 19, "F1 CTRL vs. HFD" = 86, "F1
    CTRL vs. HFDt" = 24, "F1 HFD vs. HFDt" = 5, "F2 CTRL vs. HFDt"
   = 3, "F2 HFD vs. HFDt" = 4,
"F1 CTRL vs. HFD&F1 CTRL vs. HFDt" = 10, "F1 CTRL vs. HFD&F1 HFD vs
    . HFDt" = 5, "F1 CTRL vs. HFDt&F1 HFD vs. HFDt" = 1,
"F2 CTRL vs. HFDt&F2 HFD vs. HFDt" = 2)


##print version
svg(filename = "Figure 6 - Euler diagram_v1.svg", width=9, height
    =6, pointsize = 12, family = "sans")
plot(
euler(euler.ult, shape = "ellipse"), counts = TRUE,
quantities = list(type = c("counts", "percent"), round=2, cex=0.8),
fills =list(fill=c("#377EB8", "#E41A1C", "#377EB8", "#4DAF4A", "
    #377EB8", "#4DAF4A")), alpha = 0.3, c("#377EB8", "#E41A1C", "
```

```
    #377EB8", "#4DAF4A", "#377EB8", "#4DAF4A"), alpha = 0.3,
labels = list(font = 2, cex=1),
)
dev.off()

##colorblind version
svg(filename = "Figure 6 - Euler diagram_v2.svg", width=9, height
    =6, pointsize = 12, family = "sans")
plot(
euler(euler.ult, shape = "ellipse"), counts = TRUE,
quantities = list(type = c("counts", "percent"), round=2, cex=0.8),
labels = list(font = 2, cex=1),
)
dev.off()


#código antigo
#Generation F0
GO_results_Gen0 <- read_excel("GO_results_Gen0.xlsx", col_types = c
    ("text", "text", "text", "text", "numeric", "numeric"))
GO_results_Gen0$group <- factor(GO_results_Gen0$group, levels = c("
    CTRL vs. HFD", "CTRL vs. HFDt", "HFD vs. HFDt"))
GO_results_Gen0$GO.analysis <- factor(GO_results_Gen0$GO.analysis,
    levels = c("Biological Process", "Molecular Function", "Cellular
     Component"))

#bar graph (save as 1200 x 664)
ggplot(data=GO_results_Gen0, aes(y=Term, x=norm.annot, fill=group))
    +
geom_bar(stat="identity", position=position_dodge(preserve = '
    single')) +
facet_grid(rows = GO_results_Gen0$GO.analysis, scales="free_y",
    space="free") +
scale_x_continuous(breaks = pretty(GO_results_Gen0$norm.annot, n =
```

```
    10)) +
scale_fill_brewer(palette="Set1") +
labs(x = "GO annotations (per 1000 targets)", y = "GO terms", fill
    = "Group") +
theme(axis.title = element_text(size = 12, face="bold")) +
theme(legend.title = element_text(size = 12, face="bold")) +
theme(strip.text.y = element_text(size = 12, face="bold"))

#Generation F1
GO_results_Gen1 <- read_excel("GO_results_Gen1.xlsx", col_types = c
    ("text", "text", "text", "text", "numeric", "numeric"))
GO_results_Gen1$group <- factor(GO_results_Gen1$group, levels = c("
    CTRL vs. HFD", "CTRL vs. HFDt", "HFD vs. HFDt"))
GO_results_Gen1$GO.analysis <- factor(GO_results_Gen1$GO.analysis,
    levels = c("Biological Process", "Molecular Function", "Cellular
     Component"))

#bar graph (save as 1200 x 764)
ggplot(data=GO_results_Gen1, aes(y=Term, x=norm.annot, fill=group))
    +
geom_bar(stat="identity", position=position_dodge(preserve = '
    single')) +
facet_grid(rows = GO_results_Gen1$GO.analysis, scales="free_y",
    space="free") +
scale_x_continuous(breaks = pretty(GO_results_Gen1$norm.annot, n =
    10)) +
scale_fill_brewer(palette="Set1") +
labs(x = "GO annotations (per 1000 targets)", y = "GO terms", fill
    = "Group") +
theme(axis.title = element_text(size = 12, face="bold")) +
theme(legend.title = element_text(size = 12, face="bold")) +
theme(strip.text.y = element_text(size = 12, face="bold"))

#Generation F2
```

```
GO_results_Gen2 <- read_excel("GO_results_Gen2.xlsx", col_types = c
    ("text", "text", "text", "text", "numeric", "numeric"))
GO_results_Gen2$group <- factor(GO_results_Gen2$group, levels = c("
    CTRL vs. HFD", "CTRL vs. HFDt", "HFD vs. HFDt"))
GO_results_Gen2$GO.analysis <- factor(GO_results_Gen2$GO.analysis,
    levels = c("Biological Process", "Molecular Function", "Cellular
     Component"))

#bar graph (save as 1200 x 764)
ggplot(data=GO_results_Gen2, aes(y=Term, x=norm.annot, fill=group))
    +
geom_bar(stat="identity", position=position_dodge(preserve = '
    single')) +
facet_grid(rows = GO_results_Gen2$GO.analysis, scales="free_y",
    space="free") +
scale_x_continuous(breaks = pretty(GO_results_Gen2$norm.annot, n =
    10)) +
scale_fill_brewer(palette="Set1") +
labs(x = "GO annotations (per 1000 targets)", y = "GO terms", fill
   = "Group") +
theme(axis.title = element_text(size = 12, face="bold")) +
theme(legend.title = element_text(size = 12, face="bold")) +
theme(strip.text.y = element_text(size = 12, face="bold"))

#Biological process - Transgenerational
GO_results_BP <- read_excel("GO_results_Biological process - All
    generations.xlsx", col_types = c("text", "text", "text", "text",
     "numeric", "numeric"))
GO_results_BP$group <- factor(GO_results_BP$group, levels = c("CTRL
     vs. HFD", "CTRL vs. HFDt", "HFD vs. HFDt"))
GO_results_BP$GO.analysis <- factor(GO_results_BP$GO.analysis,
    levels = c("F0 Generation", "F1 Generation", "F2 Generation"))

#bar graph (save as 1200 x 764)
```

```
ggplot(data=GO_results_BP, aes(y=Term, x=norm.annot, fill=group)) +
geom_bar(stat="identity", position=position_dodge(preserve = '
    single')) +
facet_grid(rows = GO_results_BP$GO.analysis, scales="free_y", space
    ="free") +
scale_x_continuous(breaks = pretty(GO_results_BP$norm.annot, n =
    10)) +
scale_fill_brewer(palette="Set1") +
labs(x = "GO annotations (per 1000 targets)", y = "GO terms", fill
    = "Group") +
theme(axis.title = element_text(size = 12, face="bold")) +
theme(legend.title = element_text(size = 12, face="bold")) +
theme(strip.text.y = element_text(size = 12, face="bold"))

#other options:
#ylab("GO terms") + xlab("Number of annotations") +
#geom_text(aes(label=Annotated), vjust=1.0, color="black", position
     = position_dodge(0.0), size=3.5) +
#+ scale_fill_discrete(name="Type", breaks=c("TRUE", "FALSE", "NA")
    , labels=c("existing", "not existing", "missing values")) +
    scale_x_discrete(labels = c("bad", "good"))
#facet_wrap(~GO.analysis, scales="free_y") +
#theme_minimal()
#coord_flip()

#Generation F0 vs. F1 - CTRL vs. HFD
GO_results_Gen0vs1_HFD <- read_excel("GO_results_Gen0_Gen1-HFD.xlsx
    ", col_types = c("text", "text", "text", "text", "numeric", "
    numeric"))
GO_results_Gen0vs1_HFD$group <- factor(GO_results_Gen0vs1_HFD$group
    , levels = c("Generation F0", "Generation F0"))
GO_results_Gen0vs1_HFD$GO.analysis <- factor(GO_results_Gen0vs1_HFD
    $GO.analysis, levels = c("Biological Process", "Molecular
    Function", "Cellular Component"))
```

```
#bar graph (save as 1200 x 764)
ggplot(data=GO_results_Gen0vs1_HFD, aes(y=Term, x=norm.annot, fill=
    group)) +
geom_bar(stat="identity", position=position_dodge(preserve = '
    single')) +
facet_grid(rows = GO_results_Gen0vs1_HFD$GO.analysis, scales="free_
    y", space="free") +
scale_x_continuous(breaks = pretty(GO_results_Gen0vs1_HFD$norm.
    annot, n = 10)) +
scale_fill_brewer(palette="Paired") +
labs(x = "GO annotations (per 1000 targets)", y = "GO terms", fill
    = "Generation") +
theme(axis.title = element_text(size = 12, face="bold")) +
theme(legend.title = element_text(size = 12, face="bold")) +
theme(strip.text.y = element_text(size = 12, face="bold"))
```

C.4 Paternal epigenetic inheritance of metabolic traits: a scientometric and systematic review

This annex contains scripts used in the analysis of the unpublished work:

Crisóstomo, **L.**, Oliveira, P. F., & Alves, M. G. (2022). *Paternal epigenetic inheritance of metabolic traits: A scientometric and systematic review* (Journal Article)

Sequence annotation

```
#set directory
setwd("D:/Dropbox (Personal)/UP/Artigos de revisão/2021_
    Transgenerational effects (Systematic review)/R analysis")

#libraries
library(tidyverse)
library(mixOmics)
library(igraph)

#load data
metabolites.F0 <- read.table(file = "F0_FC_metabolites.csv", header
     = TRUE, sep = ",", row.names = 1, check.names = FALSE)
lipids.F0 <- read.table(file = "F0_FC_lipids.csv", header = TRUE,
    sep = ",", row.names = 1, check.names = FALSE)
mirnas.F0 <- read.table(file = "F0_FC_miRNA.csv", header = TRUE,
    sep = ",", row.names = 1, check.names = FALSE)
pirnas.F0 <- read.table(file = "F0_FC_piRNA.csv", header = TRUE,
    sep = ",", row.names = 1, check.names = FALSE)
reprnas.F0 <- read.table(file = "F0_FC_repRNA.csv", header = TRUE,
    sep = ",", row.names = 1, check.names = FALSE)
tirnas.F0 <- read.table(file = "F0_FC_tiRNA.csv", header = TRUE,
    sep = ",", row.names = 1, check.names = FALSE)
trfs.F0 <- read.table(file = "F0_FC_tRF.csv", header = TRUE, sep =
    ",", row.names = 1, check.names = FALSE)

sample.matrix.F0 <- data.frame(sample = rownames(metabolites.F0),
    Group = as.factor(c(rep("CTRL",5), rep("HFD", 6), rep("HFDt",5))
    ))

#select the most representative samples (metabolites + lipids)
pls.F0 <- pls(metabolites.F0, lipids.F0)
plotIndiv(pls.F0, group = sample.matrix.F0$Group,
```

```
col.per.group = c("dodgerblue","red3","darkgreen"), rep.space = "XY
    -variate",
centroid = TRUE, ellipse = TRUE, abline = TRUE, size.xlabel = 12,
    size.ylabel = 12,
xlim = c(-5,5), ylim = c(-5,5), title = 'Generation F0: 2-omics PLS
    ')

#Selected: CTRL1, CTRL2, CTRL4, HFD2, HFD4, HFD5, HFDt2, HFDt3,
    HFDt4
select.F0 <- c("F0_CTRL1", "F0_CTRL2", "F0_CTRL4", "F0_HFD2", "F0_
    HFD4", "F0_HFD5", "F0_HFDt2", "F0_HFDt3", "F0_HFDt4")

#DIABLO plot (block PLS-DA)
##discard samples not available in sncRNA data
met.F0 <- metabolites.F0[(row.names(metabolites.F0) %in% select.F0)
    ,]
lip.F0 <- lipids.F0[(row.names(lipids.F0) %in% select.F0),]
groups.F0 <- data.frame(Sample = select.F0, Group = as.factor(c(rep
    ("CTRL",3), rep("HFD", 3), rep("HFDt",3))))

##change colnames of sncRNA rows
rownames(mirnas.F0) <- select.F0
rownames(pirnas.F0) <- select.F0
rownames(reprnas.F0) <- select.F0
rownames(tirnas.F0) <- select.F0
rownames(trfs.F0) <- select.F0

##compile data
data.F0 <- list(Metabolites = met.F0, Lipids = lip.F0, miRNA =
    mirnas.F0,
piRNA = pirnas.F0, repRNA = reprnas.F0, tiRNA = tirnas.F0, tRF =
    trfs.F0)
Y <- as.factor(groups.F0$Group)
keepX <- list(Metabolites = c(round(ncol(met.F0)*0.9, digits = 0),
```

```
    round(ncol(met.F0)*0.9, digits = 0)),
Lipids = c(round(ncol(lip.F0)*0.9, digits = 0), round(ncol(lip.F0)*
    0.9, digits = 0)),
miRNA = c(round(ncol(mirnas.F0)*0.9, digits = 0), round(ncol(mirnas
    .F0)*0.9, digits = 0)),
piRNA = c(round(ncol(pirnas.F0)*0.9, digits = 0), round(ncol(pirnas
    .F0)*0.9, digits = 0)),
repRNA = c(round(ncol(reprnas.F0)*0.9, digits = 0), round(ncol(
    reprnas.F0)*0.9, digits = 0)),
tiRNA = c(round(ncol(tirnas.F0)*0.9, digits = 0), round(ncol(tirnas
    .F0)*0.9, digits = 0)),
tRF = c(round(ncol(trfs.F0)*0.9, digits = 0), round(ncol(trfs.F0)*
    0.9, digits = 0)))

splsda.F0 <- block.splsda(X = data.F0, Y = Y, keepX = keepX, ncomp
    =2, near.zero.var = TRUE)

#Model performance
# perf.splsda.F0 <- perf(splsda.F0, validation = "Mfold", folds =
    3, progressBar = TRUE, nrepeat = 50)
#
# plot(perf.splsda.F0, col = color.mixo(5:7), sd = TRUE, legend.
    position = "horizontal")

#DIABLO plots
##block plot
tiff("Generation F0 - Ind. Plots.tiff", width=650, height=600,
    units="px")
plotIndiv(splsda.F0, ind.names = FALSE, subtitle = c("Metabolites"
    , "Lipids", "miRNAs",
"piRNAs", "repRNA", "tiRNAs", "tRFs"),
legend=TRUE, centroid = TRUE, abline = TRUE,
pch = c(15, 16, 17), cex = c(3, 3, 3), size.title = rel(1.5), size.
    subtitle = rel(1.4),
```

```
size.xlabel = rel(1.2), size.ylabel = rel(1.2), title = "Generation
    F0", legend.title = "Group")

dev.off()

##diablo plot
tiff("Generation F0 - DIABLO plot.tiff", width=650, height=600,
    units="px")
plotDiablo(splsda.F0, ncomp = 1)
dev.off()

##plot Loadings
plotLoadings(splsda.F0, ncomp = 1, contrib = 'max', method = "
    median")

##ROC figures
ROC.F0 <- auroc(splsda.F0, roc.block = c("Metabolites", "Lipids", "
    miRNA",
"piRNA", "repRNA", "tiRNA", "tRF"), roc.comp = 2)



#Generation F1
#load data
metabolites.F1 <- read.table(file = "F1_FC_metabolites.csv", header
    = TRUE, sep = ",", row.names = 1, check.names = FALSE)
lipids.F1 <- read.table(file = "F1_FC_lipids.csv", header = TRUE,
    sep = ",", row.names = 1, check.names = FALSE)
mirnas.F1 <- read.table(file = "F1_FC_miRNA.csv", header = TRUE,
    sep = ",", row.names = 1, check.names = FALSE)
pirnas.F1 <- read.table(file = "F1_FC_piRNA.csv", header = TRUE,
    sep = ",", row.names = 1, check.names = FALSE)
reprnas.F1 <- read.table(file = "F1_FC_repRNA.csv", header = TRUE,
    sep = ",", row.names = 1, check.names = FALSE)
tirnas.F1 <- read.table(file = "F1_FC_tiRNA.csv", header = TRUE,
```

```
    sep = ",", row.names = 1, check.names = FALSE)
trfs.F1 <- read.table(file = "F1_FC_tRF.csv", header = TRUE, sep =
    ",", row.names = 1, check.names = FALSE)

sample.matrix.F1 <- data.frame(sample = rownames(metabolites.F1),
    Group = as.factor(c(rep("CTRL",6), rep("HFD", 6), rep("HFDt",6))
    ))

#select the most representative samples (metabolites + lipids)
pls.F1 <- pls(metabolites.F1, lipids.F1)
plotIndiv(pls.F1, group = sample.matrix.F1$Group,
col.per.group = c("dodgerblue","red3","darkgreen"), rep.space = "XY
    -variate",
centroid = TRUE, ellipse = TRUE, abline = TRUE, size.xlabel = 12,
    size.ylabel = 12,
xlim = c(-8,8), ylim = c(-8,8), title = 'Generation F1: 2-omics PLS
    ')

#Selected: CTRL3, CTRL6, HFD5, HFD6, HFDt1, HFDt2
select.F1 <- c("F1_CTRL3", "F1_CTRL6", "F1_HFD5", "F1_HFD6", "F1_
    HFDt1", "F1_HFDt2")

#DIABLO plot (block PLS-DA)
##discard samples not available in sncRNA data
met.F1 <- metabolites.F1[(row.names(metabolites.F1) %in% select.F1)
    ,]
lip.F1 <- lipids.F1[(row.names(lipids.F1) %in% select.F1),]
groups.F1 <- data.frame(Sample = select.F1, Group = as.factor(c(rep
    ("CTRL", 2), rep("HFD", 2), rep("HFDt", 2))))

##change colnames of sncRNA rows
rownames(mirnas.F1) <- select.F1
rownames(pirnas.F1) <- select.F1
rownames(reprnas.F1) <- select.F1
```

```
rownames(tirnas.F1) <- select.F1
rownames(trfs.F1) <- select.F1

##compile data
data.F1 <- list(Metabolites = met.F1, Lipids = lip.F1, miRNA =
    mirnas.F1,
piRNA = pirnas.F1, repRNA = reprnas.F1, tiRNA = tirnas.F1, tRF =
    trfs.F1)
Y <- as.factor(groups.F1$Group)
keepX <- list(Metabolites = c(round(ncol(met.F1)*0.9, digits = 0),
    round(ncol(met.F1)*0.9, digits = 0)),
Lipids = c(round(ncol(lip.F1)*0.9, digits = 0), round(ncol(lip.F1)*
    0.9, digits = 0)),
miRNA = c(round(ncol(mirnas.F1)*0.9, digits = 0), round(ncol(mirnas
    .F1)*0.9, digits = 0)),
piRNA = c(round(ncol(pirnas.F1)*0.9, digits = 0), round(ncol(pirnas
    .F1)*0.9, digits = 0)),
repRNA = c(round(ncol(reprnas.F1)*0.9, digits = 0), round(ncol(
    reprnas.F1)*0.9, digits = 0)),
tiRNA = c(round(ncol(tirnas.F1)*0.9, digits = 0), round(ncol(tirnas
    .F1)*0.9, digits = 0)),
tRF = c(round(ncol(trfs.F1)*0.9, digits = 0), round(ncol(trfs.F1)*
    0.9, digits = 0)))

splsda.F1 <- block.splsda(X = data.F1, Y = Y, keepX = keepX, ncomp
    =2, near.zero.var = TRUE)

#Model performance
# perf.splsda.F1 <- perf(splsda.F1, validation = "Mfold", folds =
    2, progressBar = TRUE, nrepeat = 50)
#
# plot(perf.splsda.F1, col = color.mixo(5:7), sd = TRUE, legend.
    position = "horizontal")
```

```
#DIABLO plots
##block plot
tiff("Generation F1 - Ind. Plots.tiff", width=650, height=600,
    units="px")
plotIndiv(splsda.F1,  ind.names = FALSE, subtitle = c("Metabolites"
    , "Lipids", "miRNAs",
"piRNAs", "repRNA", "tiRNAs", "tRFs"),
legend=TRUE, centroid = TRUE, abline = TRUE,
pch = c(15, 16, 17), cex = c(3, 3, 3), size.title = rel(1.5), size.
    subtitle = rel(1.4),
size.xlabel = rel(1.2), size.ylabel = rel(1.2), title = "Generation
     F1", legend.title = "Group")

dev.off()

##diablo plot
tiff("Generation F1 - DIABLO plot.tiff", width=650, height=600,
    units="px")
plotDiablo(splsda.F1, ncomp = 1)
dev.off()

##plot Loadings
plotLoadings(splsda.F1, ncomp = 1, contrib = 'max', method = "
    median")

##ROC figures
ROC.F1 <- auroc(splsda.F1, roc.block = c("Metabolites", "Lipids", "
    miRNA",
"piRNA", "repRNA", "tiRNA", "tRF"), roc.comp = 2)

#Generation F2
#load data
metabolites.F2 <- read.table(file = "F2_FC_metabolites.csv", header
     = TRUE, sep = ",", row.names = 1, check.names = FALSE)
```

```
lipids.F2 <- read.table(file = "F2_FC_lipids.csv", header = TRUE,
    sep = ",", row.names = 1, check.names = FALSE)
mirnas.F2 <- read.table(file = "F2_FC_miRNA.csv", header = TRUE,
    sep = ",", row.names = 1, check.names = FALSE)
pirnas.F2 <- read.table(file = "F2_FC_piRNA.csv", header = TRUE,
    sep = ",", row.names = 1, check.names = FALSE)
reprnas.F2 <- read.table(file = "F2_FC_repRNA.csv", header = TRUE,
    sep = ",", row.names = 1, check.names = FALSE)
tirnas.F2 <- read.table(file = "F2_FC_tiRNA.csv", header = TRUE,
    sep = ",", row.names = 1, check.names = FALSE)
trfs.F2 <- read.table(file = "F2_FC_tRF.csv", header = TRUE, sep =
    ",", row.names = 1, check.names = FALSE)

sample.matrix.F2 <- data.frame(sample = rownames(metabolites.F2),
    Group = as.factor(c(rep("CTRL",6), rep("HFD", 6), rep("HFDt",6))
    ))

#select the most representative samples (metabolites + lipids)
pls.F2 <- pls(metabolites.F2, lipids.F2)
plotIndiv(pls.F2, group = sample.matrix.F2$Group,
col.per.group = c("dodgerblue","red3","darkgreen"), rep.space = "XY
    -variate",
centroid = TRUE, ellipse = TRUE, abline = TRUE, size.xlabel = 12,
    size.ylabel = 12,
xlim = c(-8,8), ylim = c(-8,8), title = 'Generation F2: 2-omics PLS
    ')

#Selected: CTRL5, CTRL2, CTRL6, HFD5, HFD6, HFDt1, HFDt2, HFDt6
select.F2 <- c("F2_CTRL5", "F2_CTRL2", "F2_CTRL6", "F2_HFD1", "F2_
    HFD2", "F2_HFD3", "F2_HFDt1", "F2_HFDt2", "F2_HFDt6")

#DIABLO plot (block PLS-DA)
##discard samples not available in sncRNA data
met.F2 <- metabolites.F2[(row.names(metabolites.F2) %in% select.F2)
```

```
    ,]
lip.F2 <- lipids.F2[(row.names(lipids.F2) %in% select.F2),]
groups.F2 <- data.frame(Sample = select.F2, Group = as.factor(c(rep
    ("CTRL", 3), rep("HFD", 3), rep("HFDt", 3))))

##change colnames of sncRNA rows
rownames(mirnas.F2) <- select.F2
rownames(pirnas.F2) <- select.F2
rownames(reprnas.F2) <- select.F2
rownames(tirnas.F2) <- select.F2
rownames(trfs.F2) <- select.F2

mirnas.F2 <- mirnas.F2[ order(rownames(mirnas.F2)) , ,drop=F]
pirnas.F2 <- pirnas.F2[ order(rownames(pirnas.F2)) , ,drop=F]
reprnas.F2 <- reprnas.F2[ order(rownames(reprnas.F2)) , ,drop=F]
tirnas.F2 <- tirnas.F2[ order(rownames(tirnas.F2)) , ,drop=F]
trfs.F2 <- trfs.F2[ order(rownames(trfs.F2)) , ,drop=F]

##compile data
data.F2 <- list(Metabolites = met.F2, Lipids = lip.F2, miRNA =
    mirnas.F2,
piRNA = pirnas.F2, repRNA = reprnas.F2, tiRNA = tirnas.F2, tRF =
    trfs.F2)
Y <- as.factor(groups.F2$Group)
keepX <- list(Metabolites = c(round(ncol(met.F2)*0.9, digits = 0),
    round(ncol(met.F2)*0.9, digits = 0)),
Lipids = c(round(ncol(lip.F2)*0.9, digits = 0), round(ncol(lip.F2)*
    0.9, digits = 0)),
miRNA = c(round(ncol(mirnas.F2)*0.9, digits = 0), round(ncol(mirnas
    .F2)*0.9, digits = 0)),
piRNA = c(round(ncol(pirnas.F2)*0.9, digits = 0), round(ncol(pirnas
    .F2)*0.9, digits = 0)),
repRNA = c(round(ncol(reprnas.F2)*0.9, digits = 0), round(ncol(
    reprnas.F2)*0.9, digits = 0)),
```

```
tiRNA = c(round(ncol(tirnas.F2)*0.9, digits = 0), round(ncol(tirnas
    .F2)*0.9, digits = 0)),
tRF = c(round(ncol(trfs.F2)*0.9, digits = 0), round(ncol(trfs.F2)*
    0.9, digits = 0)))

splsda.F2 <- block.splsda(X = data.F2, Y = Y, keepX = keepX, ncomp
    =2, near.zero.var = TRUE)

#Model performance
# perf.splsda.F2 <- perf(splsda.F2, validation = "Mfold", folds =
    3, progressBar = TRUE, nrepeat = 50)
#
# plot(perf.splsda.F2, col = color.mixo(5:7), sd = TRUE, legend.
    position = "horizontal")

#DIABLO plots
##block plot
tiff("Generation F2 - Ind. Plots.tiff", width=650, height=600,
    units="px")
plotIndiv(splsda.F2, ind.names = FALSE, subtitle = c("Metabolites"
    , "Lipids", "miRNAs",
"piRNAs", "repRNA", "tiRNAs", "tRFs"),
legend=TRUE, centroid = TRUE, abline = TRUE,
pch = c(15, 16, 17), cex = c(3, 3, 3), size.title = rel(1.5), size.
    subtitle = rel(1.4),
size.xlabel = rel(1.2), size.ylabel = rel(1.2), title = "Generation
     F2", legend.title = "Group")

dev.off()

##diablo plot
tiff("Generation F2 - DIABLO plot.tiff", width=650, height=600,
    units="px")
plotDiablo(splsda.F2, ncomp = 1)
```

```
dev.off()

##plot Loadings
plotLoadings(splsda.F2, ncomp = 1, contrib = 'max', method = "
    median")

##ROC figures
ROC.F2 <- auroc(splsda.F2, roc.block = c("Metabolites", "Lipids", "
    miRNA",
"piRNA", "repRNA", "tiRNA", "tRF"), roc.comp = 2)
```