THÈSE DE DOCTORAT

Soutenue à Aix-Marseille Université
le 22 Juillet 2022 par

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Maternal vitamin D deficiency in mice: consequences on white adipose tissue inflammation and hepatic lipid accumulation in the offspring

Discipline
Biologie Santé

Spécialité
Pathologie vasculaire et nutrition

École doctorale
ED 62- Sciences de la vie et de la santé

Laboratoire/Partenaires de recherche
C2VN
INSERM 1263
INRAE 1260

Composition du jury

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Affidavit

I, undersigned, Nicole Haroun, hereby declare that the work presented in this manuscript is my own work, carried out under the scientific direction of Jean-Francois Landrier and my co-thesis director Lourdes Mounien, in accordance with the principles of honesty, integrity and responsibility inherent to the research mission. The research work and the writing of this manuscript have been carried out in compliance with both the French national charter for Research Integrity and the Aix-Marseille University charter on the fight against plagiarism.

This work has not been submitted previously either in this country or in another country in the same or in a similar version to any other examination body.

Place Marseille, date 10 mai 2022

Affidavit

Je soussigné, Nicole Haroun, déclare par la présente que le travail présenté dans ce manuscrit est mon propre travail, réalisé sous la direction scientifique de Jean-François Landrier et mon co-directeur de thèse Lourdes Mounien, dans le respect des principes d'honnêteté, d'intégrité et de responsabilité inhérents à la mission de recherche. Les travaux de recherche et la rédaction de ce manuscrit ont été réalisés dans le respect à la fois de la charte nationale de déontologie des métiers de la recherche et de la charte d'Aix-Marseille Université relative à la lutte contre le plagiat.

Ce travail n'a pas été précédemment soumis en France ou à l'étranger dans une version identique ou similaire à un organisme examinateur.

Fait à Marseille, le 10 mai 2022

I would like to thank Prof. Marie-Christine Alessi, director of the laboratory "Nutrition, Obesity and Thrombotic Risk" laboratory for having welcomed me in her laboratory.

I would like to thank the members of the jury, Dr Patrice Darmon president of the jury, Dr Catherine Riva and Dr Jeremy Talvas, the reporters, and Dr Christine Coudray, the examiner, for having accepted to judge this work. I am happy to be able to have you among the members of our jury.

Thank you, Jean François my thesis director for your incredible presence and positive spirit even though with the absence of results where u always tell us the absence of a results is a result by itself! Thank you for being with me in every step specially during the covid-19 and the lockdowns. I admire your enthusiasm for science and how it makes you happy, as well as your small talks to reassure me when the thesis was particularly difficult. Thank you for allowing me to see only the good parts of the thesis despite the sleepless nights and the stress. I spent three years under your direction, and they were the most formative years of my academic career.

Thank you for your patience and trust over the years. I am happy and honored to have had you as a mentor for the past 3 years.

Thank you, Lourdes, my co-director for your interesting scientific presence. I really like how you and JF talk about science. Thank you for assisting me in making the transition from ignorance to knowledge.

I'd like to express my sincere thanks to my entire team for their constant support. Thank you, Julien and Charlene for your technical advice and constant presence during our laboratory work/experiments. Thanks a lot, Flavie, Laurine, Thomas, Batoul, and Thomas, and for our small talk and our lunch break under the sun (increasing our Vitamin D concentration). And a special thanks to Imene, with whom we arrived together and successfully completed all of the good and difficult parts of the thesis; we have supported each other and allowed ourselves to enjoy each step.

I hope I’m making my family proud! I would love to thank them for their support in every step during these three years. Although it was hard for us to be in a different country and facing a pandemic, but these obstacles made our bond stronger and made us closer. Always here to listen and they know what to say, best advices a person could ask for!

كتير بحبكم

Thank you, my family!

I will start first by thanking my amazing grandmother, Amal for her amazing presence and guidance in my life, teta was present during all my life supporting me specially when I took the decision to continue my studies with a PhD, same as for my grandfather, Nicolas who is in heaven now who was in Marseille years ago and still know every detail of this city. My grandfather who always said to me:

" الدكتورة نيكول هارون بنت بنتُ لنيكولا معكرون"

There you go jeddo, today I am the doctor you always wanted to see me. I know that you are very proud of me and that you take care of me from heaven I will always love you, I will always love your smile and I miss you every day!

Secondly a special thanks to my number one role model, my aunt Joumana which without her I wouldn't be here. She supports me, help me to see the best of things. No words can describe how much I am grateful for her and how much I appreciate her and I would love to be like here in the future.

My parents Imad and Yolla, which I consider my best friends, and also my number one fans. They encouraged me in my deepest weakest phases, and helped to get up and start over and over until I make it, and look! I just finished my PhD.

And last, the best family member who I really love and very thankful to have her in my life, is my little (not so little) sister, Joya thank you for everything, you were with me two out of three years of my PhD journey and u made it the best years u always supported me and let me see the best in me, even if had doubt in myself you never had and for that I am very grateful. You re an amazing person, just stay as you are.

I want to thank three of my closest friends, because I truly don't know what I would have done without you! thank you Corinne, my oldest best friend, I know you since kindergarten! you supported me during all these years, and you are always here for me no matter the distance. Christy, my smartest best friend and family doctor thank you for your positive presence. Cybele thank you for your funny messages that helped me a lot and I love how you spread joy to everyone around you.

I would like to thank all my friends; and yes, I will take the time and name some them because they have an important role in my life; so, thank you; Elie, Sarah, Marc, Joe, Johnny, Ralph, Stefano, Marc George.

Be in Love with your Life

Every minute of it!

— Jack Kerouac

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Publications accepted

Recent insights into vitamin D, adipocyte and adipose tissue biology. (Review)

Imene Bennour*, **Nicole Haroun***, Flavie Sicard, Lourdes Mounien, JF Landrier

Obes Rev. 2022 Apr 2: e13453. doi: 10.1111/obr.13453

*co-first authors

Vitamin D and obesity / adiposity; a brief overview of recent studies. (Review)

Imene Bennour*, **Nicole Haroun***, Flavie Sicard, Lourdes Mounien, JF Landrier

Nutrients 2022, *14*(10), 2049; hdoi : 10.3390/nu14102049

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Publications in progress

Article 1: Maternal vitamin D deficiency in mice increases white adipose tissue inflammation in offspring.

Nicole Haroun, Imene Bennour, Eva Seipelt, Julien Astier, Charlène Couturier, Lourdes Mounien, Jean François Landrier

Published in Cells June 2022

Article 2: Maternal vitamin D deficiency in mice sex-dependently affects hepatic lipid accumulation in the offspring.

Nicole Haroun, Imene Bennour, Eva Seipelt, Julien Astier, Charlène Couturier, Lourdes Mounien, Jean François Landrier

To be submitted

Oral presentations

5th SF-DOHAD congress, November 17-19, 2021, Jouy-en-Josas, France: "Maternal vitamin D deficiency in mice: consequences on inflammation of white adipose tissue in the offspring."
N Haroun, L Mounien**,** JF Landrier

Poster presentations

JFN congress, November 10-12, 2021, Lille, France: **"**The impact of maternal vitamin D deficiency on white adipose tissue inflammation in offspring."
N Haroun, L Mounien**,** JF Landrier

JFN congress, November 10-12, 2021, Lille, France: **"**The impact of maternal vitamin D deficiency on lipid metabolism in the liver in the offspring."
N Haroun, L Mounien**,** JF Landrier

5th SF-DOHAD congress, November 17-19, 2021, Jouy-en-Josas: **"**Maternal vitamin D deficiency in mice: consequences on hepatic steatosis in the liver of the offspring."
N Haroun, L Mounien**,** JF Landrier

List of training courses attended

Word for long documents - Producing your thesis - December 10, 2021

Discovering and Using LaTeX to Write Your Dissertation - November 22, 2021

Scientific Publication: Writing an article - December 17, 2020

People Management and Labor Relations - December 18, 2020

Introduction to Project Management - November 30, 2020 - December 1, 2020

Research Integrity in the Scientific Profession - November 16, 2020

Working in the consulting industry after your PhD: WORKSHOP - November 06, 2020

Working in the Consulting Industry after your PhD: CONFERENCE - November 03, 2020

Zetetic and intellectual self-defense - November 02, 2020

Writing Successful Grant Proposals - October 29, 2020

A quick and efficient reading - Marseille session - October 02, 2020

Certificate of Advanced University Studies (CESU) Level B: Design of animal experimentation projects Level B - September 01, 2020

Research Ethics - April 20, 2020

Scientific Publication: Learn to structure your discourse to publish effectively - March 04, 2020

Valuing Your Skills - February 10, 2020

After (and/or during) your PhD: conducting research at the European level - 06 February, 2020

Managing stress through guided breathing - February 04, 2020

Writing your thesis bibliography with Zotero - December 18, 2019.

Good practices in animal research: new entry day - December 11, 2019.

Databases in Science and Technology - November 25, 2019

List of abbreviation

AFLD: Alcoholic fatty liver disease
AI: Adiposity index
ALT: Alanine aminotransferase
ANSES: The French Agency for Food, Environmental and Occupational Health & Safety
APL: Alkaline phosphatase
ApoB: Apolipoprotein B molecule
ASAT: aspartate aminotransferase
AT: Adipose tissue
ATGL: Adipose triglyceride lipase
BAT: Brown adipose tissue
BMI: Body mass index
CD36: Cluster of differentiation 36
Che Et Co: Cholesteryl Ester Coenzyme
ChREB-P: Carbohydrate response element-binding protein
CNV: Copy number variation
CVD: Cardiovascular Disease
CYP: Cytochrome P450
DG: Diacylglycerol
DOHaD: Developmental origins of health and diseases
DNL: De novo lipogenesis
EFSA: European Food Safety Agency
ENNS: Expanded National Nutrition Survey
ER: Endoplasmic reticulum
FA: Fatty acid
FABP: Fatty acid binding protein
FATP: Fatty acid transport protein
FAO: Food and Agriculture Organization
FFA: Free fatty acid
FGF: Fibroblast growth factor
FLI: Fatty liver index
G3-P: Glucose-3-phosphate
GDM: Gestational diabetes mellitus
GF: Growth factor
GGT: Gamma-glutamyl transpeptidase
GO: Gene ontology
GR: Glucocorticoid receptor
GWAS: Genome-wide association study
HCC: Hepatocellular carcinoma
HDL: High density lipoproteinHF: High fat
HOMA-IR: Homeostasic model assessment of insulin resistance
HPLC: High-performance liquid chromatography
HS: Hepatic steatosis
hsCRP: High sensitivity C-reactive protein
HSL: Hormone-sensitive lipase
IFN-γ: Interferon-gamma
IκBα: Inhibitor of nuclear factor Kappa-B Kinase
IL: Interleukin
IR: Insulin resistance
IU: International unit
INCA: Individual and national study on food consumption
IPA: Ingenuity Pathway Analysis
KO: Knock-out
LC/MS: Liquid chromatography–mass spectrometry
LDL: Low-density lipoprotein

LF: Low fat
LPC: Lysophosphatidylcholine
LPE: Lysophosphatidylethanolamine
LPL: Lipoprotein lipase
LPS: Lipopolysaccharide
MAPK: Mitogen-activated protein kinases
MCP1: Monocyte chemoattractant protein 1
MFE: Meat fish eggs
MG: Monoacylglycerol
MtS: Metabolic syndrome
miR: MicroARN
MVB: Multi-vesicular body
NAFLD: Non-alcoholic fatty liver disease
NAFL: Non-alcoholic fatty liver
NASH: Non-alcoholic steatohepatitis
NCEP: National cholesterol education program
NEFA: Non-esterified fatty acid
NF-κB: Nuclear factor-kappa B
NIK: NF-κB-inducing kinase
NK: Natural killer
NPC1L1: Niemann-Pick C1 like 1
NRV: Nutrient Reference Value
PCOS: Polycystic ovary syndrome
PC: Phosphatidylcholine
Pdia3: Protein disulfide isomerase family A member 3
PE: Phosphatidylethanolamine
PG: Phosphatidylglycerol
PI: Phosphatidylinositol
PGC1-α: Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PNR: Population Nutrition Reference
POHaD: Paternal origin of health and diseases
PPARγ: Peroxisome proliferator-activated receptor gamma
PS: Phosphatidylserine
PTH: Parathyroid hormone
RXR: Retinoid X receptor
SGA: Small for gestational age
SM: Sphingomyelin
SNP: Single-nucleotide polymorphism
SR-B1: Scavenger receptor class B type 1
SREBP-1: Sterol regulatory element binding protein-1
SVF: Stroma-Vascular Fraction
TG: Triglycerides
TGFβ: Transforming growth factor-beta
TLR: Toll like receptor
TNF-α: Tumor necrosis factor alpha
TNFR: TNF receptor
T2D: Type 2 diabetes
UCP1: Uncoupling protein
UVB: Ultraviolets B
VD: Vitamin D
VDD: Vitamin D deficiency
VDBP: Vitamin D binding protein
VDR: Vitamin D receptor
VDRE: Vitamin D element reponse
VLDL: Very Low-density lipoprotein
WAT: White adipose tissue
WHO: World health organisation
25(OH)D: 25-hydroxyvitamin D
1,25(OH)2D: 1,25-dihydroxyvitamin D

ABSTRACT AND RÉSUMÉ

Abstract

Maternal nutrition during the perinatal period plays a decisive role in maintaining a constant supply of vital metabolites for fetal growth and development. Vitamins play a crucial role in this development, in particular vitamin D, which is a fat-soluble hormone that regulates a wide range of physiological processes and has pleiotropic effects in several tissues. However, Vitamin D deficiency has become a global public health concern, particularly among women of childbearing age, breastfeeding, and pregnant. In addition, the link between vitamin D and adipose tissue physiology, its primary storage location, and the liver physiology is suggested in the literature, since low serum 25(OH)D level is linked to obesity and hepatic steatosis.

The goal of this thesis is to emphasize the interactions that exist between maternal vitamin D deficiency (VDD) in obesogenic context and 1.) the potential programming of an inflammation in white adipose tissue and 2.) the presence of excessive lipid accumulation leading to non-alcoholic fatty liver disease in the offspring.

In adult offspring born from control or VDD mice and fed a normal or obesogenic diet, the adiposity of white adipose tissue, RNA and miRNA expression levels, and signaling pathways were investigated. In males, a high-fat diet combined with maternal VDD increased adiposity and inflammation-related RNA and miRNA expression, resulting in an over-representation of inflammatory pathways and associated with an activation of the NF-kB signaling pathway. In contrast to males, there was no significant modification of inflammatory pathways in females born from VDD mice.

We then investigated the effect of maternal VDD on the development of hepatic lipid accumulation in the offspring. In males and females, the morphological, histological, and lipid profiles (lipidomics) of the liver were studied. To summarize, our findings revealed a relationship between increased total lipid mass, a reduction in TG amount, and an increase in lipid membranes in males born from VDD mice and fed an HF diet, whereas females lipid profile was not significantly altered.

Therefore, maternal VDD combined with a high-fat diet activated inflammatory pathway in adipose tissue of the offspring and resulting in hepatic lipid metabolism programming, in a sex-dependent manner.

Key words: Maternal deficiency, vitamin D, white adipose tissue inflammation, hepatic steatosis, offspring.

Résumé

La nutrition maternelle pendant la période périnatale joue un rôle décisif dans le maintien d'un apport constant de métabolites vitaux pour la croissance et le développement du fœtus. Les vitamines jouent un rôle crucial dans ce développement, en particulier la vitamine D, qui est une hormone liposoluble qui régule un large éventail de processus physiologiques et a des effets pléiotropes dans plusieurs tissus. Cependant, la carence en vitamine D est devenue un problème de santé publique mondial, en particulier chez les femmes en âge de procréer, allaitantes et enceintes. De plus, le lien entre la vitamine D et la physiologie du tissu adipeux, son principal lieu de stockage, et la physiologie du foie est suggéré dans la littérature, puisqu'un faible taux sérique de 25(OH)D est lié à l'obésité et à la stéatose hépatique.

L'objectif de cette thèse est de souligner les interactions qui existent entre la carence maternelle en vitamine D et un régime obésogène et 1.) la programmation potentielle d'une inflammation dans le tissu adipeux blanc et 2.) la présence d'une accumulation excessive de lipides conduisant à une stéatose hépatique non alcoolique chez la progéniture.

Tout d'abord, chez la progéniture adulte nourrie avec un régime normal ou obésogène, l'adiposité, les niveaux d'expression des ARN et des miARN et les voies de signalisation ont été étudiés dans le tissu adipeux blanc. Chez les mâles, un régime riche en graisses combiné à une carence maternelle en VD a augmenté l'adiposité et l'expression des ARN et des miARN liés à l'inflammation, associé à une surexpression des voies inflammatoires, notamment de la voie de signalisation NF-kB. Contrairement aux mâles, aucune modification significative des voies inflammatoires n'a été observée chez les femelles en présence de la carence en VD.

Nous avons ensuite étudié l'effet de la carence maternelle en VD sur le développement de l'accumulation des lipides hépatiques chez la progéniture. Chez les mâles et les femelles, les profils morphologiques, histologiques, et lipidiques (lipidomique) du foie ont été étudiés. En résumé, nos résultats révèlent une relation entre une augmentation de la masse lipidique totale, une réduction de la quantité de TG et une augmentation des lipides membranaires chez les mâles nés de souris issus des mères carencés et nourris avec un régime HF, alors que le profil lipidique des femelles n'était pas significativement modifié.

Ainsi, la carence maternelle en VD associé à un régime riche en graisses active la voie inflammatoire dans le tissu adipeux de la progéniture et entraîne une programmation du métabolisme lipidique hépatique, d'une manière dépendante du sexe.

Mots clés : Carence maternelle, vitamine D, inflammation du tissu adipeux blanc, stéatose hépatique, progéniture.

INTRODUCTION

1 The Vitamin D

1.1 Discovery of vitamin D

The discovery of vitamin D (VD) is linked to rickets that affecting children living in poor areas with little sunlight. In this disease, skeletons were poorly mineralized and deformed. Much later, towards the end of the 18th century, Percival suggested that the administration of cod liver oil prevented the disease and even improve the health of children suffering from rickets. Then in 1865, Trousseau, stated that the sun also had a beneficial effect. But it was not until 1920 that Mac Collum and Mellanbourg made the connection between the effectiveness of cod liver oil in the prevention and treatment of rickets and the presence in this oil of a substance which they called VD. And finally, Windaus elucidated the structure of VD in 1928 and isolated VD2, a form of VD of plant origin (ergocalciferol), then VD3, a form of VD of animal origin (cholecalciferol).

VD is a fat-soluble hormone belonging to the sterol family, more specifically to the class of secosteroids, as cholesterol. It is found in 6 main forms in the body (Figure 1): ergocalciferol, cholecalciferol (native and majority form) 25-hydroxyVD2 and D3 (25(OH)D2, 25(OH)D3) or calcidiol (circulating form) and 1,25-dihydroxyVD2 and D3 ($1,25(OH)_2D2$, $1,25(OH)_2D3$) or calcitriol (active form). The VD is most often stored in the form of cholecalciferol.

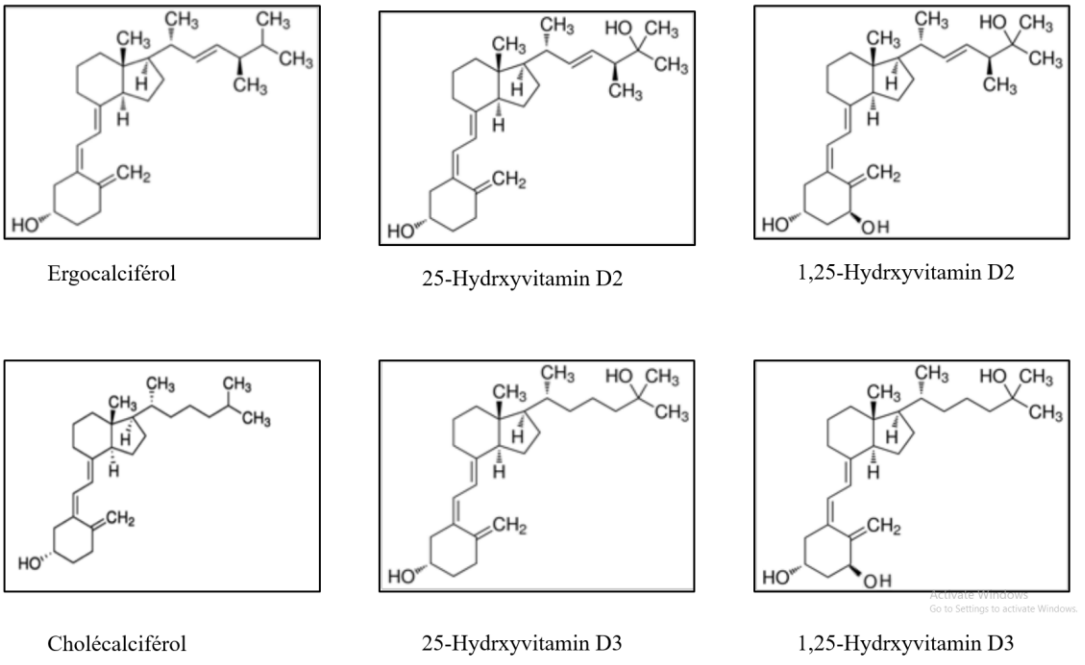

Figure 1: Structure of the different forms of VD (Landrier et al., 2012).

The term "vitamin", *i.e.* "vital" product that the body cannot produce, is inappropriate for VD, contrary to its name. It has a double origin: endogenous *via* skin exposure to sunlight and exogenous *via* dietary intake.

1.2 Metabolism of vitamin D

1.2.1 Endogenous source of VD

Endogenous synthesis is carried out by the skin following exposure to ultraviolet B (UVB wavelengths 290-315 nm (B. Lehmann & Meurer, 2010)). It is possible because in the epidermis and dermis, 7-dehydrocholesterol, a cholesterol derivative, is present in the cell membranes. UVB radiations provide the necessary energy to transform it into pre-VD3, which is rapidly converted into VD3 under the effect of heat. The VD3, is released into the circulation. This synthesis by the skin is limited by various parameters including skin pigmentation, age, incidence of the angle formed by the sun, latitude, season, air quality and body surface area of exposure (Battault et al., 2013). An exposure between 7 and 30 minutes per day between April and October provides 50 to 70% of VD requirements (Bouillon, 2017). However, endogenous intake is largely questioned and in order to obtain a sufficient intake of VD, nutritional intake is necessary.

1.2.2 Exogenous sources of VD

VD can also be provided by the diet in the form of VD2 of vegetable origin, or VD3 of animal origin, but its absorption is slow, about 40% over 12 hours. The VD2 and VD3 forms are fat-soluble and relatively stable to heat. Few foods contain VD3, it is mainly found in fish liver oil, in some fatty fish, in egg yolk or in fortified foods up to 1.25µg/100g, and VD2 is mainly found in mushrooms (Cardwell et al., 2018) (Table 1).

Food	Vitamin D_3 (µg/100g)	Vitamin D_3 (IU/100g)
Meat, eggs, fish		
Cod liver oil	250	10000
Horse mackerel	41-48	1640-1920
Herring, salmon	8-22	320-880
Tuna, mackerel, sardine	7-10	280-400
Anchovies	2.5	100
Egg yolk	2.1	84
Fruits, vegetables, legumes, oil seeds		
Peanut	10.3	412
Mushroom (cep, morel, chanterelle)	3.1-5.3	124-212
Milk, dairy products		
Low-fat soft cheese 13	15	600
Dairy specialties, snack type	2.4	96
Yoghurt enriched with VD	1.4	56
Butter	1.1	44

Table 1: Major dietary sources of VD3 (1µg = 40 IU). (From the Food Quality Information Center table, Ciqual, 2017)

A widely underestimated parameter in the calculation of dietary intake of VD is the contribution of 25(OH)D, naturally present in food. In fact, the latter is never considered in the calculation of exogenous VD intakes. However, this metabolite is present in variable but not in negligible quantities in a large number of commonly consumed foods (Ovesen et al., 2003; Schmid & Walther, 2013). In addition, it appears that the absorption of 25(OH)D is more efficient than that of VD (Desmarchelier et al., 2017). However, the actual contribution of this molecule in the maintenance of plasma levels, particularly in humans, should be the subject of in-depth studies (Ovesen et al., 2003), in order to eventually take it into account in the calculation of vitamin intakes.

1.2.3 Absorption and transport of VD

Dietary VD is incorporated into mixed micelles and absorbed in the proximal part of the small intestine. This absorption process was for a long time considered as exclusively passive until the involvement of cholesterol transporters was demonstrated. Thus, Cluster of Differentiation 36 (CD36), Niemann-Pick C1-Like 1 (NPC1L1) and Scavenger Receptor class

B type 1 (SR-B1) will also participate in the absorption of VD (Reboul et al., 2011). Because of its liposolubility, it will be more efficient in the presence of bile salts and in the presence of dietary fats in the intestinal lumen (Reboul, 2015) and it covers 10 to 25% of VD needs. After its absorption, the plasma transport seems to be dependent on its incorporation into chylomicrons, within which VD is transported to the liver. VD2 and VD3 have an almost identical metabolism and depend on the same enzymatic complexes in humans. The neosynthesized VD seems to be mostly bound to the vitamin D binding protein (J G Haddad et al., 1981) (VDBP). This protein binds both VD and its metabolites (25(OH)D and 1,25$(OH)_2$D).

The VD3, brought either by endogenous synthesis or by the food is released into the bloodstream, linked to the VDBP where it will be able to exert its direct effects. It can also be stored in the adipose tissue (AT) or transported to the organs where successive hydroxylation processes will allow it to become biologically active.

1.2.4 Hydroxylation of VD

25-hydroxylation

In the liver, endogenous or dietary VD is enzymatically hydroxylated to 25(OH)D (Figure 2). This reaction is catalysed by microsomal cytochrome P450 enzyme CYP2R1, which is considered as a key enzyme (J. B. Cheng et al., 2003; J. Zhu & DeLuca, 2012; J. G. Zhu et al., 2013). Nevertheless, several other enzymes display a 25-hydroxylation activity towards VD, including CYP27A1, CYP2C11, CYP3A4, and CYP2J2 in human (Aiba et al., 2006; Guo et al., 1993; Gupta et al., 2004).

If the 25-hydroxylation is classically considered as poorly regulated, it is noteworthy that Bell et al. reported that hepatic 25-hydroxylation was inhibited by 1,25$(OH)_2$D and parathyroid hormone (PTH) in human (Bell et al., 1985). In addition, several recent studies depicted an inhibition of *Cyp2r1* mRNA levels and/or 25-hydroxylation activity in the liver of obese/diabetic mice (Aatsinki et al., 2019; Bonnet et al., 2021; J. M. Park et al., 2015; Roizen et al., 2019), *via* mechanisms involving several transcription factors including Peroxisome proliferator-activated receptor-gamma coactivator (PGC1-α) and glucocorticoid receptor (GR). The produced 25(OH)D corresponds to the major circulating form of VD, with a half-life of about 15 days. It is classical used as biomarker of VD status (K. S. Jones et al., 2012). 25(OH)D circulates bounded to VDBP (Daiger et al., 1975), a serum α2-globulin, encoded by the *Gc* gene and synthetized by the liver. It is considered as the major plasma protein carrier of VD and its metabolites (Bouillon et al., 2020; Speeckaert et al., 2006). Indeed, around 80% of the plasma 25(OH)D is linked to VDBP. In a lesser extent, 25(OH)D is linked to albumin (19%), whereas

the remaining part is a free fraction, considered as biologically active (Haddad et al., 1988). In agreement, VDBP null mice remained normal under VD sufficient diet, suggesting that free 25(OH)D levels cover requirement for physiological functions (Safadi et al., 1999).

1α-hydroxylation

25(OH)D bounded to VDBP is then transported to the kidneys and various other organs and tissues, where 1α-hydroxylation is performed. In renal proximal tubule cells, the VDBP-25(OH)D complex enters by endocytosis, preventing thus urinary loss of VDBP-25(OH)D (Dusso et al., 2005; Nykjaer et al., 1999). This step requires the presence of megalin and cubilin (Kozyraki & Cases, 2020). Cubilin is responsible for the sequestration of the complex VDBP-25(OH)D before internalisation by megalin. After internalisation into vesicles, VDBP is degraded by lysosomes and 25(OH)D is handled by intracellular VDBP (Gacad et al., 1997). 25(OH)D is then either secreted into circulation or delivered to mitochondria to be metabolized into $1,25(OH)_2D$, the biologically active form of VD. This reaction is catalyzed by 1α-hydroxylase CYP27B1 and stimulated by PTH, low calcium and phosphorus concentrations, inhibited by Fibroblast Growth Factor 23 (FGF23) and self-regulated by $1,25(OH)_2D$ *via* a negative feed-back mechanism (Landrier et al., 2016a).

24-hydroxylation

Finally, VD metabolism is auto-regulated *via* an inactivation pathway which involves a 24-hydroxylation mediated by CYP24A1, leading to the conversion of 25(OH)D and $1,25(OH)_2D$ into 24,25(OH)D and $1,24,25(OH)_3D$, catabolized into inactive calcitroic acid (G. Jones et al., 2014). Such inactivation is induced by $1,25(OH)_2D$ itself *via* an induction of CYP24A1.

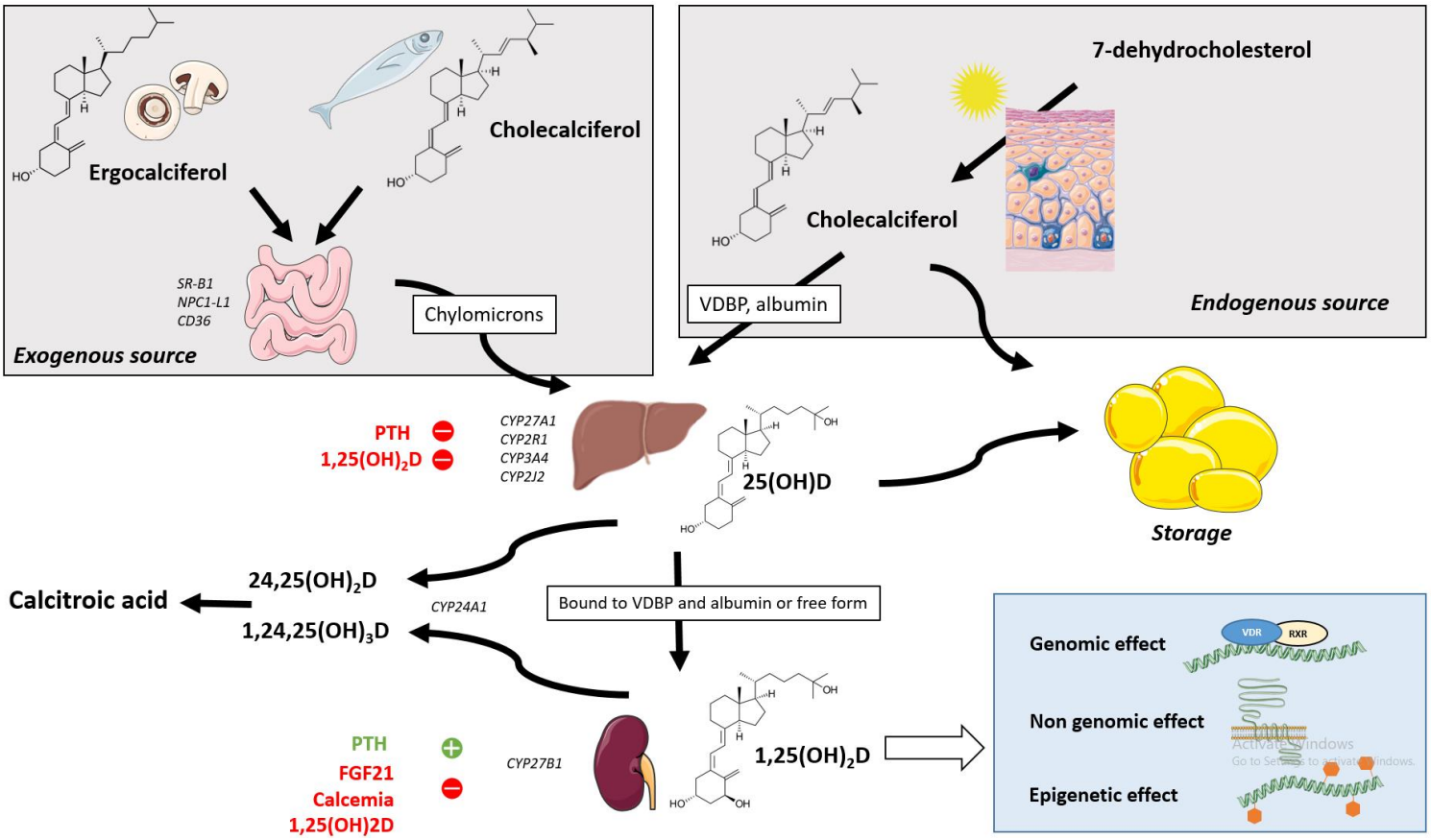

Figure 2: General metabolism of VD (Bennour & Haroun, 2022).
Vitamin D comes from food, but mainly from exposure to UVB which cause the conversion of 7-dehydrocholesterol into native vitamin D3 in the epidermis. Native vitamin D undergoes 25- and then 1α-hydroxylation to become biologically active, respectively in the liver and kidney. The metabolism of VD is autoregulated via an inactivation pathway involving CYP24A1. Calcitriol exerts its effects by binding to a specific receptor called Vitamin D Receptor (VDR). The VDR-1,25(OH)$_2$D complex is translocated to the cell nucleus where it associates with the retinoic acid receptor, the Retinoid x Receptor (RXR). The RXR-VDR heterodimer in the presence of ligand binds to DNA at sites called vitamin D response elements (VDRE), in the promoter regions of genes whose expression is thus activated or repressed.

1.3 Vitamin D storage

VD is mainly stored in AT in native form (cholecalciferol) or in the form of 25(OH)D (Landrier et al., 2012) as well as in the muscle. In 1971 the team of Rosenstreich et al. was the first to show that VD was stored in AT (Rosenstreich et al., 1971). Indeed, radiolabeled VD3 having been administered orally to weaned VDD rats, it was shown that a significant amount of radioactivity appeared in the AT 24 hours after its administration. After treatment, the AT releases the VD very slowly as shown by the progressive decrease of the radioactivity present in the AT. This release is proportional to the concentration of VD already present in the AT. Visceral AT has 20% more VD than subcutaneous AT, according to research (Beckman et al., 2013).

VD can also be stored in muscle (Table 2) in addition to AT (Heaney et al., 2009). It is also a site of local calcitriol synthesis due to the presence of 1α-hydroxylase and a target of VD due to the presence of VDR. Furthermore, VD3 accounts for 65% of the total amount of VD in the body (73% in AT and 13% in muscle). In terms of 25(OH)D, 34% is located in AT, 30% is found in serum, and 20 % is found in muscle.

Tissue	Vitamin D (UI)	$25(OH)_2D$ (UI)	Total (UI)
Adipose tissue	6960	1763	8723
Muscle	1527	1055	2581
Liver	168	214	382
Serum	271	1559	1830
Other	571	578	1149
Total	9426	5169	14665

Table 2: Main storage sites for VD (Heaney et al., 2009).

1.4 Metabolism of Vitamin D in white adipose tissue

Most of the enzymes required for VD metabolism (Figure 3) are found in adipocyte tissue (Wamberg, Christiansen, et al., 2013).

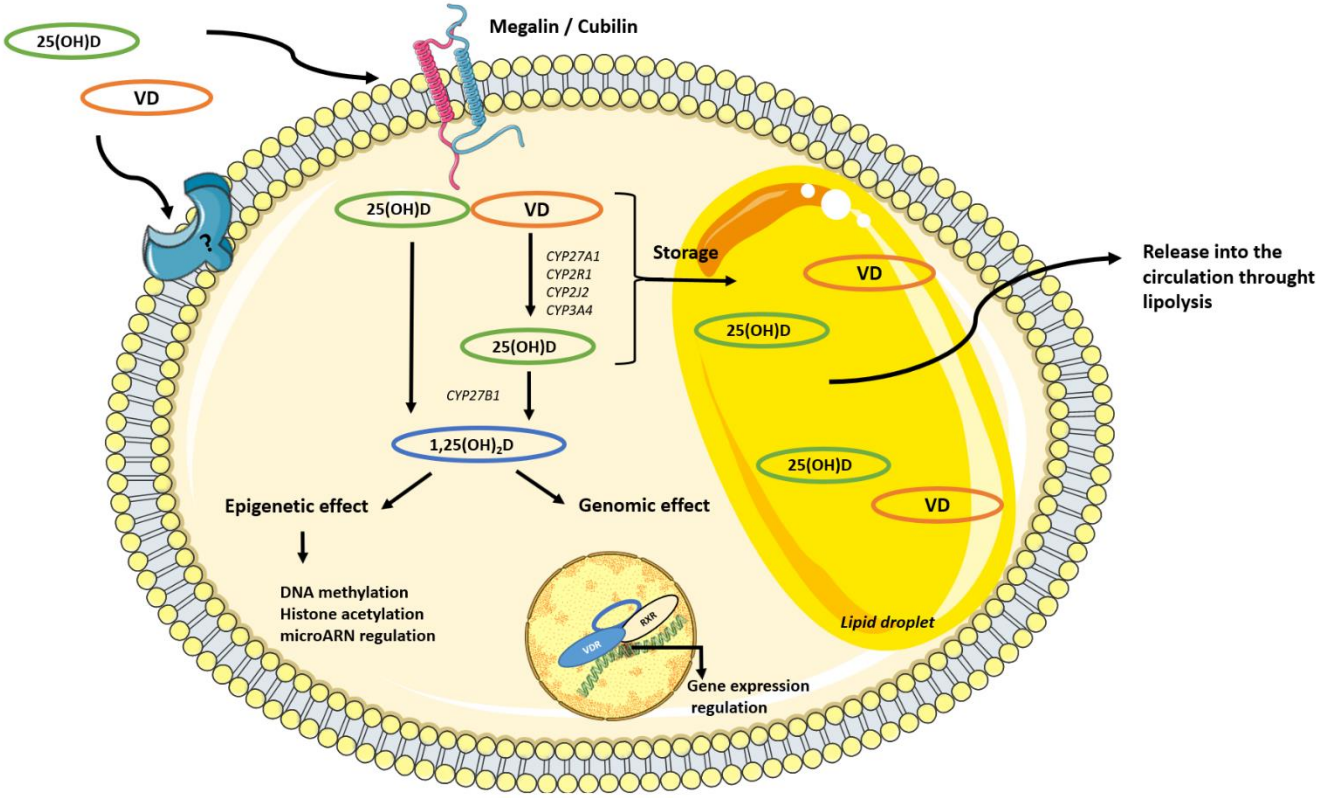

Figure 3: Metabolism of VD in AT (Landrier et al., 2022).
VD is 25- and 1α-hydroxylated in AT. Its metabolites are inactivated by CYP24A1. 1,25(OH)$_2$D can bind to VDR, be translocated into the nucleus of the adipocyte and heterodimerize with the RXR to regulate the expression of its target genes.

Our team has demonstrated that: *i)* all the enzymes necessary for VD metabolism are present in murine AT and adipocytes and some are regulated by 1,25(OH)$_2$D (Bonnet et al., 2018); *ii)* the uptake of 25(OH)D at the murine 3T3-L1 adipocytes is mediated in part by cubilin, a protein of endocytosis (Bonnet et al., 2018); *iii)* AT and the adipocyte in particular play an active role in the production and storage of 25(OH)D, through modulation of the expression of enzymes involved in 25- and 1,25-hydroxylation (Bonnet et al., 2019). Overall, active metabolites are produced by adipocytes and mediate biological effect on adipocyte and more generally on the AT itself.

Hydroxylations of vitamin D and metabolites in adipose tissue

Interestingly, all hydroxylations and associated enzymes involved in the VD metabolism have been demonstrated to be expressed in adipocytes and in AT (Table 3). Indeed, the addition

of VD in a culture medium containing adipocytes promotes the secretion of 25(OH)D, by conversion of VD and involves a functional 25-hydroxylation function (Zoico et al., 2014). 25(OH)D can be converted to $1,25(OH)_2D$ by 1α-hydroxylase, which is expressed in AT and functional on adipocyte culture (Ching et al., 2011; Li et al., 2008; Nimitphong et al., 2012).

Taken together these data suggest that adipose can act as a target tissue that is able to synthesize 25(OH)D and $1,25(OH)_2D$ that may be locally active *via* paracrine, autocrine or intracrine processes (Hewison, 2012) suggesting that VD regulates its own metabolism by reducing 25(OH)D uptake and 25-hydroxylation to avoid over-accumulation of 25(OH)D in AT. Cholecalciferol and $1,25(OH)_2D$ induced 1α-hydroxylation and 24-hydroxylation, respectively.

Hydroxylations	Enzymes	Adipocytes
25-hydroxylation (Wamberg, Christiansen, et al., 2013; Zoico et al., 2014)	CYP27A1	3T3-L1 Human
1α-hydroxylation (Ching et al., 2011; Li et al., 2008; Nimitphong et al., 2012; Wamberg, Christiansen, et al., 2013; Zoico et al., 2014)	CYP27B1	3T3-L1 Human
24-hydroxylation (Li et al., 2008; Nimitphong et al., 2012; Wamberg, Christiansen, et al., 2013)	CYP24A1	Murine Human

Table 3: All hydroxylations and associated-enzymes involved in the VD metabolism in adipose tissue.

The impact of obesity has been studied on VD metabolism in AT of mice. Several modulations of gene expression were reported, including an increase of CYP27A1 and CYP2J3 in visceral AT (J. M. Park et al., 2015). In addition, we reported that obesity in mice induced the expression of CYP2R1 and accumulation of 25(OH)D in AT (Bonnet et al., 2019), suggesting that diet-induced obesity could transcriptionally and not only passively as widely assume, mediates the trapping of VD and its conversion to 25(OH)D.

In addition, $1,25(OH)_2D$ induced the leptin expression and secretion by AT (Kong et al., 2013), whereas it repressed adiponectin expression (Lorente-Cebrián et al., 2012; Rühl & Landrier, 2016). Effects of $1,25(OH)_2D$ has been reported regarding lipid metabolism. Several genes related to β-oxidation and lipolytic enzymes have been described to be upregulated by $1,25(OH)_2D$, participating thus to the decrease in intracellular fat accumulation in adipocytes

(Chang & Kim, 2017; Larrick et al., 2018). Briefly, it is well-established that 1,25$(OH)_2$D displays anti-adipogenic effect in 3T3-L1 preadipocyte cells and reduces lipid accumulation (Blumberg et al., 2006; Ishida et al., 1988; Kamei et al., 1993; Kong & Li, 2006).

1.5 Mechanisms of action of the vitamin D

1.5.1 Genomic effect

The nuclear receptor VD receptor (VDR) is known to mediate a large part of biological effects of 1,25$(OH)_2$D (Carlberg, 2019). Its ubiquitous distribution explains that a large number of genes (more than 1000) are regulated directly or indirectly by 1,25$(OH)_2$D (Carlberg, 2019). VDR heterodimerizes with retinoid X receptor (RXR), and binds to DNA at sites called VD response elements (VDRE), located in the promoter regions of regulated genes. In absence of ligand this heterodimer complexes with co-repressors and histones deacetylases, whereas in presence of 1,25$(OH)_2$D co-activators and histone acetyltransferases are recruited leading to transcriptional activation (Tuoresmäki et al., 2014). This genomic effect of VD through VDR is highly suspected to display a large part of biological effects in health and disease, and notably in the context of obesity, as supported by several studies suggesting correlation between VDR polymorphism and pathological issues (Kazemian et al., 2019).

1.5.2 Non-genomic effects

The non-genomic effects of VD are characterized by very fast (seconds to minutes) activation of signalling pathways such as phospholipase C and phospholipase A2, phosphoinositide 3-kinase, protein kinase A, mitogen-activated protein kinases. It also includes the opening of Ca^{2+} and Cl^- channels. These non-genomic effects of VD are dependent on protein disulphide isomerase family A member3 (Pdia3, also known as ERp57, GRP58 and 1,25-MARRS (Nemere et al., 2004; Turano et al., 2011)), a membrane receptor described in enterocytes, in osteoblasts and hepatocytes (J. Chen et al., 2013; Nemere & Hintze, 2008; Zmijewski & Carlberg, 2020).

1.5.3 Epigenetic effects

Several epigenetic effects of the VD have been described in several models and pathophysiological contexts. These effects include DNA methylation, possibly through the modulation of DNA methyltransferases and/or DNA demethylases expression (T. C. Lawrence

et al., 2020). VD can also regulate histone acetylation, *via* activation of histone acetyltransferases and histone deacetylases, but also histone methylation and demethylation, leading thus to modulation of chromatin accessibility for transcription factors (Nur et al., 2021). Finally, VD has also reported to be involved in the regulation of the expression of micro-RNA (Nur et al., 2021) (miRNA). In accordance with this, we recently published data showing that $1,25(OH)_2D$ down-regulated inflammation-linked miRNA expression in adipocytes both *in vitro* and *in vivo* (Karkeni et al., 2018).

1.6 Recommended intakes and consumption of vitamin D in France

Since 2017, the ANSES (The French The French *Agency for Food, Environmental and Occupational Health & Safety*) uses the new name "Nutritional reference for the Population" (NRP) to define the recommended daily intake of nutrients. The NRP for VD has been set on the basis of none endogenous skin synthesis, in order to cover the nutritional needs of almost the entire French population, without geographical or socio-economic distinction. The definition of "nutritional need" is broad, but the World Health Organization (WHO) (WHO/FAO, 2003) considers it to be "the minimum amount of a nutrient that should be consumed by an individual for good health" and in the context of a micronutrient, it is defined as "the level of intake that satisfies a criterion of adequacy, thereby decreasing the risk of inadequate or excessive intake". Setting the NRP for VD on the assumption that endogenous synthesis via sun exposure is absent is an extreme assumption.

However, it was retained because it is difficult to estimate the level of endogenous synthesis of the population, which can be variable according to the individual. The NRP values for VD follow the Nutritional Reference Values (NRV) of the European Food Safety Authority (EFSA). They are set at 15 µg/day (600 IU/ day) for adults and 10 µg/day (400IU/d ay) for infants (EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA), 2016) (Table 4). EFSA established these VD intake cutoff values to improve muscle and bone health but did not consider other putative benefits of VD such as cardiovascular diseases and cancers. According to EFSA, this intake allows almost all Europeans to reach a minimum blood level of 20 ng/mL (equivalent to 50 nmol/L) in 25(OH)D.

Age	NRP (µg/jour)	NRP (IU/jour)
New born (7-11 months)	10	400
Child (1-17 years)	15	600
Adults (men and women)	15	600
Pregnant and breastfeeding women	15	600

Table 4: Nutritional references for VD for the population. (1 µg = 40 IU; Data from EFSA NRV, 2016)

Nevertheless, these values are far from the real consumption of VD in France. The INCA3 study (National Individual Study of Food Consumption 3) conducted over the period 2014-2015, has shown that the dietary intake of VD is only 3.1 µg/day in adults, and less than 3 µg/day in children, compared to the recommended 15 µg/day. In adults, the majority of VD intake comes from 39% of MFEs (meat, fish at 19% and eggs) and MFE-based products and 25% from dairy products. Respectively, 16% from MFEs and 40% from dairy products, in adolescents, 16% and 63% in children (Table 5).

Age	Average nutrient intake (µg/day)
Child (1-3 years)	5.2
Child (4-10 years)	2.6
Teenager (11-17 years)	2.9
Adults (18-79 years)	3.1
Males (18-79 years)	3.3
Women (18-70 years)	2.9

Table 5: VD consumption in France. (Values from the INCA3 study, 2017)

1.7 Vitamin D supplementation

VD supplementation remains critical to nutritional balance due to the high number of insufficiencies shown in various pathologies. Exposure to UVB increases the synthesis of 25(OH)D without risk of intoxication as excess VD3 and pre-VD3 are converted to inactive metabolites (Holick, 2007). Before pharmacological supplementation, several recommendations can be made by intervening at the nutritional and behavioral levels. For example, sun exposure of the arms and legs for 5 to 30 minutes, twice a week, between 10 to

15 hours, outside the winter period, significantly increases plasma 25(OH)D levels. If the whole body is exposed, 20,000 IU of VD can be provided. The use of UV lamps, although considered, has a limitation, because of the risk of developing melanoma (Holick, 2011).

Dietary compensation in VD also has its limits, since few foods contain it naturally and significantly. In addition, these foods often have a high fat content and are therefore not suitable for high consumption: ANSES recommends that the general population limit consumption of fatty fish to twice a week, in order to minimize the risks of exposure to certain heavy metals.

VD supplementation is widely used and the modalities of prescription have been extensively studied and debated. The VD3 and VD2 forms show similar efficacy in increasing plasma 25(OH)D levels, either following a high dose (100,000 IU) or over a short period of supplementation. In the long term, VD3 appears to be better suited than the VD2 form for maintaining plasma 25(OH)D levels above the optimal threshold, which seems more relevant for mediating both classical and non-classical effects of VD (U. Lehmann et al., 2013; Oliveri et al., 2015). A minimum dosage of 800 IU/d of VD3 appears necessary to protect bone and seems to be a useful prerequisite in terms of public health (Audran & Biot, 2010).

Cases of hypervitaminosis (or VD toxicity) are rare and occur with prolonged high-dose supplementation (Koutkia et al., 2001). Excess VD can cause, nausea, vomiting, weight loss, headache and diarrhea.

1.8 Assessment of vitamin D status

Serum 25(OH)D level, representing total VD from both skin synthesis and dietary intake, is the gold standard indicator of VD status (Seamans & Cashman, 2009). Serum 25(OH)D has a half-life of approximately 15 days (K. S. Jones et al., 2014). It can be measured by different techniques such as high-performance liquid chromatography (HPLC), liquid chromatography coupled to mass spectrometry (LC/MS) or immunoassays. Establishing cutoff values for 25(OH)D concentration to define VD deficiency, impairment, insufficiency, and sufficiency is a source of debate.

Classically, it is considered that for a serum level of 25(OH)D (Figure 4) greater than 30 ng/mL (75 nmol/L), reserves are said to be "sufficient" and that VD status can be described as "optimal". Conversely, the terms (Holick, 2007):

- Suboptimal VD status is often used when 25(OH)D is less than 30 ng/ml (75nmol/L)
- Insufficiency, defined as 25(OH)D level between 20 and 30 ng/ml (50-75 nmol/L)
- Deficiency, defined as 25(OH)D level below 10 ng/mL (25 nmol/L)

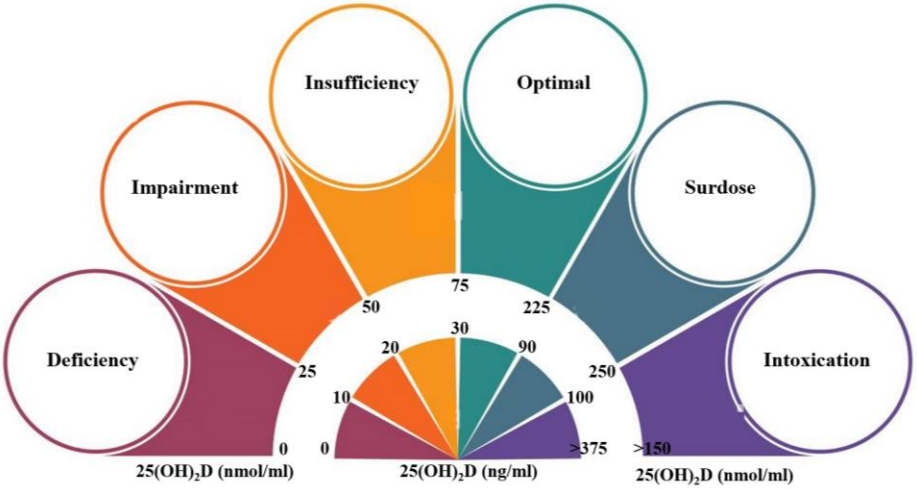

Figure 4: Values of different VD status.

25(OH)D can exist in free form in the bloodstream (D. D. Bikle et al., 1986), measurement of serum free 25(OH)D levels has recently been suggested as a new marker of plasma VD status (D. Bikle et al., 2017). It has been shown that plasma free 25(OH)D levels were strongly correlated with plasma total 25(OH)D levels in humans and that this correlation was the same across different seasons of the year (Oleröd et al., 2017). However, appropriate studies will be needed to define the clinical value of measuring free 25(OH)D, rather than total 25(OH)D, in normal subjects as well as in various pathologies (Bouillon, 2016). Several methods are listed to determine the serum concentration of free 25(OH)D free:

- Measurement of serum concentrations of total 25(OH)D, VDBP or albumin
- Measurement of the affinity between 25(OH)D and its binding proteins
- Direct measurement of equilibrium dialysis, ultrafiltration
- Immunoassay

However, these methods still need to be standardized in order to optimize the results. Other markers can be used, such as plasma concentrations of calcium, phosphorus or PTH (EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA), 2016). However, the measurement of total 25(OH)D remains the most widely used to analyze the vitamin status of an individual.

1.9 Vitamin D deficiency

1.9.1 Global population

Despite the dual origin (endogenous and exogenous) of VD, the French population is highly deficient or impaired in VD (Table 6). Indeed, at the threshold value of 20 ng/mL, we realize

that about 40% of the French population is deficient, and if we set the threshold at 30 ng/mL, nearly 80% of the population is then in a situation of insufficiency, according to the data of the National Study Nutrition Health (ENNS) conducted between 2006-2007 (Vernay et al., 2011).

25(OH)$_2$D	**Deficiency** **<10 ng/mL** **<25 nmol/L**	**Impairment** **<20 ng/mL** **<50 nmol/L**	**Insufficiency** **<30 ng/mL** **<75 nmol/L**
Men	3.6%	35.8%	78.7%
Women	5.9%	49.0%	81.4%
18-29 years	7.5%	45.9%	79.2%
30-54 years	5.2%	41.4%	79.1%
55-74 years	1.9%	41.7%	82.4%

Table 6: Plasma VD status of the French population (Vernay et al., 2011).

1.9.2 Causes of insufficiency and deficiency

These low levels of 25(OH)D are explained on the one hand by the small number of foods rich in VD and their relatively low consumption. On the other hand, while cutaneous synthesis seems thought to cover 50-70% of VD requirements, various factors related to our lifestyle caused this neosynthesis to decline. According to recent research, endogenous synthesis is thought to meet 10 to 25% of VD demands.

It has long been known that endogenous VD synthesis is influenced by season, exposure timing and latitude. Neosynthesis is almost non-existent throughout the winter season. Only a few months of the year (between April and October) UVB in France are sufficient for VD3 synthesis. Other anthropomorphic parameters tend to reduce synthesis, such as Age, skin pigmentation, obesity or overweight, sunscreen, atmospheric pollution, socio-cultural aspects.

The concentration of 7-dehydrocholesterol in the deep layers of the epidermis decreases with age since a 70 years old person produces 4 times less VD than a 20 years old person. In addition, melanin (skin pigment) acts as a natural sunscreen and the increase in this melanin pigmentation can reduce VD synthesis (Landrier, 2014). Thus, the prevalence of vitamin D deficiency (VDD) is more important in black skinned subjects (at least at the total 25(OH)D level, but not necessary at the free 25(OH)D level). Certain factors linked to the modern lifestyle also favor insufficiency, such as a sedentary lifestyle leading to less exposure to the sun as well as the increase in the use of sunscreens linked to the application of photoprotection instructions for

the prevention of skin cancers (Holick, 2007). Indeed, VD synthesis can be reduced by more than 90% by sunscreens with a protection index of 15 or more, which leads to a paradoxically higher prevalence of VDD in countries with high levels of sunlight due to high sun protection. Living in a sunny region is not necessarily synonymous with optimal VD production. Atmospheric pollution, can partially block the UVB radiation, contribute then to the reduction of VD synthesis. Finally, socio-cultural aspects, such as the wearing of covering clothes, also limit the endogenous synthesis of VD.

1.9.3 Clinical manifestations of vitamin D deficiency

Rickets in children and osteomalacia in adults are the clinical manifestations of VDD. Both are caused by defects in mineralization, due to inefficient absorption of calcium and phosphorus (Holick, 2006; Holick et al., 2012). The symptoms in adults are less pronounced than in children and may include pain and muscle weakness. VDD can cause a loss of bone density and predispose the elderly to osteoporosis without treatment.

2 Adipose tissue

AT is an organ in its own right that has evolved from being an inert deposit of excess energy to a tissue with active endocrine, paracrine or autocrine functions (Figure 5).

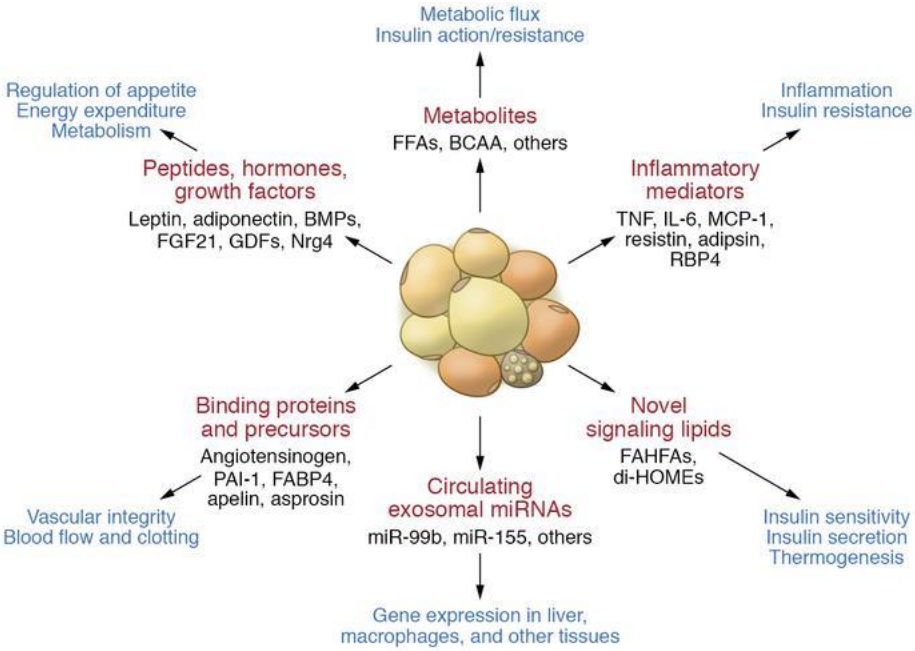

Figure 5: Adipose tissue and adipokines (Kahn et al., 2019).

Therefore, the AT, which is the major organ involved in the development of obesity and associated disorders, represents the major target of cellular and structural remodeling. This adipocyte plasticity allows the organism to adapt to a wide range of physiological and pathological conditions (Y.-H. Lee et al., 2014).

2.1 Description

In accordance with their morphology, function and location, there are two main types of AT, white adipose tissue (WAT) the majority form in adults, and brown adipose tissue (BAT). Following that, researchers discovered a novel form of brown-like adipocyte implanted in WAT termed beige or bright cells, which are activated in response to cold exposure, β3-adrenergic stimulation, and peroxisome proliferator-activated receptor-gamma (PPAR-δ) agonist treatment in a process called fat browning. Finally, during pregnancy and lactation, a female-specific cell type known as the pink adipocyte forms in the mammary glands from breast

subcutaneous WAT for milk secretion (Cinti, 2019) (Table 7). These pink adipocytes were discovered before the beige adipocyte lineage was discovered.

	White adipocytes	**Brown adipocytes**	**Beige adipocytes**	**Pink adipocytes**
Adipocyte	Unilocular	Multilocular	Multilocular	Alveolar milk cells
Size	25-200 µm	15-60 µm	15-60 µm	
Mitochondria	Few	Abundant	Abundant	Few
Lipidic vacuole	Unique	Multiple	Multiple	Unique
Nucleus	Peripheral	Central	Central	Peripheral

Table 7: Main characteristics of white, brown and beige adipocytes.

The phenomenon of browning allows white adipocytes to acquire a brown phenotype and therefore to be able to dissipate energy in the form of heat (Aldiss et al., 2018; Cinti, 2009). Conversely, in the case of consumption of an obesogenic diet, BAT can become white, a phenomenon called whitening (Bartelt & Heeren, 2014) (Figure 6). In addition to these changes, adipocyte plasticity can lead to structural changes including an increase in the size and number of adipocytes present within the AT.

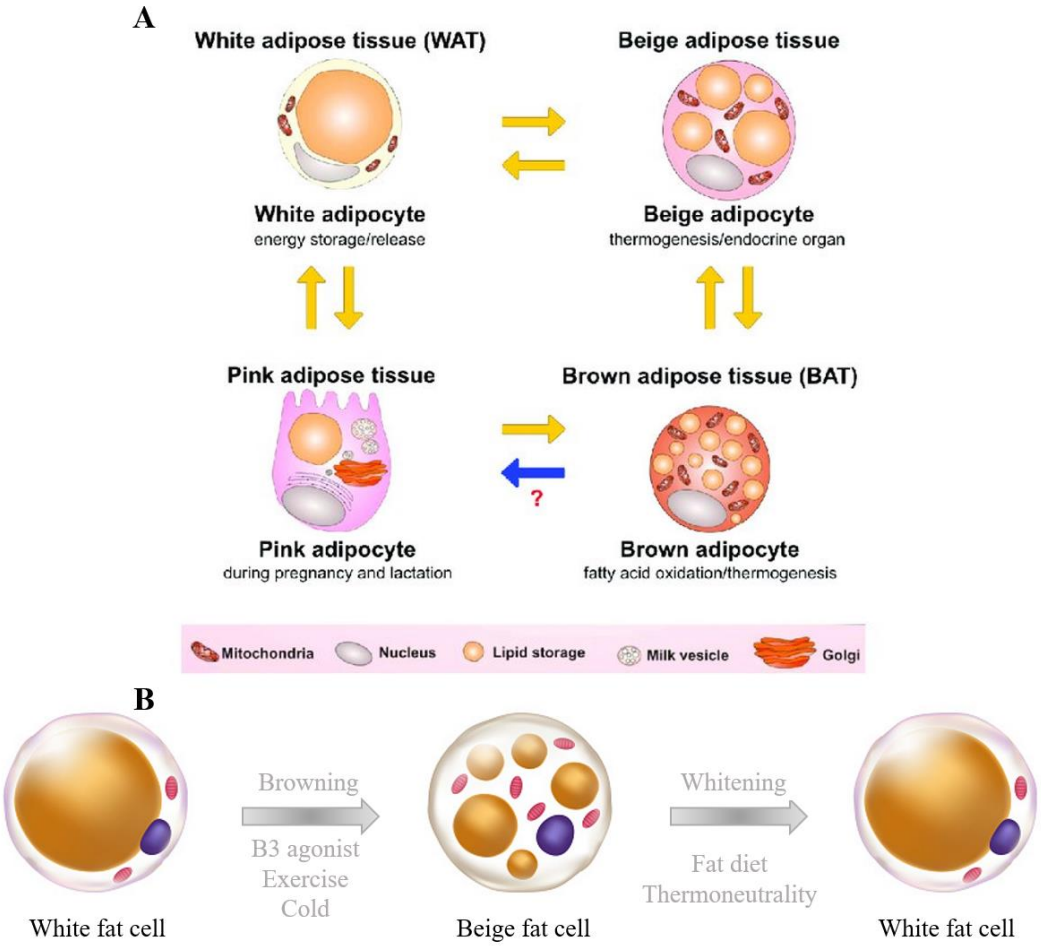

Figure 6: Phenotypic changes in adipocytes in response to environmental environment A (Zorena et al., 2020) and B (adapted from (Bartelt & Heeren, 2014)).

Adipocytes and the stroma-vascular fraction (SVF) are the two cell fractions that mainly compose WAT. Adipocytes are large cells that originate from mesenchymal cells and are composed of a large unilocular lipidic droplet (containing triglycerides) and cytoplasm. The SVF contains leukocytes, macrophages, fibroblasts, preadipocytes, endothelial cells (Figure 7).

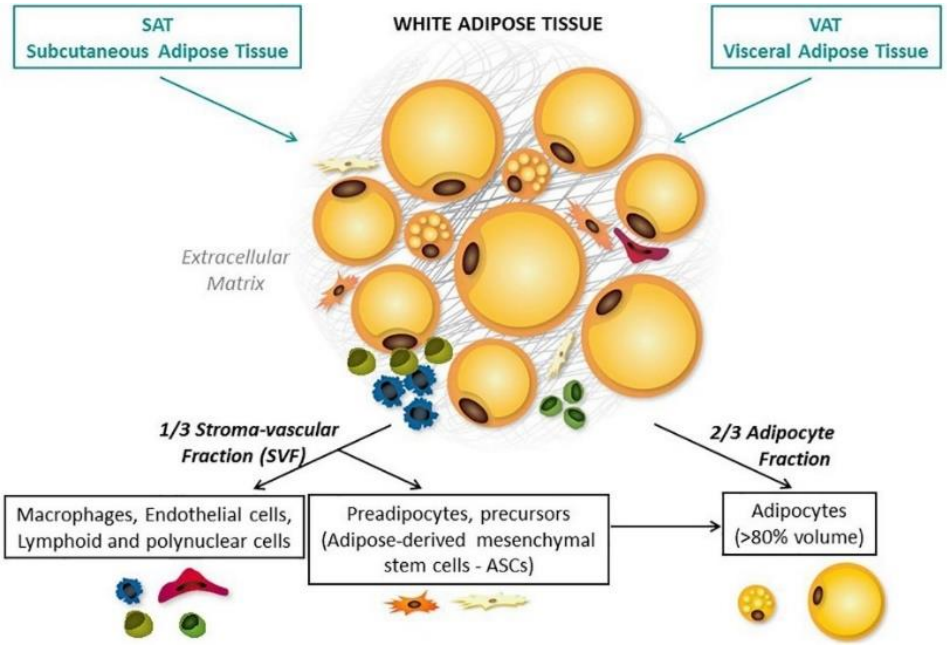

Figure 7: Structure and composition of adipose tissue (Bourgeois et al., 2019).

The WAT represents between 15 to 25% of the weight of an individual. Its location varies it can be either be subcutaneous or visceral (Figure 8).

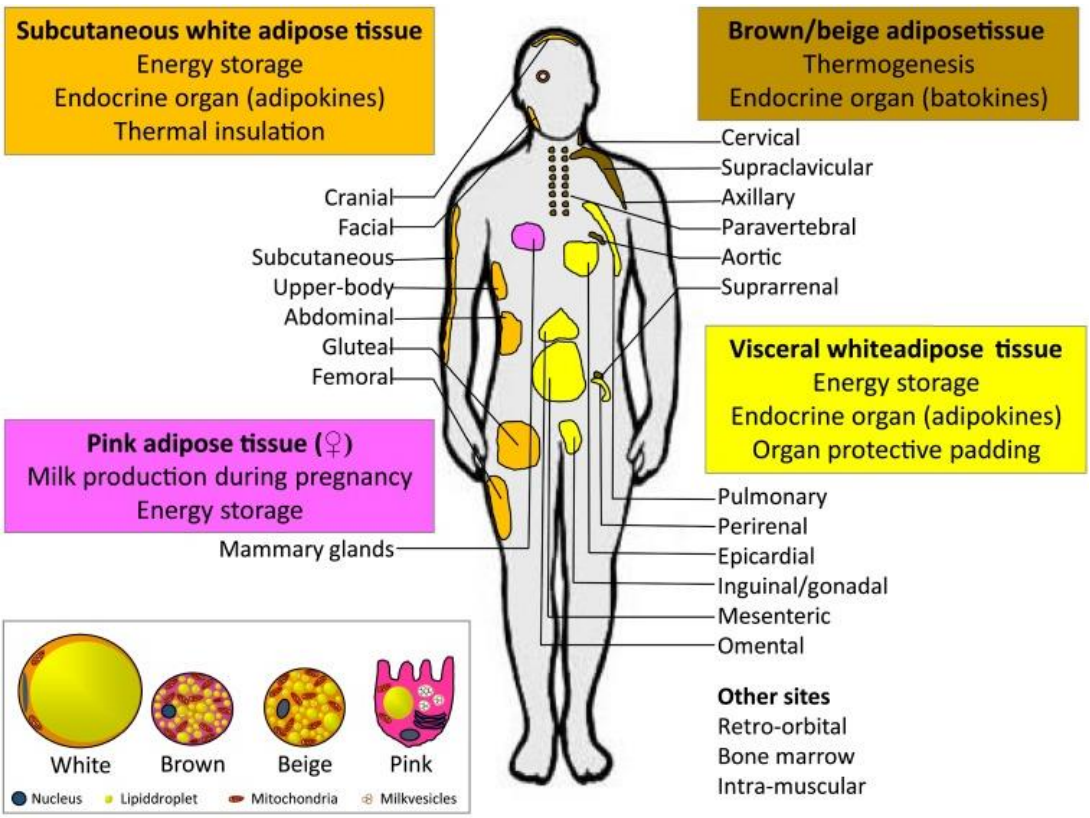

Figure 8: Distribution of different adipose tissues in humans (Rodríguez et al., 2020).

The WAT is located at 80% in subcutaneous position and at 20% in the intra-abdominal position (Lafontan & Berlan, 2003) (deep tissue). The intra-abdominal tissue is further subdivided into visceral AT (80%) and retroperitoneal AT (20%). The distribution of AT is sex-dependent with an accumulation approximately 2 times more important in women at the lower level of the body (thighs, hips, pelvis), it is called "gynoid" adiposity. Alternatively, the accumulation found in the in the abdomen and torso mainly in men is called “android" adiposity (Figure 9).

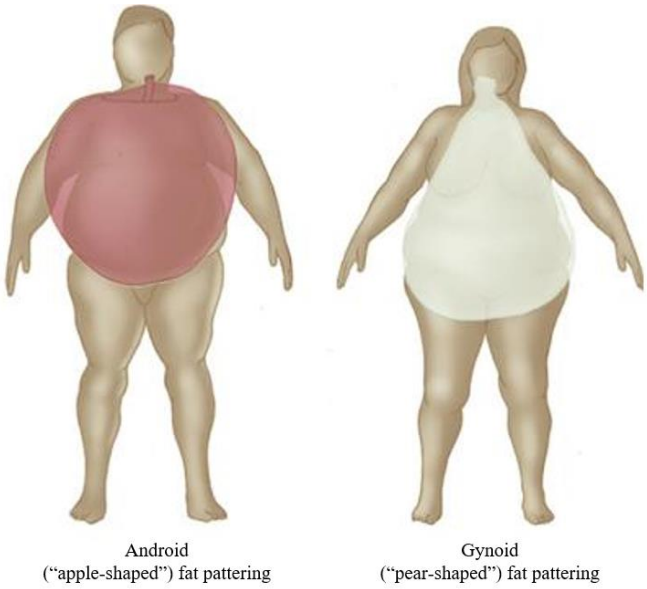

Figure 9: Distribution of adipose tissue according to sex: gynoid and android adiposity.

Long considered as non-existent in adults and very present in neonates and hibernating mammals, BAT has recently been detected in the supra-clavicular level and in the cervical region (Cypess et al., 2009).

In rodents, the WAT has the same locations as in humans. Visceral fat deposits are found at the epicardial mesenteric, perirenal, retroperitoneal and additionally at the gonadal level (Bartelt & Heeren, 2014). Subcutaneous fat is found at the abdominal and inguinal level (A. Park et al., 2014). In contrast, BAT is organized into deposits localized at the interscapular, cervical, mediastinal (Cheong & Xu, 2021) (pericardial, perirenal, peri-aortic) (Figure 10).

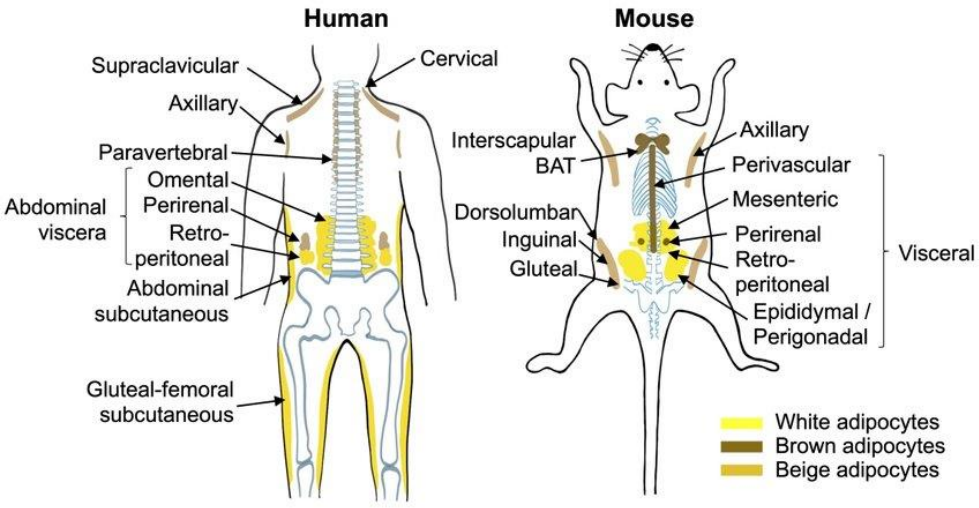

Figure 10: Distribution of adipose tissues in the human and mouse (Cheong & Xu, 2021). Upper body subcutaneous (cervical, supraclavicular, axillary, paravertebral, and abdominal subcutaneous), abdominal viscera, and lower body subcutaneous are the types of adipose depots in humans (gluteal-femoral subcutaneous). Adipose depots in mice are divided into three types: anterior subcutaneous (axillary, BAT), visceral, and posterior subcutaneous (dorsolumbar, inguinal and gluteal).

Beside their different composition, WAT and BAT have different roles (Table 8). The storage of lipids in adipocytes is in the form of triglycerides (TG). The main source of these lipids comes from circulating plasma lipids which are either non-esterified fatty acids (NEFA) bound to albumin or TG incorporated into lipoproteins from the diet. Another part of the TGs may come from de *novo* lipogenesis (DNL) in the liver, mainly from very-low-density lipoprotein (VLDL). The TGs contained in both chylomicrons and VLDL are hydrolyzed by lipoprotein lipase (LPL), enzyme secreted by adipocytes and anchored on the membrane of endothelial cells of blood capillaries, into 2-monoglycerol (2-MG). The transport proteins fatty acid transport protein (FATP) allow the capture of free fatty acid (FFA) by the adipocytes and these fatty acids (FA) then bind to cytoplasmic proteins fatty acid binding protein (FABP) to be activated into acyl-CoA by an acyl-CoA synthase and can be re-esterified to TG in the presence of glyceraldehyde 3-phosphate (G3-P). The FA neosynthesized by the lipogenic pathway ensure the storage of lipids in the form of TG. DNL contributes to the storage of TG in a minority way. Mainly, the regulation of FA storage is subject to nutritional control (glucose, NEFA) but it is also hormones such as insulin, steroids or catecholamines (Jaworski et al., 2007; Reilly et al., 2020).

	White adipocytes	Brown adipocytes
Functions	Energy storage Adipogenesis Lipogenesis Lipolysis Adipokines secretions	Thermogenesis
Fatty acid oxidation	+	+++
Respiratory chain	+	+++

Table 8: Role of white and brown adipocytes.

Lipolysis, on the other hand, is the process that allows the release of NEFAs by the hydrolysis of TG contained in the lipid droplet. This mechanism allows the body to meet its needs in case of energy restriction. Lipolysis is under the action of two lipases, the hormone-sensitive lipase (HSL) and adipose triglyceride lipase (ATGL). These enzymes catalyze the successive reactions of TG hydrolysis into diacylglycerol (DG), MG and glycerol. Finally, at each step there is release of FA molecules into the bloodstream, which are then used by the other cells of the body for energy purposes (Figure 11). As lipogenesis, lipolysis is subject to nutritional and hormonal control (insulin, adrenalin, noradrenalin).

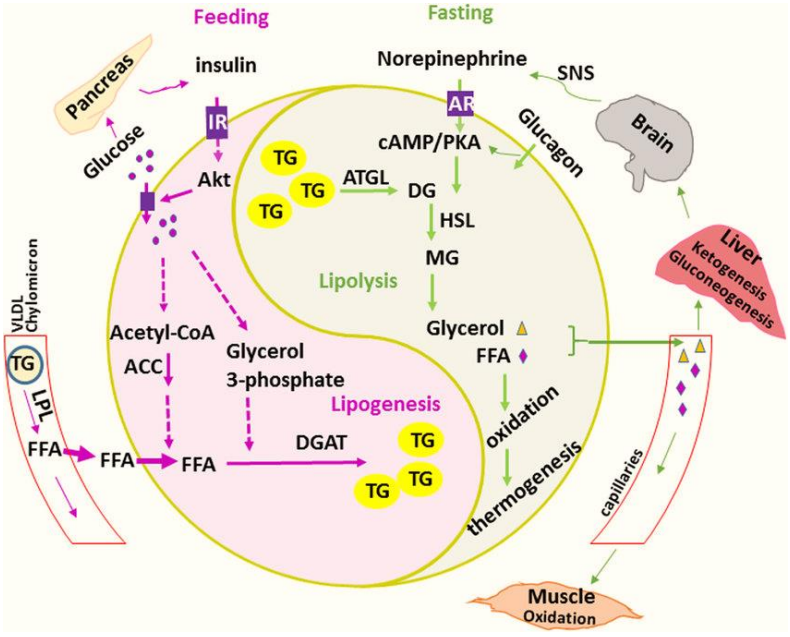

Figure 11: Stages of lipogenesis and lipolysis in the adipocyte (L. Luo & Liu, 2016).

BAT regulates the so-called non-shivering thermogenesis mediated by the sympathetic nervous system. This thermogenic capacity is enabled by the abundant presence in the presence in the mitochondrial inner membrane of an uncoupling protein 1 (UCP1). Following an exposure to cold or food ingestion, a release of noradrenalin produced by the sympathetic nervous system leads to an increase in sympathetic nervous system leads to an increase in the number of brown adipocytes, mitochondria and consequently UCP1 expression (Merlin et al., 2016) (Figure 12).

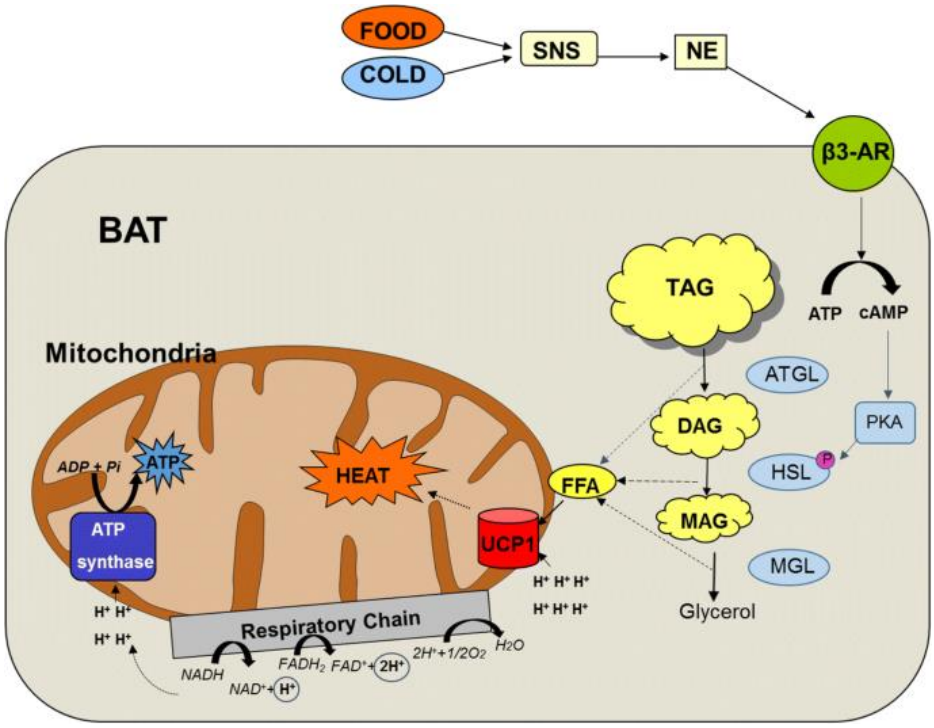

Figure 12: Thermogenesis in the brown adipocyte (Bourgeois et al., 2019)

The adipose secretome is partly composed of the secretory products of the adipocyte but also of those of the stroma-vascular fraction (Esteve Ràfols, 2014) or from both, such as TG and FFA (Pogodziński et al., 2022), and the relative proportions of these FA in the body depend primarily on diet. In the secretome we also find, exosomal miRNAs (Botello-Flores et al., 2022) (exo-miRNAs, miRNA will be discussed in details in section 2.3.1), and exosomes that are mostly multi-vesicular body (MVB). The secretory products of AT are called "adipocytokines" or "adipokines". By extension, the word "adipokine" is widely used to designate the secretions of AT, whatever the cellular origin of these adipokines. Among these adipokines, we can distinguish hormones, cytokines, chemokines or growth factors (GF) (Figure 13) (Shi et al.,

2009; Vernon et al., 2001). These factors, whose list is constantly growing are capable of influencing behavior, energy regulation, lipid oxidation, immune function, vascular function, hormonal status, etc.

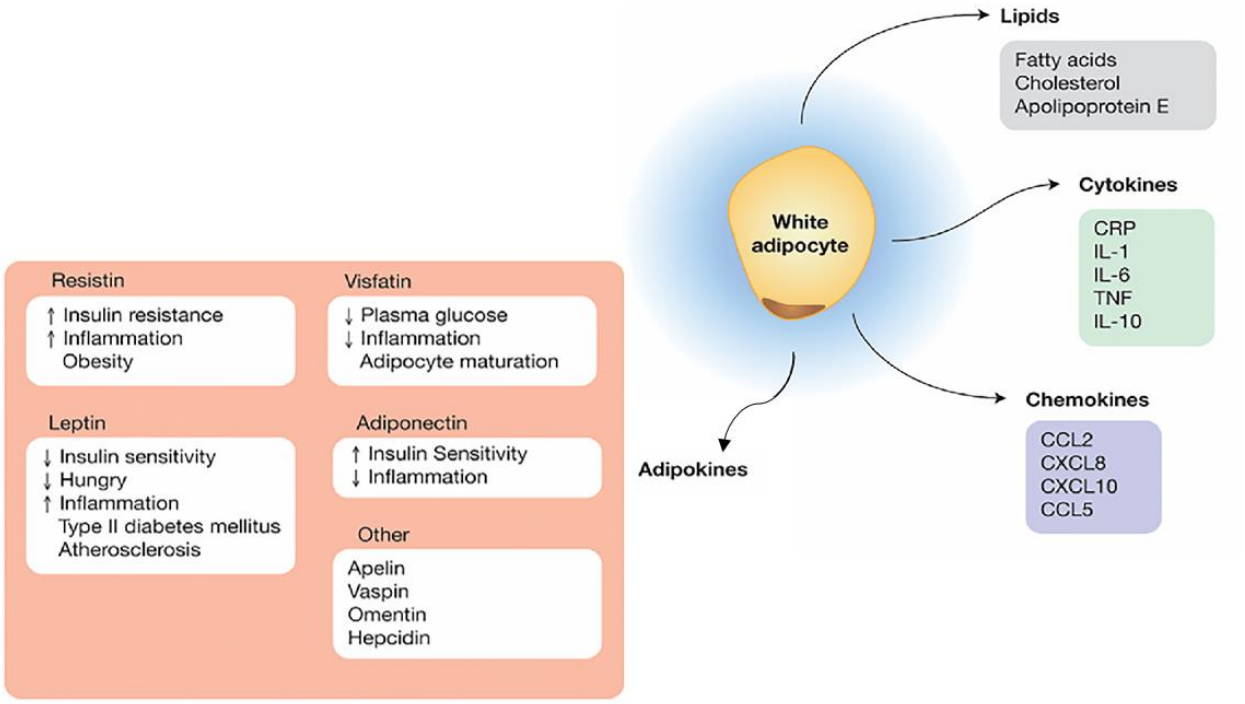

Figure 13: WAT has various endocrine functions (Halabí et al., 2020).
Adipokine production and secretion, lipid uptake, storage, and synthesis, glucose homeostasis, and inflammatory state are all WAT activities. Adipokines include leptin, resistin, and adiponectin, which are only synthesized and produced by adipocytes.

2.2 Adipose tissue and obesity

During obesity, the WAT can increase in mass by two phenomena hyperplasia, *i.e.* an increase in the number of adipocytes by differentiation and hypertrophy (the predominant phenomenon) which corresponds to an increase in size of the adipocytes(Spalding et al., 2008) resulting from an accumulation of TG. The AT and the adipocyte, in the context of obesity, will be confronted with hypoxia, inflammation, endoplasmic reticulum (ER) stress, leukocyte infiltration, accumulation of FFA and oxidative stress.

According to the WHO, obesity is defined as "an abnormal or excessive accumulation of body fat constituting a risk to health". This pathology is caused by an increase in energy intake

associated with a decrease in energy expenditure, leading to an increase in the mass of AT. In 2016, according to WHO data, more than 1.9 billion adults (>18 years) in the world are overweight (1 in 5 individuals), of which 650 million are obese. This corresponds to 39% of adults over the age of 18 years for overweight (38% of men and 40% of women) and 13% for those related to obesity (11% of men and 15% of women). Obesity and overweight constitute the 5th leading cause of death worldwide, which corresponds to 2.8 million deaths each year. More worryingly, it was estimated in 2016 that nearly 50 million girls and 75 million boys aged 5 to 19 years are affected by obesity worldwide (Abarca-Gómez et al., 2017). Children who are overweight or affected by obesity have an increased risk of developing T2D, insulin resistance (IR) (Hannon et al., 2005), and cardiovascular disease in adulthood with an increase in associated risk factors (Umer et al., 2017), such as hypertension and hypercholesterolemia.

The calculation of the BMI for adults, allows an estimate of the fat mass of a person, according to the formula: weight (in kilograms, Kg) divided by the square of the height (in meters, m). This data allows us to distinguish different categories ranging from thinness to massive obesity (Figure 14), but it remains indicative because it can be biased by certain physiological situations such as aging, pregnancy, high-level sports practice, etc. In addition, a waist circumference greater than 102 cm in men and 88 cm in women is an indicator of obesity and is associated with a high risk of diabetes and cardiovascular disease.

Figure 14: Classification of individuals according to their BMI.

2.2.1 Complications associated with obesity

Most of the mortality linked to obesity is due to the complications associated with it. These complications are multiple, frequent and often severe. They are influenced by the degree of obesity, the duration of the disease and the distribution of the AT. The most well-known

complications associated with obesity are then IR which can lead to T2D, hepatic steatosis (HS) and metabolic syndrome (MtS).

Insulin-resistance

Insulin-resistance (IR) is defined as an insulin target cell's inability to respond to insulin at physiological concentrations as a result of a deficiency in glucose absorption, a large rise in hepatic glucose synthesis and a change in lipid metabolism.

IR is a well-known obesity complication that can lead to the development of T2D. Intra-abdominal fat accumulation in visceral AT contributed to glucose intolerance and hyperinsulinemia, which can lead to IR (Lafontan & Berlan, 2003). The visceral AT becomes resistive to additional lipid accumulation leading to the development of systemic IR. Indeed, excess of circulating lipids resulted in ectopic lipid deposition in the liver, muscle, and heart (Figure 15).

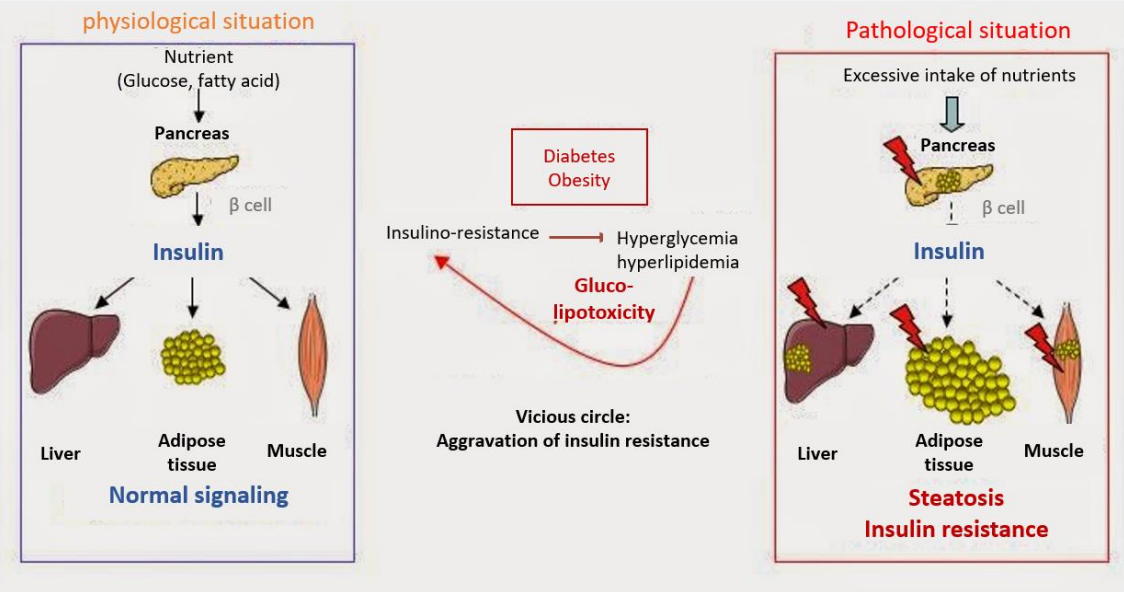

Figure 15: Insulin response under normal and pathological conditions (adapted from (Zarjevski, 2014)).

Hepatic steatosis

Fat deposits can also be detected in the liver, in addition to fat storage in the AT. Liver diseases can develop as a result of this impairment. The most common is non-alcoholic fatty liver disease (NAFLD), which is on the rise in Europe (Gehrke et al., 2019) and is closely linked to the epidemic of obesity. NAFLD refers to a group of liver diseases (Willebrords et al., n.d.). The initial stage is steatosis or simple HS and due to peripheral IR, there is an accumulation of

lipids in the liver during this stage (This is an important section of my thesis and it will be enlarged upon in Chapter 3).

Metabolic syndrome

Insulin signaling disruption is a crucial factor in the development of the metabolic syndrome (Figure 16). In 1988, the MtS was first characterized as a collection of metabolic and cardiovascular abnormalities (Reaven, 1993). The metabolic syndrome is a clinical and biological entity characterized by the presence of many risk factors such as adiposity, impaired glucose metabolism, dyslipidemia, and hypertension (DeFronzo & Ferrannini, 1991; Haffner et al., 1992; Reaven, 1993). All of these traits are influenced by environmental influences, physical activity, food habits and genetic susceptibility factors (Mayer et al., 1996). WHO (Alberti & Zimmet, 1998) and the National Cholesterol Education Program (NCEP, 2001) updated the metabolic syndrome screening in 2001, and it is based on the following criteria:

- Abdominal obesity (waist circumference >88 cm in women and >102 cm in men)
- Systolic blood pressure >130 mmHg and diastolic blood pressure >85 mmHg
- HDL (high-density lipoprotein) cholesterol <1 mmol/l for men and <1.3 mmol/l for women
- TG >1.7 mmol/l
- Fasting blood glucose >6.1 mmol/L

This definition makes it possible to realize that abdominal obesity plays an important role in the development of metabolic abnormalities related to obesity (Després & Lemieux, 2006).

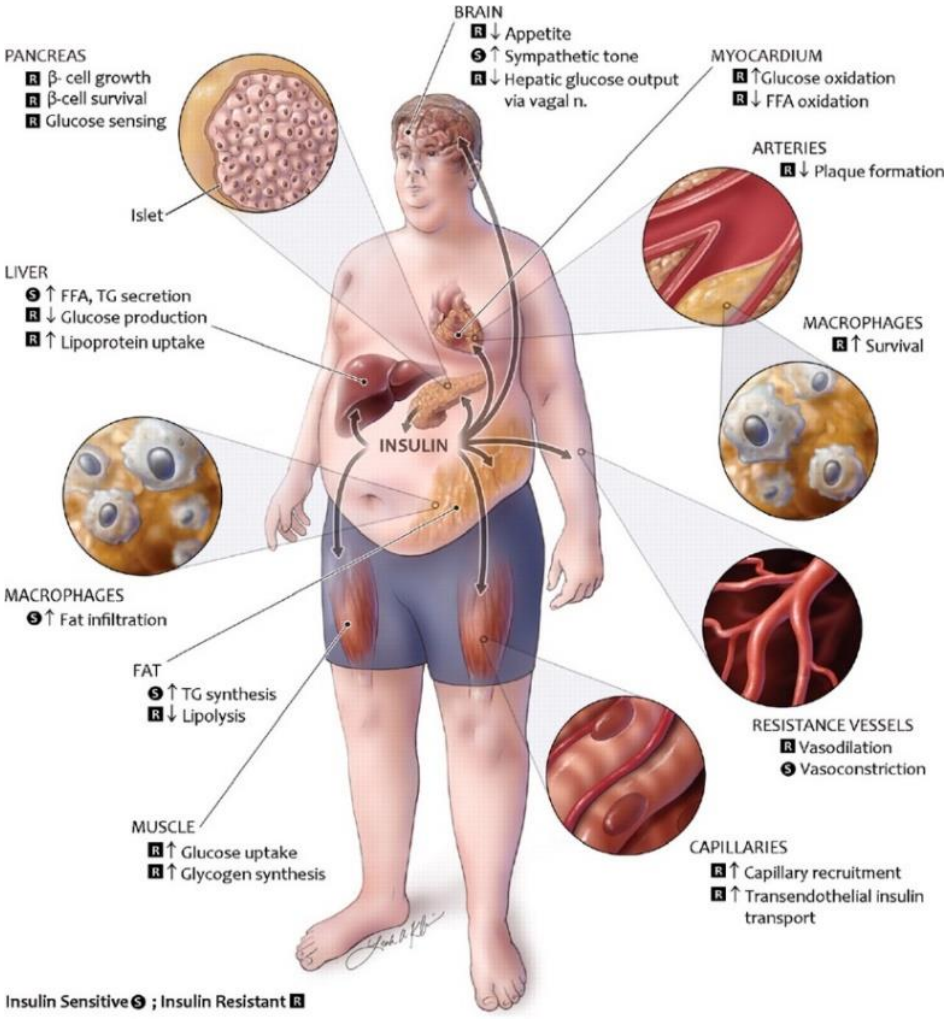

Figure 16: Physiologie de la signalisation de l'insuline dans le syndrome métabolique (Rask-Madsen & Kahn, 2012).

2.3 Adipocyte inflammation driven by obesity

In addition to structural changes, obesity induces adipocyte dysfunction that will favor the occurrence of several disorders including chronic low-grade inflammatory status. All these events will lead to an imbalance in homeostasis resulting in a modification of the secretion of adipokines (adiponectin, leptin) as well as inflammatory mechanisms (Gregor & Hotamisligil, 2011) (Figure 17).

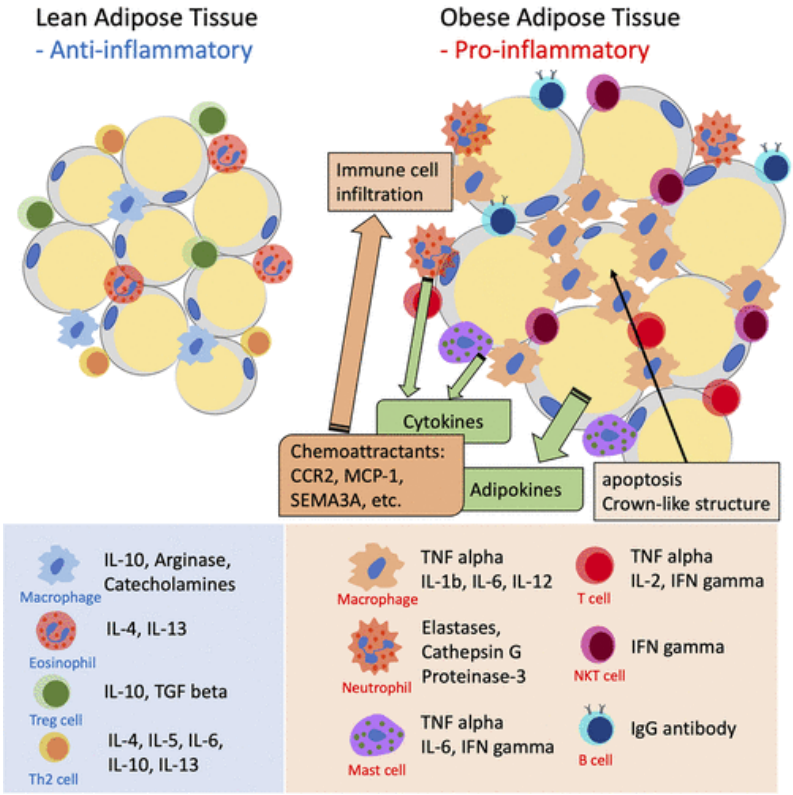

Figure 17: The inflammatory phenotype of expanding AT.
Hypertrophic adipocytes and tissue-resident immune cells undergo phenotypic alterations that include the switch from secreting anti-inflammatory, protective cytokines to secreting inflammatory adipokines and cytokines that act locally and systemically to cause peripheral insulin resistance (Kawai et al., 2021).

Inflamed adipocytes disrupt the normal function of AT by secreting both locally and systemically pro-inflammatory cytokines. The studies on the link between obesity and inflammation date back about 30 years. The first indication of inflammation in obese mice AT was the detection of an increase in the pro-inflammatory cytokine Tumor Necrosis Factor-α (TNF-α) (Hotamisligil et al., 1995), which was associated with an increase in M1 type macrophage infiltration (Weisberg et al., 2003). This has been followed by numerous studies describing the phenotypic differences observed between obese and non-obese WAT in both humans and animals. It is now known that not only TNF-α but a whole network of inflammatory cytokines is induced during obesity (Table 9). Indeed, AT produces numerous proinflammatory cytokines Transforming growth factor beta (TGF β) and Interferon gamma (IFNγ), interleukins (IL1, IL6, IL10 and IL8), procoagulant factors (PAI-1, fibrinogen) and chemokines (Berg & Scherer, 2005; Shoelson et al., 2006; Tourniaire et al., 2013) (monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein 1 α).

Among these substances produced specifically by the adipocyte (such as adiponectin and leptin (H.-K. Park & Ahima, 2015; Trayhurn & Wood, 2004)) and others produced in

abundance by this tissue but not specifically by it, such as inflammatory proteins such as IL-6, which is primarily produced by the SVF (Fain, 2006) or IL-1β and IL-10 which are primarily secreted by macrophages (Sims & Smith, 2010; Toita et al., 2016). Obesity is also associated with a decrease in the production of adiponectin, which is usually considered as a factor with anti-inflammatory (Trayhurn & Wood, 2004) and insulin-sensitizing properties, as well as leptin (R. C. Lee et al., 1993; Unger, 2003).

Group	Name	Functions
Hormones	Adiponectin	• Insulin-sensitive • Stimulates the oxidation of GA • Anti-inflammatory action
	Leptin	• Satiety signal by direct effect on the hypothalamus • Stimulates lipolysis, inhibits lipogenesis, stimulates the oxidation of GA
	Resistin	• Induces insulin resistance • Adipocyte differentiation
Acute phase proteins	PAI Plasminogen Activator/ Inhibitor	• nhibition of fibrinolytic system • Induces insulin resistance
	Haptoglobin	• Antioxidant properties
Cytokines	IL-6 Interleukin 6	• Pro-inflammatory • Decreases leptin and insulin pathways insulin pathways
	IL-10	• Inhibition of cytokine production • Decreased immune response
	TNF-α Tumor Necrosis Factor α	• Pro-inflammatory • Induces insulin resistance • Increased lipolysis in adipocytes
Chemokines	CCL5 C-C motif ligand 5	• Leukocyte recruitment to inflammatory sites
	MCP1 Monocyte Chemoattractant Protein 1	• Recruitment of macrophages to inflammatory sites • Increased lipolysis and secretion of leptin
Growth factor	Angiopoietin	• Stimulation/proliferation of endothelial cells • Involved in angiogenesis
	TGF-β Transformation Growth Factor β	• Anti-inflammatory • Effect on proliferation, differentiation differentiation and apoptosis
miRNAs	miR-14, miR-218, let-7	• Lipid and carbohydrate metabolism
	miR-17/20/93, miR21/590-5p, miR-200b/c, miR221/222, let-7/miR-98, miR-203	• Regulation by diet
	miR-21	• Correlation with BMI
	miR-132, miR-126, miR-193b, miR-145	• Regulation of the expression of inflammatory markers
	miR-155	• Adipocyte inflammation

Table 9: Functions of different inflammatory mediators.

2.3.1 MicroRNAs in white adipose tissue inflammation

Discovery of microRNAs

MiRs were first discovered in 1993 in the nematode Caenorhabditis elegans, where these miRs are involved in embryonic development (R. C. Lee et al., 1993). It was until the early 2000s that miRs were recognized as a distinct class of regulators. Subsequently, hundreds of miRs were found in worms, flies, plants and vertebrates. Rapidly, numerous discoveries have allowed us to understand the roles of these miRs as well as the different stages of their biosynthesis.

Biosynthesis of microRNAs

MiRs are small RNAs (19-25 nucleotides) transcribed from non-protein coding regions of the genome. They are considered as major elements of the control of gene expression at the post-transcriptional level. MiRs are derived from primary transcripts (pri-miRNAs), usually hundreds to thousands of nucleotides long, which undergo a series of events leading to a final molecule (Figure 18). Currently, in humans, there are 1881 miRs in the databases (according to miRBase, V21, http://www.mirbase.org). It is important to specify that each miR can regulate about a hundred genes and can inversely be the target of several miRs. In this way, miRs can regulate more than 30% of all protein-coding genes. Functional studies of miRs indicate that these small RNAs control many physiological processes (Bartel, 2004) (development, cell differentiation and proliferation, apoptosis development, or stress response) and the alterations in their expression level is associated with pathologies (obesity, cancers, cardiovascular diseases...).

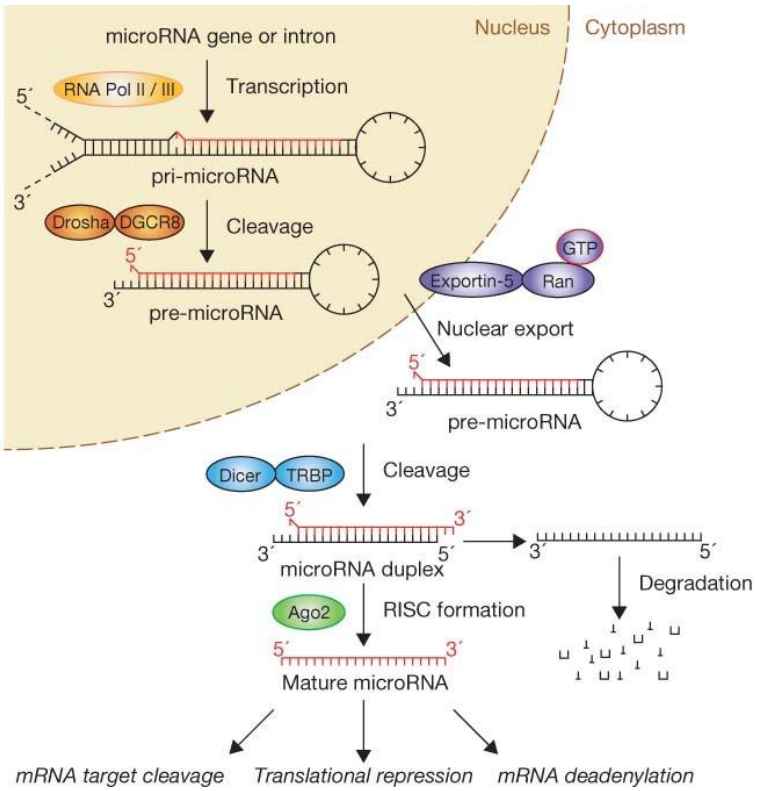

Figure 18: Microrna processing follows a 'linear' canonical mechanism (Winter et al., 2009). For a long time, the miRNA processing pathway was thought to be linear and universal to all mammalian miRNAs. This normal maturation includes RNA polymerase II or III producing the primary miRNA transcript (pri-miRNA) and the microprocessor complex Drosha–DGCR8 (Pasha) cleaving the pri-miRNA in the nucleus. Exportin-5–Ran-GTP exports the resulting precursor hairpin, the pre-miRNA, from the nucleus. The RNAse Dicer, in collaboration with the double-stranded RNA-binding protein TRBP, cleaves the pre-miRNA hairpin to its mature length in the cytoplasm. The functional strand of mature miRNA is loaded into the RNA-induced silencing complex (RISC) with Argonaute (Ago2) proteins, where it directs RISC to silence target miRNAs via miRNA breakage, translational repression, or deadenylation, while the passenger strand (black) is destroyed.

MiRs can act intracellularly, but they can also be secreted in bloodstream. In this case, their stability is warranted by their transport which protects them from endogenous RNase activity. The miRs are indeed transported in microvesicules (exosomes, microparticles, apoptotic bodies) or associated with protein, lipid or high-density lipoprotein (HDL) complexes (Creemers et al., 2012) (Figure 19).

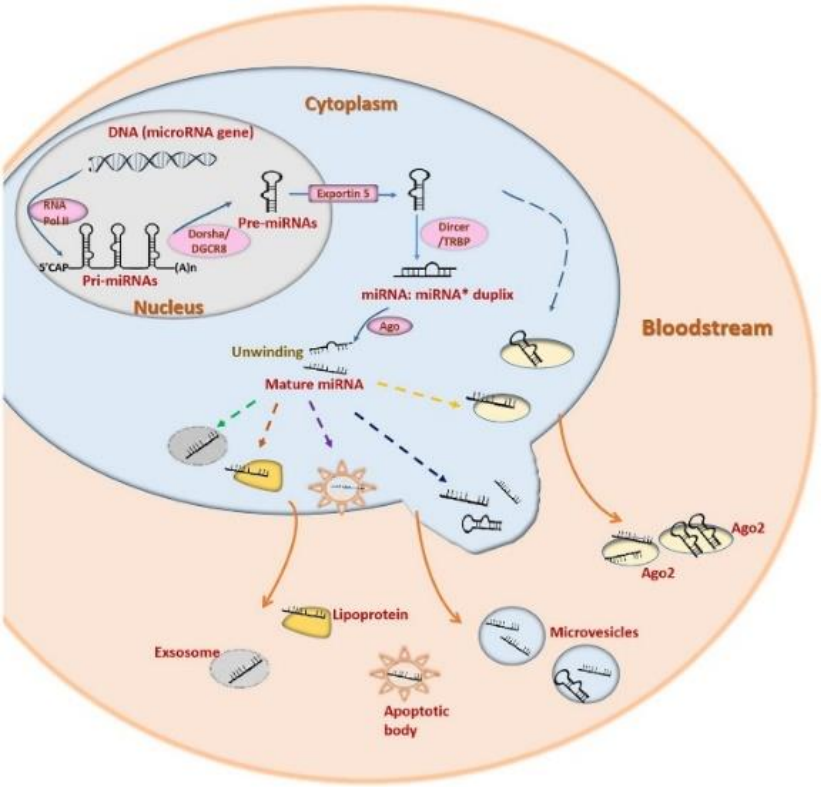

Figure 19: The biogenesis, cellular release, and circulation of mirna and their underlying mechanisms (L. Zhang et al., 2020). RNA polymerase II generates pri-mirnas from miRNA genes, which are then cleaved by Drosha/DGCR8 to create pre-miRNAs. Pre-miRNAs are then carried into the cytoplasm, where Dicer/TRBP cleaves them. After that, the miRNA duplexes are converted into mature miRNAs. The mature miRNA is solely translated from one strand of the miRNA duplex. Several mechanisms can release pre-miRNAs and mature miRNAs into the bloodstream. MiRNAs can be released by interacting with particles such as HDL and RNA-binding proteins (Ago2), or by being bundled in vesicles (exosomes, microvesicles, and apoptotic bodies).

Role of microRNAs in adipocyte inflammation

Numerous studies have shown that miRs regulate the expression of certain cytokines involved in inflammation in AT as well as in human adipocytes (Amri & Scheideler, 2017; E. Arner et al., 2012; Strum et al., 2009). For example, it was recently shown that the transcription of a miR (1275), involved in the maturation of human preadipocytes, was regulated by inflammatory factors (IL-6 and TNF-α) through the involvement of the nuclear factor-kappa B (NF-κB) pathway in human adipocytes (Zhao et al., 2016). Conversely, inflammation, induced by TNF-α or lipopolysaccharide treatment, also regulates the expression of certain miRs (L. Zhu et al., 2014). Other studies have focused on the deregulated miRs in AT during obesity and their associations with obesity parameters: 21 miRs are differentially expressed in epididymal WAT of lean and obese mice (Chartoumpekis et al., 2012) and 50 miRs were quantified in subcutaneous WAT of normo-weighted and obese subjects. Among these miRs (Table 10), 17

are strongly correlated with BMI and metabolic parameters (Ortega et al., 2010) (glucose and TG levels). These results are consistent with those obtained by Kloting et al. who showed significant correlations between the expression of certain miRs and the morphology and metabolic parameters (glucose levels, amount of visceral fat mass and circulating levels of inflammatory markers (Klöting et al., 2009) (IL-6, leptin and adiponectin). In addition, miR-155 has been identified by our team as being involved in adipocyte inflammation mediated by the NF-κB signaling pathway (Karkeni et al., 2016).

miRs	Regulations
miR-132	• Increase IL8 and MCP1 production • Activate the NFkB
miR-126 and miR-193b	• Regulate the secretion of MCP1 • Decrease its expression and TNF secretion
miR-145	• Increase the expression and secretion of TNF-α
miR-335	• Its expression increases with leptin, resistin, TNF-α and IL-6 treatment
miR-130, miR-146b and miR-222	• Its expression increases with TNF-α treatment
miR-103 and miR-143	• Its expression decreases with TNF-α treatment
miR-155	• Its expression increases with LPS or TNF-α treatment

Table 10: List of deregulated miRs during adipocyte inflammation.

2.3.2 NF-κB signaling pathway activation

The NF-κB pathway plays an essential role in the regulation of inflammation with TNF-α, IL-6 and MCP-1 as target genes and is found to be increased in the case of obesity. The NF-κB family of inducible transcription factors regulates a number of genes involved in immune and inflammatory response biological processes (Oeckinghaus & Ghosh, 2009). NF-κB1 (also known as p50), NF-κB2 (also known as p52), RelA (also known as p65), RelB, and c-Rel are members of the NF-κB family of structurally related proteins. These proteins bind to specific DNA regions known as B-enhancers and regulate the transcription of their target genes by

forming homo- or hetero-dimers (S.-C. Sun et al., 2013). Depending on the availability of negative or positive regulatory factors, the canonical and non-canonical signaling pathways (Figure 20) are implicated in the activation of NF-κB.

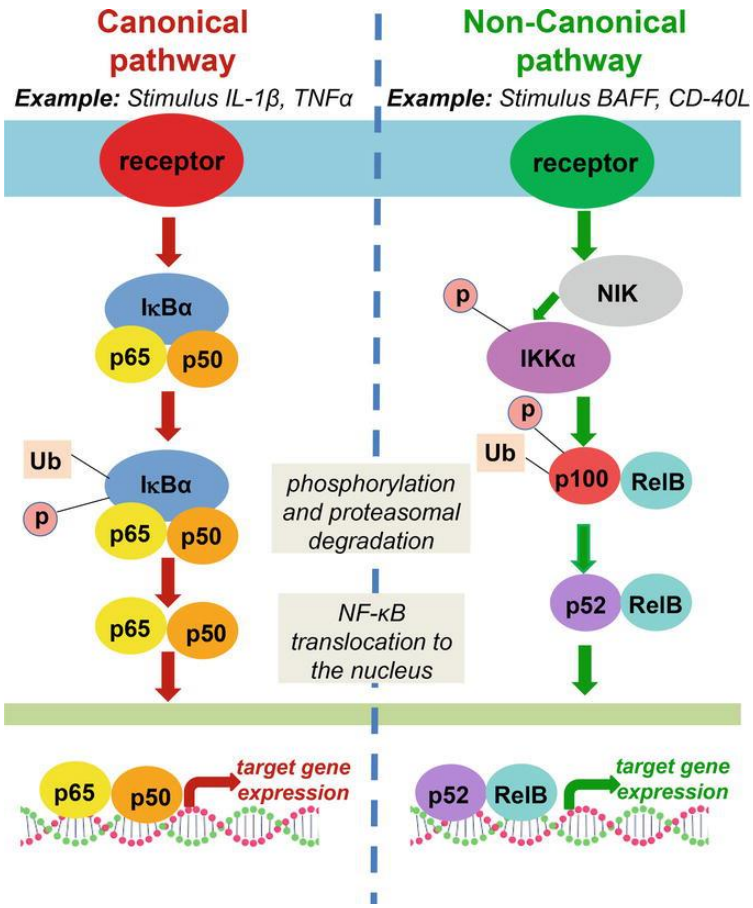

Figure 20: Inflammatory Signaling Controlled by Intracellular Pathways (Martin et al., 2019). FFAs and proinflammatory cytokines activate the NF-κB pathways in obese patients AT, promoting IR and the transcription of target genes encoding inflammatory mediators.

The key mechanism for canonical pathway activation is based on inhibitor kappa B α (IκBα) negative regulation, which results in p65/p50 heterodimers remaining in a latent state in the cytoplasm, preventing nuclear translocation and DNA binding. Several stimuli increase the activation of the IKK kinase complex, including cytokines, GF, mitogens, microbial components ROS and stress agents (T. Lawrence, 2009; S.-C. Sun & Ley, 2008). IKK phosphorylates IκBα on its serine residues when activated, causing ubiquitination and destruction of IκBα by the proteasome.The p65/p50 complex is rapidly translocated into the nucleus. Its binding to the enhancer or promoter regions induces transcription of target genes (T. Lawrence, 2009).

The non-canonical pathway is activated by a subset of TNF receptor (TNFR) superfamily members (LTBR, BAFFR, CD40, and RANK) and relies on NF-κB -inducing kinase to respond to a specific set of stimuli NF-κB-inducing kinase (NIK). To induce p100 phosphorylation, NIK activates the IKKα complex. This causes p100 to be ubiquitinated and its C-terminal IκB-like structure to be degraded by the proteasome, culminating in the formation of mature NF-κB2 p52 and nuclear translocation of the non-canonical NF-κB complex p52/ReIB (Senftleben et al., 2001; Vallabhapurapu & Karin, 2009). Although the signaling mechanisms of the two NF-κB routes differ, the canonical NF-κB is engaged in practically all aspects of immune responses, whereas the non-canonical pathway governs certain adaptive immune system functions (Cereijo et al., 2018; Ota, 2014; S.-C. Sun & Liu, 2011).

2.3.3 P38/MAPK pathway activation

The p38 mitogen-activated protein kinase (MAPKs) pathway was first discovered in myeloid cells as a master regulator of pro-inflammatory cytokine release. Different members of the p38 family are involved in the production of pro-inflammatory mediators in macrophages and increase monocyte recruitment, intensifying the pro-inflammatory response of adipocytes (Cereijo et al., 2018; Ota, 2014). p38 inhibition partially inhibits adipocyte TNF-α induced IL-6 secretion, implying that the regulatory actions of p38 seen in macrophages may also be present in adipocytes (Trujillo et al., 2006) (Figure 21).

Activation of the p38 pathway is strongly triggered by environmental stress stimuli (UV, radiation, mainly inflammatory cytokines), by inflammatory cytokines and less stimulated by GF. Once the stress is detected or the ligands on their receptors, the signal will be transmitted to GTPases of the Rho family which are responsible for the activation of MAP3Ks (Cuenda & Rousseau, 2007; S. Zhang et al., 1995). MAP3Ks, will then activate MAP2Ks. It is the MAP2Ks that will finally transmit the extracellular signals to the p38 and activating them in turn. This activation is done by phosphorylation of the threonine-X-tyrosine residues which will lead to a change of conformation allowing both the accessibility of the substrates and the approach of the site kinase activity, the N-terminal and C-terminal domains.

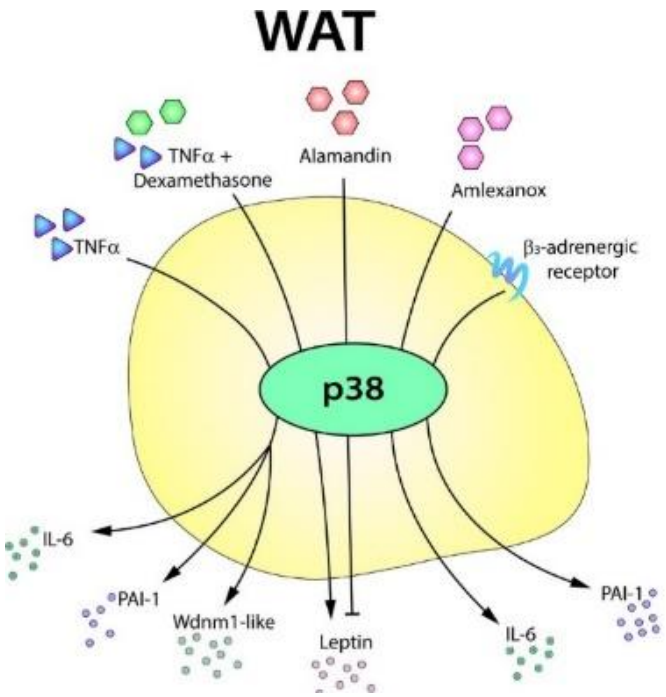

Figure 21: The secretome of AT mediated by p38 (Leiva et al., 2020).
In WAT the p38 pathway regulates the production and secretion of several adipokines. Following TNF α stimulation, p38 activation is necessary for the generation of IL-6, PAI-1, and Wdnm1-like in white adipocytes. In response to TNF-α, p38 signaling regulates leptin secretion, with a positive role in the presence of dexamethasone but an inhibitory role in the presence of alamandin (Uchiyama et al., 2017) (decrease leptin expression) IL-6 production in response to amlexanox (Möller et al., 2020) (anti-inflammatory mediator) and PAI-1 activation after stimulation are both mediated by p38.

2.4 White adipose tissue expandability hypothesis and ectopism

WAT expandability is one concept that links obesity to the development of HS. This hypothesis is appealing because it explains a number of clinical and epidemiological facts about the development of HS. Individuals with HS do not all have the same BMI. According to the WAT expandability hypothesis, various people have distinct intrinsic limits on their ability to expand their WAT depots. They develop IR and then HS when they approach their limits at various levels of obesity (Azzu et al., 2020).

Adipocyte dysfunction, aggravated by systemic IR, will block lipogenesis, slow down the anti-lipolytic action of insulin and thus induce an increase in the flow of FFA, no longer managed by the adipocytes. Each individual has his own intrinsic limit of capacity to store excess lipids in the AT. When the body is overwhelmed by the overload of dietary FA, other organs will be affected and fat deposits will be redirected to the liver, muscles and heart. These deposits constitute the ectopic fat and can be distinguished in two types: either by the formation

of AT around the organs or by the accumulation of intracellular TG called steatosis (Figure 22). In addition, these ectopic deposits will have a local or systemic action.

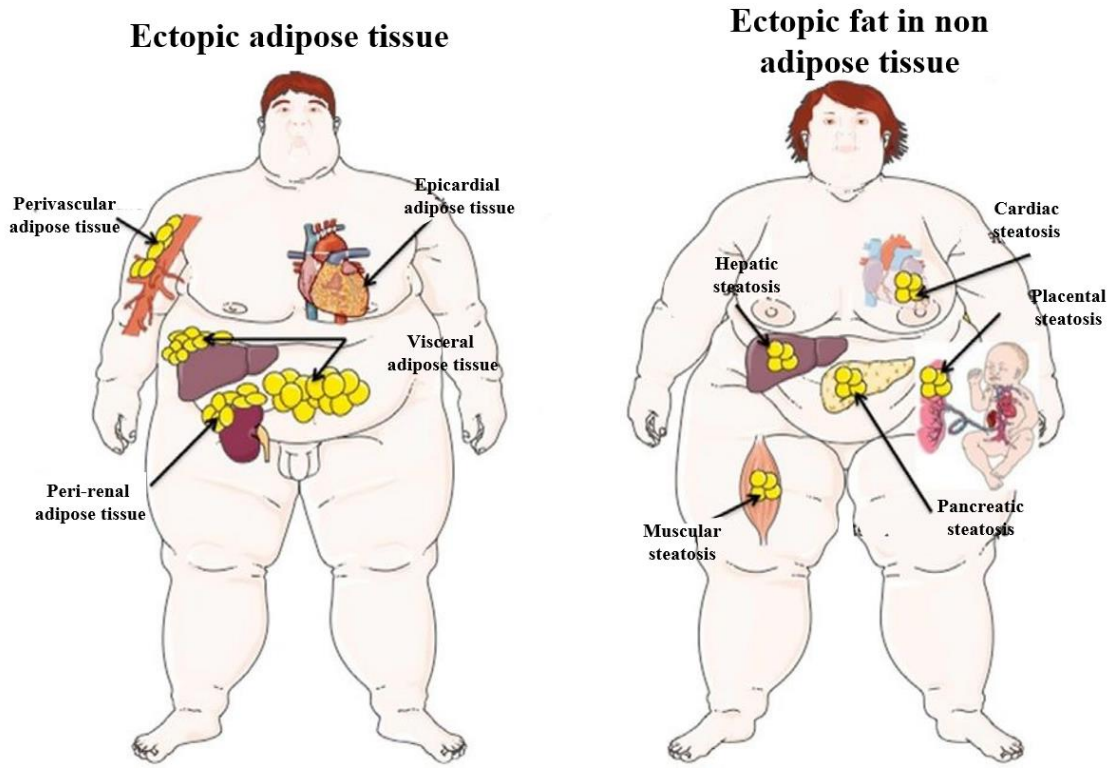

Figure 22: Different types of ectopic deposits (Gaborit et al., 2013).

The excess of nutrients in the body will promote the overload of FFA which can be stored in the liver (Azzu et al., 2020). This accumulation of lipids in the liver protects the other organs from this lipid overload when the AT is no longer able to ensure its storage function. It can be transient as well as permanent when associated with obesity. We speak of HS and it is an early marker of the syndrome of ectopic lipid deposits syndrome. Molecules called hepatokines are released by the liver and have an action on the other organs. At the adipose level, these hepatokines will also regulate lipolysis and fat mass accumulation.

3 Non-alcoholic liver disease

3.1 Historical background

The recognition of the presence of HS occurring without alcohol consumption was reported as early as 1938 in diabetic subjects (Connor, n.d.) and in 1958 in obese subjects (Westwater & Fainer, 1958). The authors observed that this disease is often associated with obesity and T2D (Ludwig et al., 1980).

Several decades later, the acronym NASH for nonalcoholic steatohepatitis was credited to Ludwig et al. (Ludwig et al., 1980) following a Mayo Clinic study published in 1980 reporting histological liver characteristics. In 1999, Matteoni et al (Matteoni et al., 1999). expanded the concept from its most benign to the most malignant and in chronological order of probable onset. NAFLD appears to progress from simple HS (nonalcoholic fatty liver (NAFL)) to NASH characterized by thepresence of steatosis and inflammation (Figure 23).

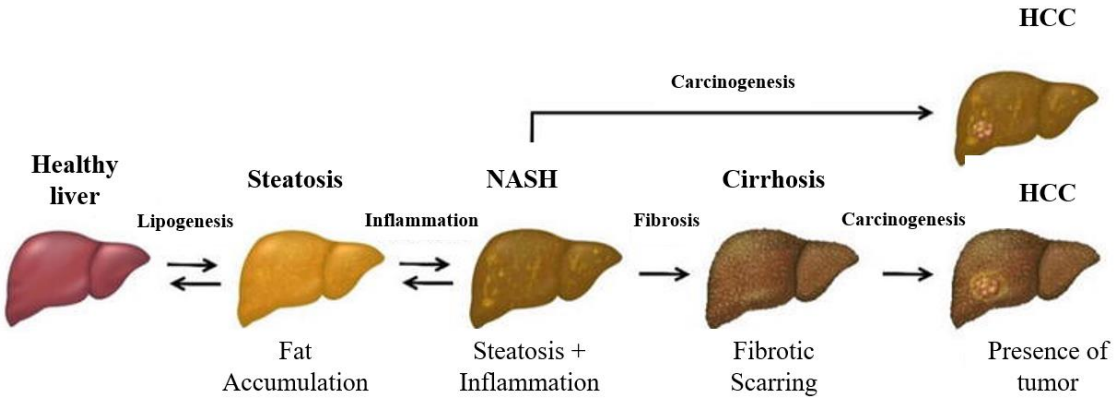

Figure 23: Diagram illustrating the progressive continuum of NAFLD.
Abbreviations, HCC: Hepatocellular carcinoma (adapted from (Tsochatzis, 2022).

3.2 Definition

Biochemically, NAFLD is considered once hepatic fat exceed 5% of its weight (Rodes, 2003). The lipids most often concerned are TG. Histologically, it is characterized by the existence of fat droplets in the hepatocytes.

NAFLD includes two distinct entities which are isolated steatosis and steatohepatitis. Isolated steatosis affected more than 5% of hepatocytes without necroinflammation lesions and without fibrosis. Steatohepatitis, when steatosis impacted more than > 5% of hepatocytes with lesions lobular inflammation or hepatocyte ballooning with or without fibrosis.

HS can be classified as being of primary or secondary origin (Falck-Ytter et al., 2001). Briefly, the primary type is associated with MtS, obesity, IR, and T2D. Secondary HS is thought to be due to the side effects of certain drugs and surgical procedures, the ingestion of toxins and various disorders (Jl et al., 1995).

3.3 Epidemiology and prevalence

NAFLD is currently the most common cause of chronic liver disease in children and adults worldwide. Studies on the natural history of NAFLD have shown that some patients with isolated steatosis or NASH appear to have a non-progressive clinical course (Marengo et al., 2016) (Figure 24).

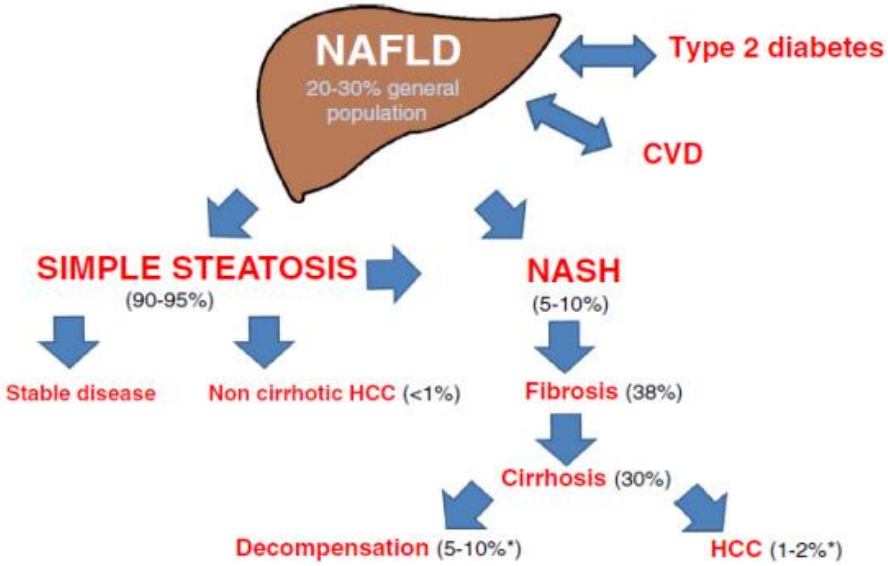

Figure 24: Prevalence of NAFLD in the general population (Buzzetti et al., 2016).
A high prevalence of NAFLD in the general population (20 - 30%) where the vast majority of patients have simple steatosis. Only 5-10% of diagnosed NAFLD patients will develop NASH and 30% of them will develop cirrhosis. Abbreviations, CVD: Cardiovascular Disease.

The prevalence of NAFLD worldwide varies from 6.3% to 33% (Chalasani et al., 2012), 5.4% to 24% (Clark, 2006), in the general population depending on the geographic region and the diagnostic method used. In industrialized countries the prevalence is estimating between 20 and 40% (Bedogni et al., 2005).

In the United States, it is estimated that one third of the adult population has NAFLD (Browning et al., 2004). The prevalence in Europe and Middle East (Figure 25) varies between 20% and 30% (Babusik et al., 2012; Bedogni et al., 2005; Caballería et al., 2010). More recent

studies in Japan and China find a prevalence similar to that found in Europe (20-30% in Japan and 15-30% in China (Fan et al., 2005)). Due to the adoption of sedentary lifestyles and dietary habits in Western countries the prevalence of NAFLD has significantly increased in developing countries

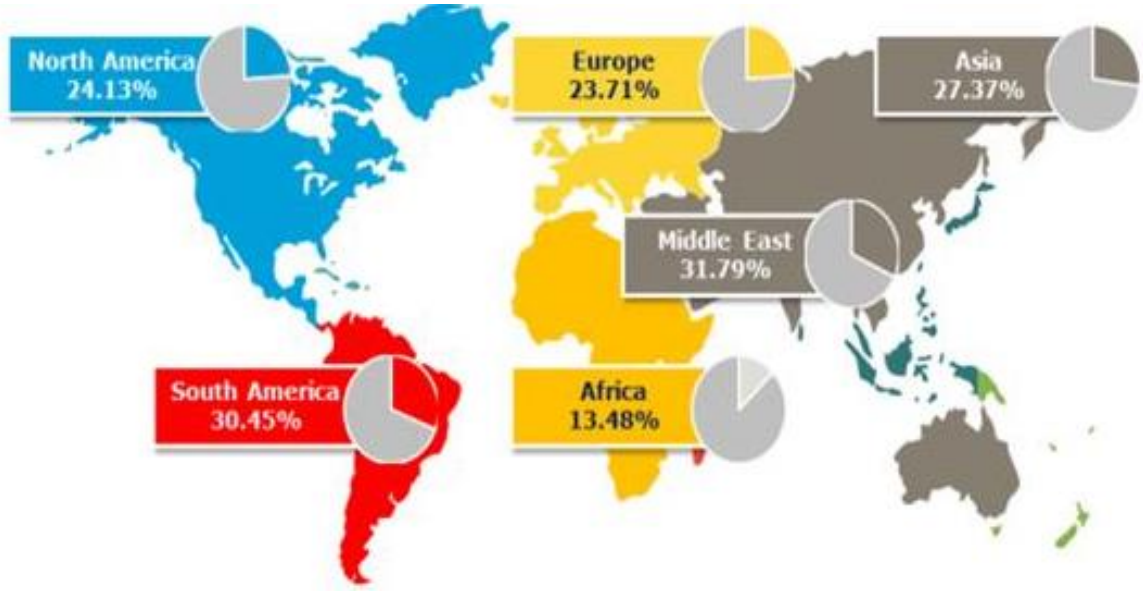

Figure 25: The prevalence of NAFLD in the world population.
Europe 23.71%, Asia 27.37%, Africa 13.48%, Middle East 31.79%, South America 30.45%, North America 24.13% (Younossi et al., 2018).

3.4 Risk factors

The prevalence of NAFLD increases with age and is greater than 40% in subjects aged 70 years or more who are at the highest risk of developing fibrosis or cirrhosis (prevalence of 40% and 14% respectively (Frith et al., 2009)). The addition of metabolic cardiovascular risk factors, mitochondrial dysfunction and redistribution of AT, are factors that increase IR and explain the high prevalence of NAFLD in the elderly.

Browning et al. (Browning et al., 2004) showed that NAFLD was more common in males than in women, with a ratio of 1.1:1. But the majority of patients in many of the initial NAFLD trials were women. Ludwig et al. found that 65% of patients were women (Ludwig et al., 1980). A large study in Hong Kong found a significant increase in the prevalence of NAFLD in women over 50 years of age (Wai-Sun Wong et al., 2012). These results may suggest a role for sex hormones in the pathogenesis of the disease. This hypothesis is supported by the high prevalence of NAFLD in polycystic ovary syndrome (PCOS) (Kelley et al., 2014).

IR has a major role in the pathogenesis of NAFLD and explains the close links between the MtS and its components, particularly obesity, T2D, dyslipidemia, and hypertension (Marchesini et al., 1999, 2001, 2003). NAFLD is currently considered to be the hepatic manifestation of the MtS (Marchesini et al., 2003).

The prevalence of NAFLD varies considerably according to ethnicity and is often accompanied by particular clinical phenotypes. Recent studies have described a high prevalence of IR (59%) as well as an increase in TG content of the liver by 2 to 3 times in East Coast Asian Indians compared to Caucasian subjects (Petersen et al., 2006). In contrast, African subjects, despite a high rate of obesity and diabetes, appear to be protected from NAFLD (Browning et al., 2004).

These inter-ethnic differences in prevalence cannot be attributed solely to differences in socioeconomic level, culture or lifestyle (level of physical activity, dietary habits) and highlight the potential role of genetic.

Among the genetic factors, the best studied is the polymorphism of the adiponutrin (PNPLA3-adiponutrin/patatin-like phospholipase 3). Genome-wide association studies (GWAS) have shown that the I148M genetic variant of adiponutrin or PNPLA3 is a potential marker of HS. Its expression is associated with an increase in triglycerides in patients with non-alcoholic fatty liver disease(Anstee & Day, 2013).

3.5 Diagnosis

Given the global impact of NAFLD in terms of prevalence, economic costs and morbidity-mortality, it is essential to detect and diagnose the disease in its early stages before patients develop advanced fibrosis.

By now, a significant proportion of patients escape diagnosis for various reasons: lack of knowledge of the disease, its silent evolution, normal transaminases levels, the presence of multiple metabolic and/or cardiovascular comorbidities, which means that these patients are often followed by endocrinologists and/or cardiologists but are rarely referred to the hepatologist.

Symptoms in NAFLD patients are generally non-specific and constitutional. Hepatomegaly (enlarged liver) is present in 75% of cases, however due to the high incidence of obesity in NAFLD patients, it can be difficult to diagnose (Diehl et al., 1988).

Methods of hepatic steatosis detection

It is difficult to assess the prevalence of HS because it is often asymptomatic or have non-specific symptoms, such as fatigue or right hypochondrium pain (Day, 2011). Blood liver tests

can be completely normal, the diagnosis of NAFLD requires the exclusion of other causes of liver disease, such as excessive alcohol consumption; the threshold for heavy drinking varies across studies (Liangpunsakul & Chalasani, 2012). Limits of 21 units of alcohol per week for men and 14 units per week for women are typically used (Sanyal et al., 2011). Chronic hepatotoxic treatment or hepatitis (viral, autoimmune, drug-induced), lipodystrophy, hemochromatosis or Wilson's disease, and eating disorders must all be cleared out when assessing HS. (L. A. Adams & Feldstein, 2011; Dowman et al., 2011) (malnutrition, parenteral nutrition, and rapid weight loss).

Liver biopsy

Liver biopsy is an invasive and painful procedure. Thus, despite the analysis of histopathology is still the gold standard for the diagnosis of NAFLD (Brunt & Tiniakos, 2005).It is the only method that allows identification of the severity of the disease and as well as the stage of hepatocyte damage on the NAFLD continuum and thus to discriminate steatohepatitis from HS.

Serum markers

SteatoTest (Poynard et al., 2005) and Fatty Liver Index (FLI) (Bedogni et al., 2006) have been independently validated as a non-invasive method. In the absence of advanced disease, blood liver tests are normal or show moderate elevation of transaminases, alkaline phosphatase (APL) and gamma-glutamyl transpeptidase (GGT) at 1.5-3 times the upper of normal limit (Bacon et al., 1994; Diehl, 1999; Ludwig et al., 1980; Powell et al., 1990; Sanyal & American Gastroenterological Association, 2002). Elevated Alanine aminotransferase (ALT) and aspartate aminotransferase (ASAT) (L. A. Adams & Feldstein, 2011; Pratt & Kaplan, 2000) are present in approximately 50% of patients with simple steatosis and in 80% of patients with NASH (Yan et al., 2007). Nevertheless, a significant proportion of subjects may have normal transaminases (L. A. Adams et al., 2005).

Medical imaging

Medical imaging techniques, such as ultrasound, magnetic resonance and nuclear magnetic resonance spectroscopy (Longo et al., 1995; Siegelman & Rosen, 2001; Szczepaniak et al., 2005) can be used to detect the presence of HS. These techniques have similar or better diagnostic performance than liver biopsy for the detection of <5% steatosis. The magnetic resonance has the advantage of quantifying steatosis in each liver segment and thus reduces the risk of sampling variability inherent in liver biopsy (Noureddin et al., 2013). However, the use

of these techniques in routine practice is limited by their cost and their availability for the moment to research centers. In addition, they allow a more objective assessment of steatosis than histological analysis, as well as the evaluation of a greater portion of the liver than is possible with liver biopsy, thereby eliminating a percentage of error related to sampling (Bacon et al., 1984; Szczepaniak et al., 2005).

3.6 Hepatic lipid metabolism

The pathogenesis of HS is a complex process that involves the interaction of multiple mechanisms, explaining the "systemic" character of the disease, which goes far beyond the liver. It is essential to understand the mechanisms responsible for the development and progression of the disease, as this not only allows to identify patients at risk but also to detect potential therapeutic targets.

Logically, an increase of intrahepatic lipids necessarily results from an imbalance between the pathways that promote the accumulation of hepatic lipids and those that promote their elimination. The majority of researchers who have conducted studies on NAFLD conclude that the major and primary dysfunction leading to the accumulation of hepatic lipids is the increase in circulating FFA, followed by a stimulation of hepatic lipogenesis and mis-adjustment of oxidation and secretion of accumulated lipids by the organ (Kraegen et al., 1991; Matteoni et al., 1999; Petersen et al., 2002).

3.6.1 Normal hepatic lipid metabolism

Dietary lipids (>90% TG) are digested in the gastrointestinal system and lipolytic products are released into the enterocyte. TG, cholesterol, phospholipids, and apolipoproteins are packed into chylomicrons within enterocytes. FFA enter adipocytes, muscle cells and hepatocytes, where they are esterified to G3-P and eventually stored as TG (D. H. Adams, 2007; Barrows & Parks, 2006; Donnelly et al., 2005).

In the liver FA are either stored as TG or undergo β-oxidation in the mitochondria or peroxisomes of hepatocytes, depending on the energy status, and hence do not contribute to energy storage. FA are converted to TG for storage or to be delivered into the circulation by VLDL when they are not required for energy production. Lipolysis in AT is the primary source of FA for VLDL formation. DNL liver accounts for just 8% of the FA incorporated into the VLDL particle in the feeding state (4% in the fasting state), while AT, chylomicrons, and dietary acids account for 44%, 15%, and 10% (Barrows & Parks, 2006), respectively. The

ability of the liver to form and produce VLDL particles has a substantial impact on the lipid steady state within the liver.

3.6.2 Lipid accumulation and lipid elimination by the liver

The accumulation of lipids (mainly TG) in the liver seen in NAFLD is the outcome of a lipid turnover imbalance in the liver. HS can be caused by changes in numerous areas of normal lipid metabolism, including the transport of FFA to the liver, DNL in hepatocytes, the rate of β-oxidation within the liver, and the export of TG through the formation and secretion of VLDL (Harrison & Day, 2007) (Figure 26).

Influx of FFA: There is a pool of FA in plasma that are not esterified and are referred to as FFA. Insulin does not completely reduce HSL within adipocytes in people with IR. As a result, greater lipolysis in the AT occurs in these people, leading in an increased influx of FFA into the liver (Sanyal et al., 2001).

Hepatic lipogenesis: DNL is elevated in patients with NAFLD and produces around a quarter of the TG in the liver of NAFLD patients, compared to about 5% in normal situation (Donnelly et al., 2005). Two transcriptional factors, Sterol regulatory element-binding transcription factor 1 (SREBP- 1) and Carbohydrate-responsive element-binding protein (ChREB-P), have elevated activity in NAFLD animal models (Dentin et al., 2006; Shimomura et al., 1999). Both of these factors stimulate DNL by regulating gene expression. Increased lipogenesis is accompanied with a decrease in fatty acid oxidation.

Lipid export: VLDL particles carry TG out of the liver. Each VLDL particle is 30-100 nm in diameter and contains a single apolipoprotein B molecule (ApoB). Within the hepatocyte, ApoB synthesis is a rate-determining step in the generation of VLDL. Hyperinsulinemia, which is associated with IR, can affect ApoB production (Dashti et al., 1989). ApoB synthesis is reduced in NAFLD patients, which could indicate that decreased ApoB synthesis is a key role in the development of HS (Charlton et al., 2002).

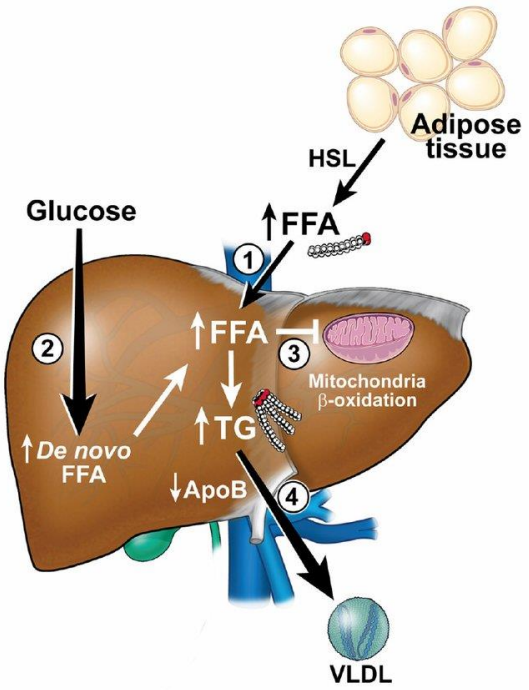

Figure 26: Pathways of lipid accumulation and elimination by the liver (Q. Liu et al., 2010; Shimomura et al., 2000).
1)An increase in circulating FFA from adipocyte lipolysis and spillover of muscle LPL action on intestinal chylomicrons increases hepatocyte chylomicrons from intestinal origin results in an increase in GLA uptake by hepatocytes. 2) An de novo lipogenesis activity increases intrahepatic FFA uptake. 3) An increase in the amount of ingested lipids and thus of chylomicrons of intestinal origin is likely to increase the uptake of residual chylomicrons by the liver. 4) secretion of VLDL, will lead to the development of HS.

3.7 Histopathology of NALFD

There are four levels of steatosis in the liver (Table 11). TG production and fat buildup in the liver were thought to be hepatoprotective at first, but high intrahepatic fat content is now a risk factor for disease development (Fartoux et al., 2005). Simple HS is a reversible condition that can be reversed *via* lifestyle changes such as increased physical activity and dietary changes.

Grade	Lesions
Grade 0: Healthy	Lower than 5% of lobules, hepatocyte
Grade 1: Minimal Steatosis	10-30% of lobules, hepatocyte ballooning, minimal lobular inflammation, no portal inflammation
Grade 2: Moderate Steatosis	34 to 66% of lobules, hepatocyte ballooning, moderate intralobular inflammation, minor to moderate portal inflammation
Grade 3: Severe Steatosis	Diffuse steatosis greater than 66% of lobules, intralobular hepatocyte ballooning, and periportal inflammation intralobular and portal inflammation

Table 11: Grading of HS (Brunt et al., 1999; Nassir et al., 2015).

There are two types of steatosis: macrovacuolar steatosis (most often benign), where the fat is concentrated in a large vacuole that pushes the nucleus to the periphery of the cell, and microvacuolar steatosis, where the fat is located in small intracellular vacuoles without changing the position of the nucleus (R. G. Lee, 1995) (Figure 27).

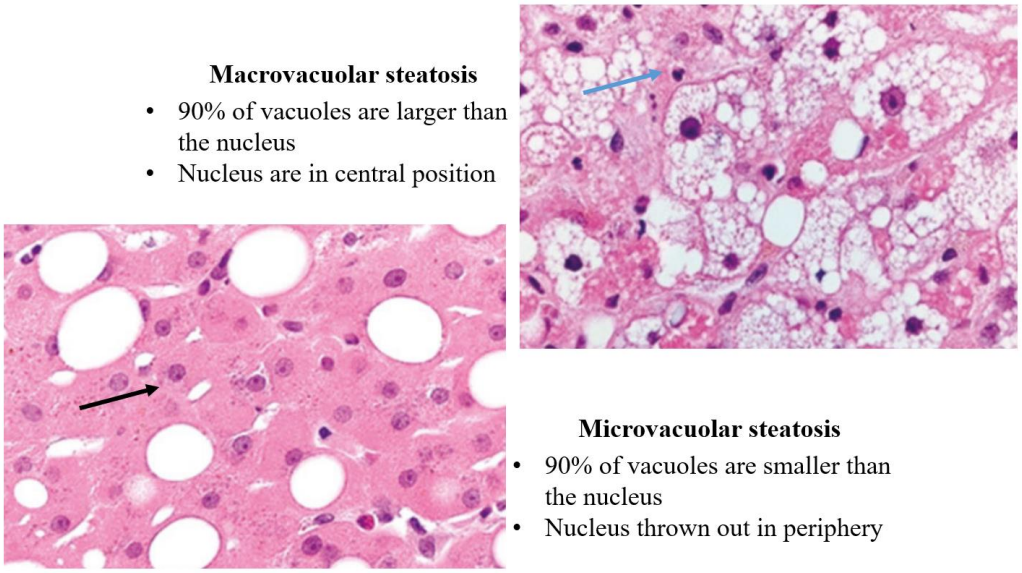

Figure 27: Comparison between the microvacuolar and microvacuolar steatosis. A black arrow points area of macrovesicular and a blue arrowhead indicates microvesicular steatosis.

3.8 The development of NAFLD: the "multi-hit model"

Several pathophysiological mechanisms could be at the origin of NAFLD. The "multi-hit" (initially "double-hit") (Figure 28) hypothesis is the best-known theory that could explain the pathogenesis of NAFLD.

According to the theory of Day and James, first put forward in 1998, the pathogenesis of NAFLD occurs in 2 "hits" (Day & James, 1998). The subsequent theory explains the progression of NAFL to NASH. HS, according to the "multiple-hit" model (Nadeau et al., 2005; Wiegand et al., 2010), may be an epiphenomenon of several different injurious mechanisms (Manco et al., 2008).

The first hit represents the development of simple HS, with accumulation of FFAs and TG in hepatocytes is primarily due to IR, (Nadeau et al., 2005). The liver becomes extremely vulnerable after the initial development of steatosis (Day & James, 2003).

The second hit is a combination of oxidative stress, mitochondrial dysfunction, and adipocytokine imbalance that leads to steatohepatitis and fibrosis (Manco et al., 2008). NASH has been linked to NF-κB activation (Franzese et al., 1997; Pacifico et al., 2010), which can result in increased transcription of many proinflammatory genes. Reactive oxygen species cause hepatocytes to release cytokines, then oxidative stress is induced by a combination of hyperinsulinemia and lipid peroxidation , which can lead to mitochondrial dysfunction in NASH, TG accumulation, and eventually cell necrosis (Brecelj & Orel, 2021).

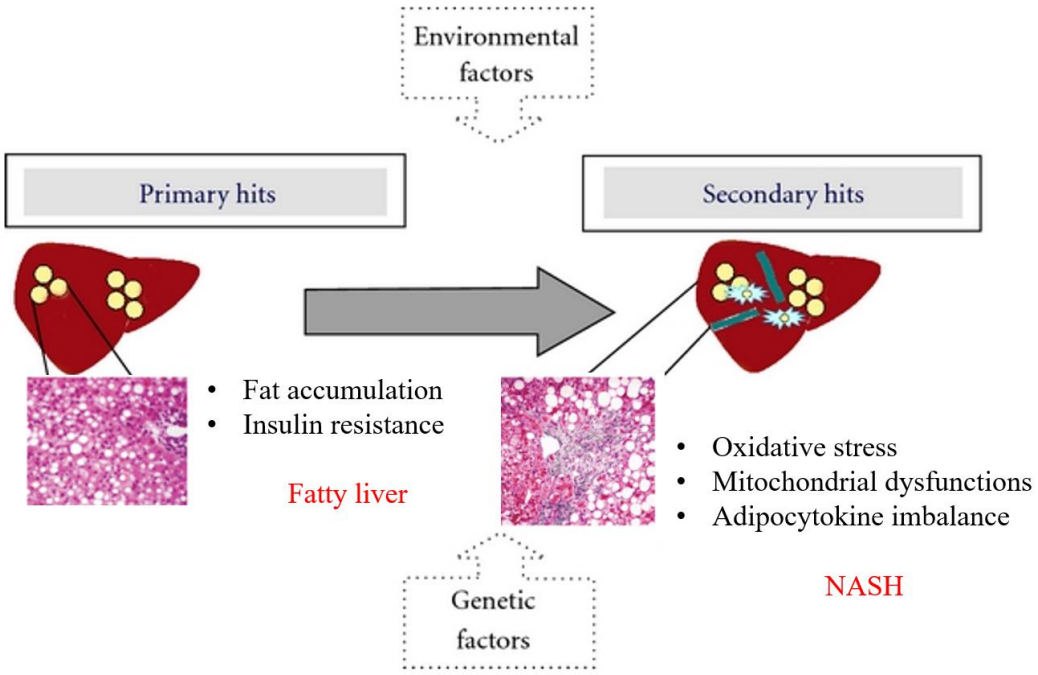

Figure 28: Simplified representation of 2 hit model of NASH pathogenesis (Alisi et al., 2011).

4 Effects of vitamin D on the biology of white adipose tissue and hepatic steatosis

4.1 Vitamin status in a context of obesity

A large set of cross-sectional studies have pointed out the inverse relationship between low serum 25(OH)D and obesity (S. Cheng et al., 2010). Such observations have been found in adults but also in children (Fu et al., 2020) and in elderly people (Abarca-Gómez et al., 2017; Perna, 2021). Three hypotheses seem to be able to explain this association: *i)* by sequestration of VD in the AT (Wortsman et al., 2000); *ii)* the volumetric dilution of VD in plasma, which seems to be the preponderant hypothesis (Drincic et al., 2012); *iii)* the metabolism of VD is altered in the AT of obese patients (Wamberg, Christiansen, et al., 2013).

Plasma 25(OH)D concentrations are also inversely correlated with obesity-related parameters such as body fat percentage (García et al., 2009; Gilbert-Diamond et al., 2010; Vilarrasa et al., 2007; Vitezova et al., 2017), BMI, Homeostatic Model Assessment of Insulin Resistance **(**HOMA-IR), waist circumference, and concentrations of markers of inflammation (Jackson et al., 2016; McGill et al., 2008) (such as IL-6, high sensitivity C-reactive protein (hsCRP)). In addition, plasma VD levels are inversely correlated with levels of lipoproteins and circulating TG, as well as higher IR (Mirhosseini et al., 2017; Reddy Vanga et al., 2010; Seida et al., 2014).

Several studies have shown that weight loss and a decrease in percentage of adiposity, are associated with an increase in circulating 25(OH)D levels to variable degrees in obese adults (Wamberg, Christiansen, et al., 2013).

In obese person (Landrier et al., 2016b), weight loss combined with VD supplementation appears to enhance VD status. It seems that 25(OH)D levels are linked to thermogenesis and lipid oxidation (Teegarden et al., 2008) and therefore low 25(OH)D concentrations are associated with an imbalance in energy balance.

Because of the VDD observed in obese people, many studies have used VD supplementation in weight loss programs. Different methodologies have been applied, using different doses and different modalities of administration. In the majority of studies, VD supplementation did not induce a difference in weight loss (Al-Daghri et al., n.d.; Farag et al., 2018; Mai et al., 2017; Mason et al., 2014; Rosen et al., 2012; Salehpour et al., 2012; Sneve et al., 2008; Wamberg, Kampmann, et al., 2013; Zhou et al., 2010; Zittermann et al., 2009). However, with a certain

dose (50,000 IU per week or 100,000 IU every 2 weeks), weight loss is reported (Khosravi et al., 2018; Zittermann et al., 2009).

Experimentally, few studies have been conducted in the context of weight loss. However, data are available in the prevention of the onset of obesity, where VD supplementation limits the expansion of AT and weight gain despite the consumption of a diet rich in sugar and fat for 10 weeks in C57BL/6J mice (Marcotorchino et al., 2014). This is associated with an increase in energy expenditure due to oxidation of lipids. Indeed, in mice supplemented with VD the expression of genes involved in mitochondrial metabolism and in oxidation is strongly induced in muscle, liver and BAT. Numerous studies have confirmed these results and highlighted a limitation of weight gain. A study on Sprague-Dawley rats subjected to the diet rich in sugar and fat associated with a VD supplementation for 8 weeks also reported a limitation in weight gain (Yin et al., 2012). Similar results have been observed in mice (Sergeev & Song, 2014). In addition, an increase in body fat has been observed in VDD rats (Chanet et al., 2017).

4.2 Effect of vitamin D on adipose tissue inflammation

Both innate and adaptive immunological responses are known to be modulated by VD (Lemire, 2000). While initial studies suggested that $1,25(OH)_2D$ induced the expression of several pro-inflammatory cytokines and decreased the expression of anti-inflammatory cytokines in human and mouse adipocytes (X. Sun et al., 2008; X. Sun & Zemel, 2007, 2008), further studies did not confirm these conclusions.

Indeed, several groups consistently reported anti-inflammatory effects since $1,25(OH)_2D$ significantly reduced the basal release of MCP-1, IL-8 and IL-6 from human pre-adipocytes as well as monocyte recruitment by the same type of cells (Gao et al., 2013) and MCP-1 by human adipocytes (Lorente-Cebrián et al., 2012). Our team reported that $1,25(OH)_2D$ inhibited the expression of inflammatory markers (IL-6, MCP-1 and IL-1β) under basal and TNF-α stimulated conditions in human adipocytes and in 3T3-L1 adipocytes (Marcotorchino et al., 2012). We observed that NF-κB signaling was involved in the anti-inflammatory effect of $1,25(OH)_2D$ but also p38 dephosphorylation resulting from the induction of Dusp10 under $1,25(OH)_2D$ effect in 3T3-L1 (Marcotorchino et al., 2012) (Figure 29). In addition to the classical impact of $1,25(OH)_2D$ on cytokines and chemokines, we recently described a new anti-inflammatory effect, mediated by the inhibition of the several inflammatory-linked miRNAs, including miR-146a, miR-150 and miR-155 (Karkeni et al., 2018), known to be related to inflammatory process in adipocytes (Karkeni et al., 2016).

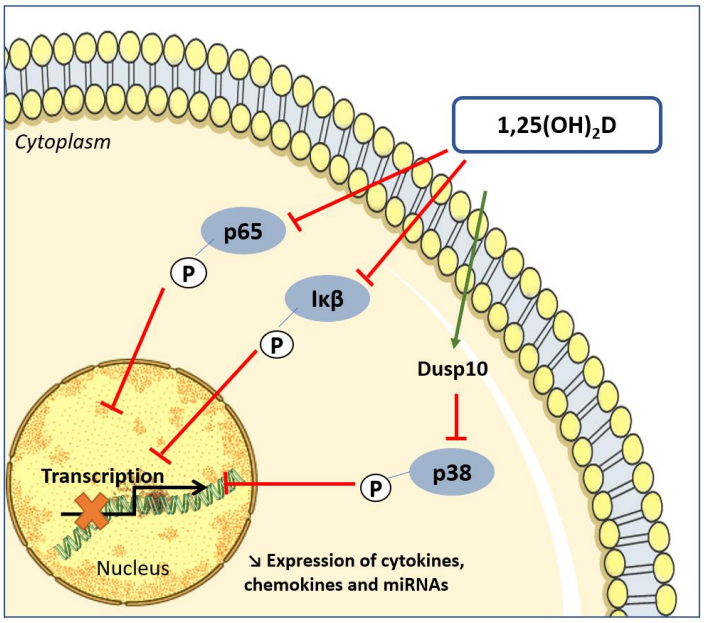

Figure 29: Anti-inflammatory effects of 1,25(OH)$_2$D (Landrier et al., 2022).
This effect involves interference with NF-kb signaling through inhibition of p65 and Iκβ, it also involves p38 dephosphorylation resulting from the induction of Dusp10, leading to the down-regulation of the expression of inflammatory markers (cytokines, chemokines, miRNA).

1,25(OH)$_2$D has been found then in human and mouse adipocytes to reduce inflammatory chemokine and cytokine release. 1,25(OH)$_2$D inhibits downstream pro-inflammatory gene transcription pathways and decreases the activation of NF-κB and MAPK signaling mediators in inflammatory pathways (Figure 30) and a modulation of pro-inflammatory miRNAs (Karkeni et al., 2018).

The anti-inflammatory effect of VD has also been investigated *in vivo*. Importantly, an upregulation of several cytokines mRNA levels were observed in epidydimal AT of adipocyte-specific VDR knock-out mice (Yimagou et al., 2020). Concerning the effect of VD supplementation, Palaniswamy et al. recently reported that VD supplementation do not support a beneficial role on obesity-related inflammation at systemic level (Palaniswamy et al., 2020) and Wamberg et al. reported that VD supplementation in subjects with obesity showed no decrease of AT expression of MCP-1, IL-6 or IL-8(Wamberg, Cullberg, et al., 2013). Nevertheless, several other studies are more in favour of an anti-inflammatory effect of VD *in vivo*.

A recent study demonstrated that VD repletion in subjects with overweight or obesity and a characterized 25(OH)D deficiency reduced TNF-α, IL-6, iNOS and PAI-1 expression in subcutaneous AT and in AT macrophages (Yimagou et al., 2020). Several experimentations in rodents confirmed the ability of VD or 1,25(OH)$_2$D to blunt inflammatory process in AT. Thus IL-6 protein levels were reduced in epididymal AT of high fat (HF) fed mice supplemented

with 1,25$(OH)_2$D (Lira et al., 2011). In addition, we published data showing that VD supplementation in a HF diet model leading to metabolic inflammation, reduced the expression and protein levels of pro-inflammatory cytokines and chemokines, but also inhibited macrophage infiltration in AT of obese mice(Karkeni et al., 2015). These effects were confirmed in an acute inflammation model mediated by intraperitoneal injection of Lipopolysaccharide (Karkeni et al., 2015) (LPS). Similarly, VD supplementation down-regulated epididymal fat MCP-1 and CCL5 and reduced the number of macrophages and Natural Killer (NK) cells within AT in HF fed mice (C. Y. Park et al., 2020). In rats, VD supplementation also limited the concentrations of TNF-α and MCP-1 in AT (Farhangi et al., 2017). Recently, we tested the curative effect of a VD supplementation and showed a reduced expression of chemokines in obese mice (Marziou et al., 2020, 2021).

Oral supplementation with 1,25$(OH)_2$D of 7000 IU per day for 26 weeks inhibited IL-1ß driven production of MCP-1, IL-6, and IL-8 in an *in vitro* culture model of subcutaneous AT fragments in a randomized controlled study with fifty-five obese people (Wamberg, Cullberg, et al., 2013). The *in vivo* 1,25$(OH)_2$D administration, on the other hand, had no effect on inflammation markers in the circulation or AT (Wamberg, Cullberg, et al., 2013). In 3T3-L1 cell line activated by LPS, 1,25$(OH)_2$D had similar effects (Lira et al., 2011).

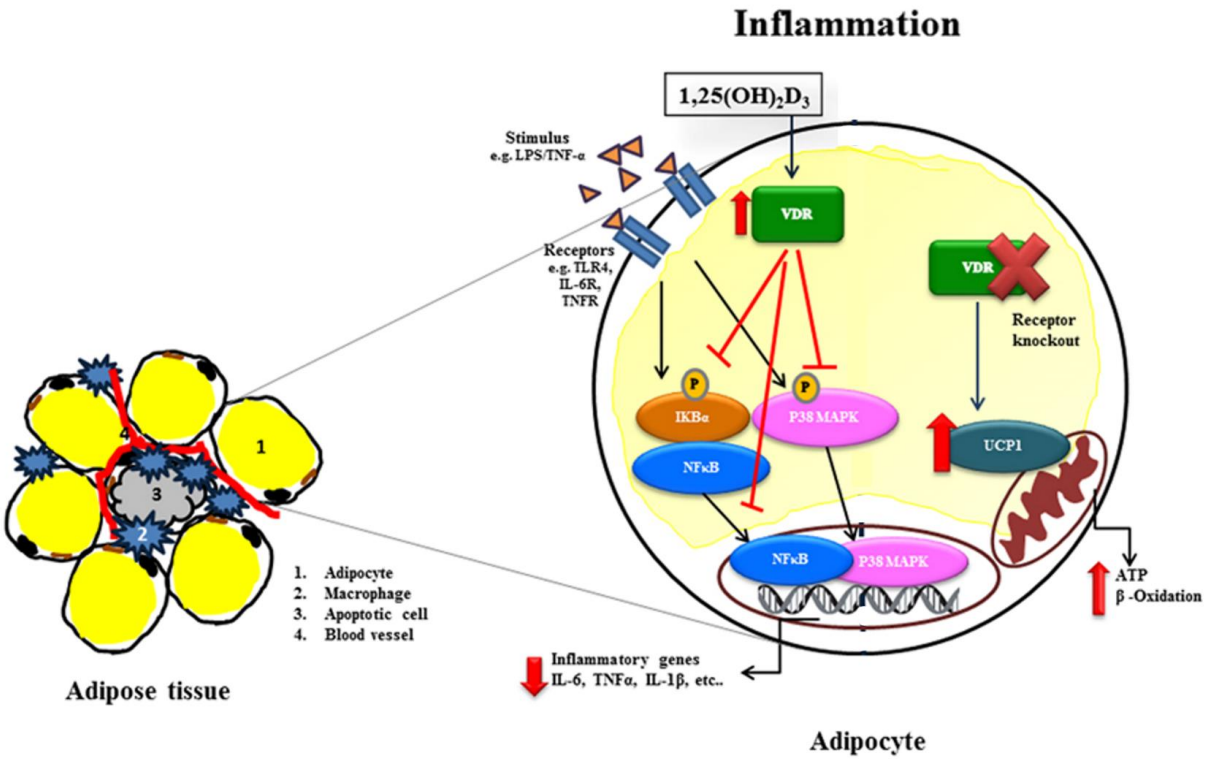

Figure 30: The molecular effects of 1,25(OH)$_2$D3 on inflammation in adipocytes. Adapted from review (Mutt et al., 2014). Stimulation of inflammatory genes such as IL-6, TNF-α, and IL-1β, via specific receptors such as TLR, IL-6 receptors (IL-6R), activates NF-κB or p38MAPK signaling dependent transcription. Ik-κB phosphorylation and translocation of NF-κB as well as P38/MAPK into the nucleus are inhibited by 1,25(OH)$_2$D3. In addition, 1,25(OH)$_2$D3 has an effect on energy homeostasis. Uncoupling proteins in VDR/mice increases energy consumption (UCPs). Activation is indicated by the arrows in the picture, whereas inhibition is indicated by the blunted line. The effect of 1,25(OH)$_2$D3 on the inflammatory signaling pathway is indicated by the red lines and arrows.

4.3 Effect of vitamin D on hepatic steatosis

As previously mentioned, obesity is strongly associated with fatty liver. Because of the importance of VD in these pathologies, the question of the effect of VD on liver diseases has arisen. In humans and animals, several studies have found a relationship between plasma VD concentration and HS.

In a clinical study including adults with normal serum liver enzymes, low plasma 25(OH)D concentrations were correlated with the presence of HS (Barchetta et al., 2011), independently of MtS, obesity and IR profile (Barchetta et al., 2011). This observation was then confirmed in a study where patients with NAFLD had low 25(OH)D levels (Eliades et al., 2013) and thus suggested a role for VD in the pathogenesis of NAFLD (Cimini et al., 2017; S. Liu et al., 2019).

Epidemiological studies revealing the association between VD and liver disease have thus led to the use of VD as a therapeutic strategy for liver damage (Nobili & Reif, 2015). Numerous

studies have followed, the majority of which have not reported a beneficial effect on HS (Sangouni et al., 2019). Indeed, the pilot study on NASH conducted by Kitson and his team (Kitson et al., 2016), did not show an effect of VD on intrahepatic fat accumulation, inflammation, or fibrosis. In agreement with these data, a study in patients with T2D and NAFLD did not show a corrective effect of VD supplementation for 24 weeks (Barchetta et al., 2016). However, other studies have reported effects of VD supplementation on biochemical parameters of NAFLD. For example, it has been shown that supplementation limited liver damage, with a decrease in liver inflammation but no change in the degree of steatosis (Sharifi et al., 2014). In the Lorvand Amiri study, VD supplementation and a low-calorie diet reduced the concentrations of TG and increase HDL (Lorvand Amiri et al., 2016) and also have positive effects on lipid profile and insulin sensitivity (Lorvand Amiri et al., 2017). Finally, VD improved HS in patients with hepatic fat accumulation after 4 weeks of supplementation (Papapostoli et al., 2016).

VDD has also been reported in the animal model to be associated with fat accumulation in the liver (Giblin et al., 2017) and to the development of NAFLD (Roth et al., 2012). Although the effect of VD supplementation in the treatment of HS in humans is controversial, preclinical studies in animals demonstrate a beneficial therapeutic effect (Karatayli et al., 2020). In a rat model of HS induced by HF diet, injection of VD for 2 months prevented HS by reducing the accumulation of TG by inhibiting lipogenesis and inducing lipid oxidation (Yin et al., 2012). In addition, changes in lipogenesis, β-oxidation and hepatic inflammation induced by VDD, were improved by 10 weeks of VD supplementation in mice (Ceciliano et al., 2019). This improvement was also found in an interventional study in mice where VD supplementation reduced steatosis and highlighted the preventive effect of VD on the decrease in expression of genes involved in lipogenesis and in the progression of the disease (Jahn et al., 2019).

We recently reported that in a 15 weeks VD supplementation and in HF and high-sucrose fed mice, a decrease in lipid droplets and a reduction in TG accumulation in the liver indicated that VD had a protective impact on HS. This was linked to a reduction in gene expression coding for essential enzymes involved in hepatic de novo lipogenesis and fatty acid oxidation. Overall, our findings suggest that VD supplementation may be beneficial in reducing AT inflammation and HS, and may be a promising nutritional strategy for combating obesity-related comorbidities (Marziou et al., 2020).

4.4 Maternal vitamin D deficiency

VDD became a worldwide health problem (Bendik et al., 2014; Palacios & Gonzalez, 2014), especially for females in ages of childbearing age, pregnant or breastfeeding. In France, almost 50% of women are deficient in 25(OH)D (<50nmol.L) and 6% have plasmatic concentrations <25nmol/L. A recent meta-analysis of 95 studies (Saraf et al., 2015) allowed us to define the maternal and new born 25(OH)D concentrations in different part of the world. The OMS defined the regions as: America, Europe, East-Mediterranean, South of Asia and West of the Pacific (Table 12). This study clearly depicted large deficiency all around the world. The high prevalence of maternal VDD may be attributed to changes in lifestyle (sun exposure and nutritional consumption). Additionally, because endogenous production of VD from UV radiation is constrained, women who have more skin melanin, and who are veiled or covered are thought to be at particularly high risk for deficiency (Bowyer et al., 2009; Dijkstra et al., 2007).

	Women		**New born**	
Regions	**25(OH)2D <50nmol/L**	**25(OH)2D <25nmol/L**	**25(OH)2D <50nmol/L**	**25(OH)2D <25nmol/L**
America	64%	9%	30%	14%
Europe	57%	23%	73%	39%
East-Mediterranean	N/A	79%	60%	N/A
South of Asia	87%	N/A	96%	45%
West of the Pacific	83%	13%	54%	14%

Table 12: The prevalence of 25(OH)2D <50nmol/L and <25nmol/L.in different regions of the world (Saraf et al., 2015).

4.4.1 Vitamin D deficiency consequences for the mother and child

Maternal VD status conditions fetal and neonatal VD status, since the fetus is totally dependent on nutrients from the placenta. The 25(OH)D diffuses via the placenta and there is a direct linear correlation between 25(OH)D levels in umbilical blood, which are lower than those

found in the serum of mothers (Delvin et al., 1982). In addition, the newborn has a VD stock established *in utero*, which could be reduced in case of maternal VDD during pregnancy.

VD concentrations in breast milk are higher than 25(OH)D and 1,25$(OH)_2$D concentrations because VD circulates through the bloodstream more quickly than these two metabolites. VD concentrations in milk are typically low, ranging from 0.25 to 2 µg/L. This corresponds to around 0.9 µg/day of VD secreted in milk during the first 6 months, which is insufficient to protect breastfed children from rickets (EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA), 2013).

In the mother, VDD has been associated with altered glucose homeostasis and increased incidence of gestational diabetes mellitus (GDM), and preeclampsia (Weinert & Silveiro, 2015).

The increased risk preeclampsia appear to be associated with 25(OH)D levels < 50nmol/L in pregnant women,, which is characterized by increased blood pressure and protein in the urine (Scholl et al., 2013). It is important to highlight that preeclampsia is responsible for one third of very premature births in France (between 6 and 7 months). It is also a cause of intrauterine growth retardation or Small for Gestational Age (SGA). Indeed, 25(OH)D concentrations between 25-37 nmol/L or lower appear to increase the risk of SGA (Burris et al., 2012; Gernand et al., 2013). Lowered plasma levels of VD during pregnancy (especially during the third trimester) appear to be associated with low birth weight (Horan et al., 2015), in association with an increased risk of SGA (Y. Chen et al., 2017; Yuan et al., 2015), decreased head circumference and body size (Miliku et al., 2016).

Milajerdi et al. in their review prospective cohort studies found a significant association between VDD and an increased risk of GDM (Milajerdi et al., 2021). Undoubtedly, the rising frequency of GDM is a result of the increasing rates of overweight and obesity in the general population (Ferrara et al., 2004), which puts both mothers and their newborns at risk for negative health effects (Kjos & Buchanan, 1999). Type 2 diabetes mellitus is more likely to develop in women with GDM. Congenital abnormalities, macrosomia, birth trauma, respiratory distress syndrome, jaundice, and hypoglycemia are more prevalent in children whose mothers have diabetes. Even though a number of GDM risk factors have been discovered (Ben-Haroush et al., 2004), including advanced maternal age, obesity, a family history of diabetes, and ethnicity (Bardenheier et al., 2013), the mechanism by which these risk factors predispose women to GDM is still being studied (Harlev & Wiznitzer, 2010). VDD has received more attention in recent years as a potential factor (Dror, 2011).

Furthermore, bone occurrences in newborns under one year of age are linked to concentrations of 43 nmol/L or less during pregnancy. In children, rickets develops when 25(OH)D levels are less than 30 nmol/L and calcium levels are appropriate. However, this link is not evident when 25(OH)D levels are greater than 50 nmol/L. Similarly, it does not appear that calcium absorption and 25(OH)D levels between 30 and 50 nmol/L are correlated.

VD supplementation should therefore be considered to ensure that the child has sufficient VD levels for their development. A meta-analysis showed (Gallo et al., 2020), that maternal dietary supplements daily dose of 400 to 120 000 IU of VD during pregnancy were associated with a significant increase in maternal 25(OH)D concentrations, but a daily dose of 4 000 to 120000 IU had no effect on the development of preeclampsia. During pregnancy a dose of 200 to 300 000 IU daily can decrease HOMA-IR, but not the plasma glucose. As for the infant the evidence showed that a daily maternal dose of 2 000 to 120 000 IU increase infant 25(OH)D concentrations, but do not affect the gestational age nor birth length but increases the infant birth weight.

In conclusion, maternal VD status is critical for the mother's health during pregnancy as well as for the health of the newborn.

4.4.2 Fetal programming and maternal vitamin D deficiency

What is DOHaD?

The “developmental origins of health and disease” (DOHaD) is a concept that has emerged over the past 50 years, linking the state of health and risk from disease in later childhood and adult life with the environmental conditions of the early life. In other words, the DOHaD corresponds to fetal programming. It was in the 1980s that epidemiological studies by Barker and his team first associated low birth weight (as an indicator of impaired fetal nutrition) and increased risk of developing type 2 diabetes (T2D) and hypertension (Barker & Osmond, 1986; Hales et al., 1991).

In addition to low birth weight, other conditions have been associated with a risk of developing a pathology in adulthood. For instance, high birth weight has been linked to an increased risk of obesity and impaired glucose tolerance in infants whose mothers have diabetes (Mccance et al., 1994) (type 1 and 2).

The term "early nutritional programming" is now widely used to refer to the impact of nutrition in its entirety on the outcome of offspring (Figure 31). This programming in humans

occurs throughout the periconceptual phase (from fertilization to blastocyst embryo stage) and lasts for the so-called 1000-day period (from conception to the second year of life).

Thus, several publications have suggested that rapid weight gain during the first months of life is a risk factor for developing obesity in the child (Botton et al., 2008; Druet et al., 2012; Stettler et al., 2003).

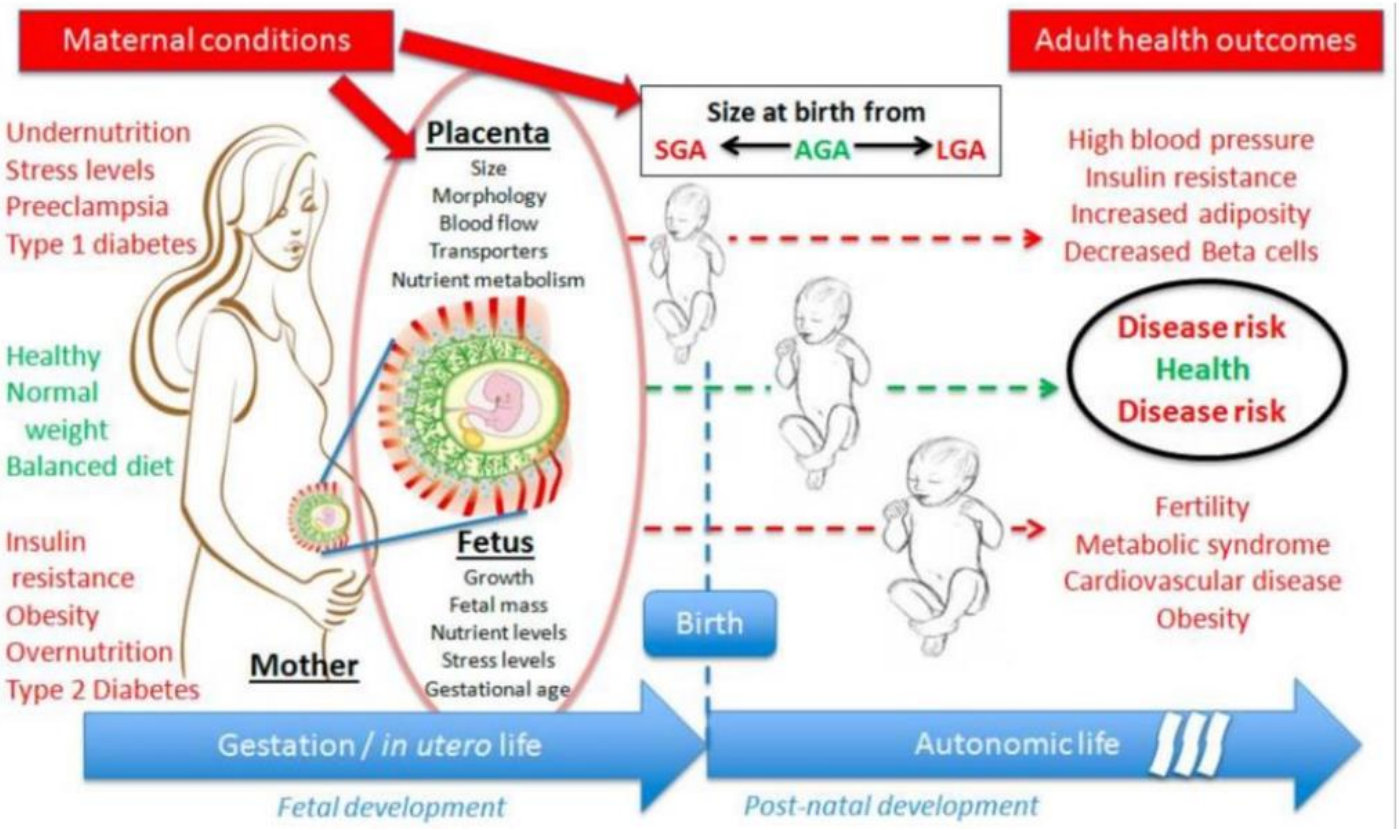

Figure 31: Schematic representation of the DOHaD concept in human.
The effects of several maternal conditions (non exhaustive) are shown during the gestation on feto-placental unit development (affected parameters), leading to particular birth size from SGA to LGA (small to large for gestational age), and the most observed outcomes in terms of health at adulthood.(Chavatte-Palmer et al., 2016).

Despite the accumulation of epidemiological studies, retrospective and prospective type, animal models have been required to validate the concept of early developmental and dietary programming.

These animal models include: *in utero* food restriction (Dahri et al., 1991; Garofano et al., 1999; Snoeck et al., 1990) (caloric or protein restriction), uteroplacental restriction (Alisi et al., 2011) and stress exposure (Moisan & Le Moal, 2012; Reinisch et al., 1978) (*e.g.*, glucocorticoids).

Reichetzeder et al. study included a clinical and preclinical trial, indicates the relevance of maternal micronutrition throughout pregnancy. It has been suggested that severe VDD in

women is linked to low birth weight and gestational growth retardation. Although there is no difference in birth weight in animals, mice from VDD females are smaller at 15 days, have a higher death rate before weaning, and have a glucose tolerance defect (Reichetzeder et al., 2014).

Epigenetics is the final aspect that has helped to solidify the concept of DOHaD. More epigenetic markers are established during the periconception period (Chavatte-Palmer et al., 2016). Indeed, the genomes inherited from parental genomes will be reprogrammed first, through demethylation of DNA and histones or acetylation of histones, to form the embryonic genome.

Furthermore, genome re-methylation (Chavatte-Palmer et al., 2016) occurs after fetal implantation. The parental environment can then alter these epigenetic markers, which will be inherited by offspring in several modes of transmission (Gabory et al., 2013) (Figure 32).

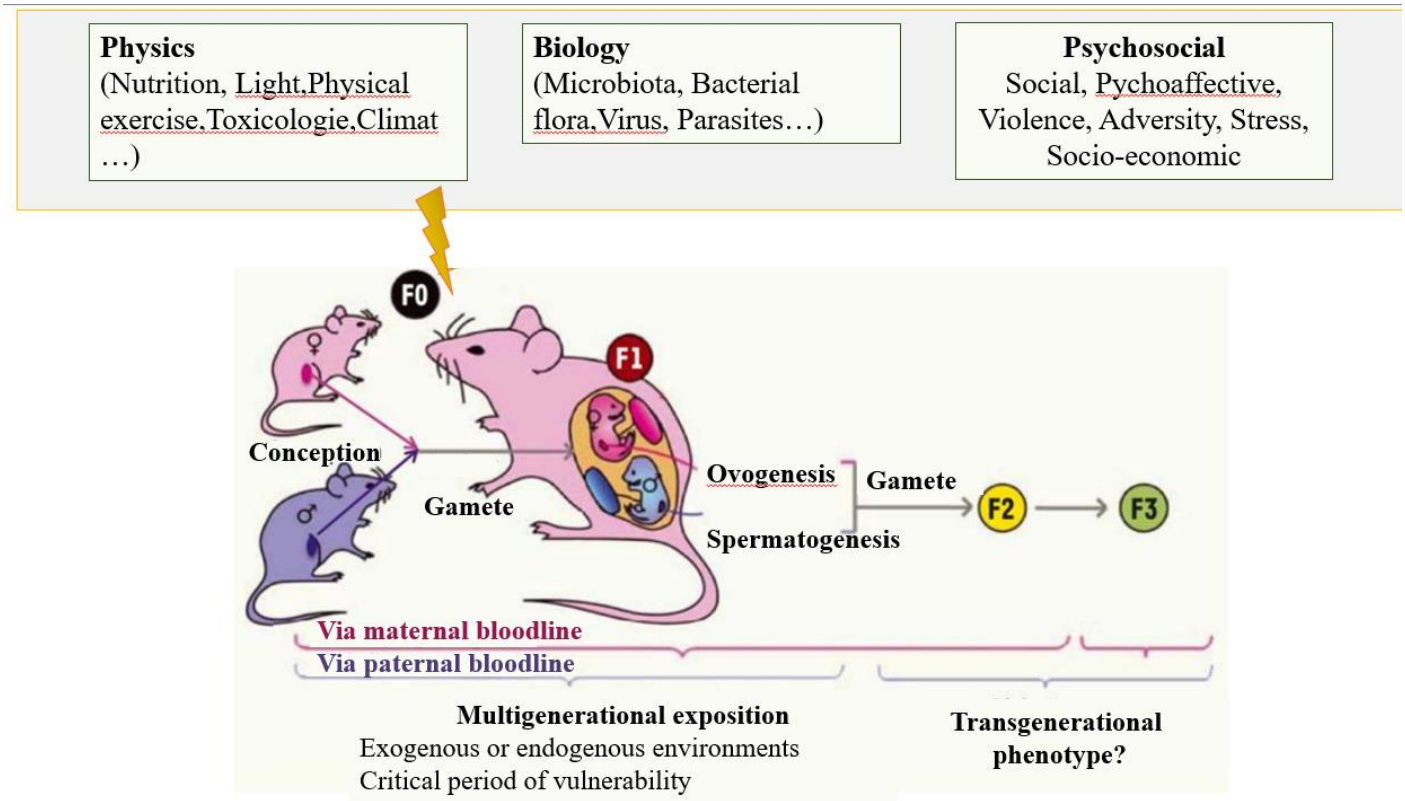

Figure 32: The multigenerational and transgenerational transmission of environmental exposures differs by parental gender (Junien et al., 2016).

Sex-specific transmission of exposure to environment to subsequent generations. Environmental factors, such as diet, psychosocial stress, pollutants, endocrine disruptors, tobacco, alcohol, and microbiota, have different effects on (F0) individual epigenetic landscapes hence gene pathways and networks in ways that differ between the sexes. For instance, preconceptional exposures to the mother and father can affect the quality of the gametes and be transmitted on to the next (F1) generation. Furthermore, sex-specific effects of maternal F0 (stress, metabolism, diet, hormonal changes) exposure during pregnancy can be passed from the maternal to the fetal compartment via the placenta and alter the formation of F1 tissue. The first generation's long-term health outcomes may shift as a result of programming somatic tissues. Furthermore, the environment of the F0 mother can influence primordial germ cells as they grow and undergo reprogramming during fetal development, passing down genetic and epigenetic information to the F2 generation. Paternal and maternal lineages have different effects on how these influences are passed down. In particular, multigenerational exposure on the maternal lineage affects the F0, F1 and F2 generations, while transgenerational phenotype is seen in the F3 generation. In contrast, multigenerational exposure on the paternal lineage affects the F0 and F1 generations, and transgenerational phenotype in F2 and F3 generations.

When it comes to maternal exposure, we talk about multigenerational transmission between generations (F0) to (F2), and paternal exposure between generations (F0) to (F1). Only the generation (F3) for a maternal lineage (F0) and for a paternal lineage (F2) shows transgenerational transmission, i.e. meiotic. Intergenerational effects are those that can be seen (Gabory et al., 2013; Junien et al., 2016).

Beyond the impact of the maternal environment, the concept of paternal origins of health and diseases (POHaD) has recently emerged and also highlights the impact of paternal and obesity on the health and development of the offspring. From studies in mice subjected to an obesity-

inducing diet have shown that paternal sperm miRNAs can be modified by this environmental change and induce epigenetic marks in the offspring (Dupont et al., 2019).

4.4.3 Maternal vitamin D deficiency and adipose tissue

In recent years, studies of large mother-child cohorts have suggested associations between maternal plasma 25(OH)D levels and various parameters characterizing obesity in children. Thus, low 25(OH)D levels during pregnancy are associated with: *i*) low percentages of fat mass at birth; *ii*) high percentages of fat mass in children aged 4 and 6 years (Boyle et al., 2017; Crozier et al., 2012); *iii*) lower percentages of lean mass in children aged 6 years and 9.5 years (Krishnaveni et al., 2011; Miliku et al., 2018); *iv*) higher IR (Krishnaveni et al., 2011). In a smaller cohort, low maternal 25(OH)D were associated with greater subcutaneous and visceral adiposity in the newborn (Tint et al., 2018). Finally, other human data associate maternal VD status with BMI, weight, and waist circumference of the child. Indeed, 25(OH)D concentrations during pregnancy are inversely correlated with BMI and waist circumference in children aged 4 and 6 years of age (Daraki et al., 2018), but also to an elevated risk of fetal and neonatal overweight (without finding such an association at age 4 years (Morales et al., 2015))

All these clinical studies thus tend to confirm the role of VD on developmental programming of obesity in offspring. However, long-term studies (until adulthood) have not yet been conducted and the early mechanisms involved in this programming have yet to be elucidated.

In rats, it was reported that maternal VDD induced before and during gestation appeared to promote the differentiation and proliferation of adipocytes and preadipocytes in VDD male offspring. This phenomenon seems to be associated with epigenetic changes (differential methylation of promoters and CpG islets), leading to an obese phenotype (increased body mass and adiposity) in offspring of VDD females (Wen et al., 2018). In mice, maternal VDD is associated with an obese phenotype in male offspring (higher body mass, adiposity, glucose intolerance) but were not observed in a second generation (Nascimento et al., 2013).In a transgenerational study, a maternal VDD induced by a VDD diet (5 weeks before mating until weaning) leads to disturbances in DNA methylation in somatic liver and germ cells (sperm) over two successive generations. These epigenetic changes were associated with differences in body weight and lean mass / fat mass ratio over the two generations (Xue et al., 2016). Indeed in a 6 weeks deficient VD diet, where the male offspring were fed a high fat diet, Li et al. have found that maternal VDD intake exacerbated the development of obesity in male offspring mice that were both obese and non-obese, as evidenced by larger adipose cells and abnormal glucose and lipid metabolisms, additionally, proinflammatory cytokine expression was elevated such as

TNF-α while anti-inflammatory cytokine expression was decreased in the maternal VDD, and they also observed a change in the immune cell profiles in WAT of both obese and non-obese male offspring (Pe et al., 2021). However, this association between maternal VDD and obese phenotype in the offspring is not systematically observed. For example, in a study conducted on male Sprague-Dawley rats from deficient mother in VD during gestation, no difference in body mass was reported. However, these animals showed persistent inflammation (H. Zhang et al., 2014) (including elevated concentrations of plasma and liver of IL-1β, IL-6, IL-8, TNF-α). Similarly, male mice from deficient mothers with intrauterine growth retardation and accelerated growth in early life, do not show higher mass in adulthood. Nevertheless, these animals are predisposed to develop adipocyte hypertrophy following to a HF diet (Belenchia et al., 2017). Interestingly, the persistently increased inflammation was explained by the continuously increased IKK expression related to methylation modifications (H. Zhang et al., 2014).

We recently reported that in juvenile males born to VDD mothers, a smaller body weight and higher energy expenditure was noted when compared with control group, while no discrepancy in body weight was observed in female (Seipelt et al., 2020), highlighted thus a strong metabolic sex-specific response which has never been pointed out before. Furthermore, we showed that challenging offspring with HF diet strongly increased adiposity index (AI) and IR in males born to VDD mice, which correlated with insulin resistance. At the opposite female born to VDD mice and subjected to HF diet displayed similar adiposity index and insulin sensitivity as female fed control diet.

All of these data therefore suggest that maternal VDD and environment in adulthood are parameters to be considered to study the establishment of the obese phenotype in the offspring. Moreover, this programming seems to depend on the models and diets used.

4.4.4 Maternal vitamin D deficiency and hepatic steatosis

There hasn't been much studies of how VD in the mother influences the development of HS in the offspring.

Current findings in mice showed that 6 weeks VDD in the mother affects the normal pattern of growth in the offspring, with metabolic changes leading to an increased BMI and fat pad in the F1 generation only, indicating that there is no diet-generation interaction (Sharma et al., 2017). In addition, structural and molecular modifications were observed in the liver and pancreas, (higher insulin secretion and TG levels). The liver displayed marked steatosis in the VDD (F1) generation but only a reduced steatosis in the (F2) generation. Indeed, in Lundy et

al. study they have found that a 5 weeks prenatal VDD modifies the gene expression profile of the liver and increases the incidence of histopathological abnormalities in the adult liver (Lundy et al., 2022).

Another study focused on the impact of 30 weeks VDD in offspring mice (Nascimento et al., 2013). Whereas serum TG levels were greater in VDD female offspring and severe steatosis was detected, male offspring displayed mild steatosis with fewer lipid metabolism abnormalities because TG levels of VDD male offspring were not significantly different.

There have been no clinical studies on the impact of maternal VDD on the offspring HS.

THESIS OBJECTIVE

In this introduction, we have discussed the importance of VD in the establishment of many mechanisms essential to the development of the organism and the maintenance of good health throughout life. Indeed, this vitamin affects a variety of organs. On the one hand, the link between vitamin D and AT, its main storage site, has been strongly documented and it appears that VDD is classically associated with obese phenotypes and AT inflammation. Obesity, on the other hand, can cause ectopic fat deposition, notably in the liver, causing HS, which is correlated with VDD. In addition, we have mentioned the maternal diet is being recognized as having a considerable impact on foetal development and metabolic programming in children. The prevalence of the number of pregnant women with inadequate levels of VD is important in our society today, as well as the high prevalence of obesity in the general population in children and adults, thus we decided to study the existing associations between mice maternal VDD and potential programming for inflammatory pathways in WAT and the lipid accumulation in the liver of the offspring, in obesogenic situation.

The goal of this study was to assess morphological, and biological changes in the adult offspring of a C57BL/6J mother mouse with a VDD in an obesity context.

We focused on the impact of VDD on the WAT inflammation according to the sex of the offspring (Article 1). In parallel, we studied maternal VDD in mice sex-dependently affects hepatic lipid accumulation in the offspring (Article 2).

MATERIALS AND METHODS

Animal experimentation (Article 1 & 2)

Male and female C57BL/6J mice (Janvier Lab, Le Genest-Saint-Isle, France) fed ad libitum during the 1-week acclimation period with control food (chow diet A04 from Safe-diets, Augy, France) and full access to drinking water. The animals had a controlled photoperiod of 12 h of light and 12 h of darkness and were maintained at a constant temperature of 22°C. Female mice (15 per group) were randomly assigned to one of two experimental groups: control (CTRL, AIN-93G with vitamin D3, 1.0 IU/g) or vitamin D deficient (VDD, AIN-93G without vitamin D3, 0.0 IU/g) for 8 weeks, and then were mated with the males. Body mass was measured once a week and food intake were measured at 3 weeks of diet (pre-mating), 5 days and 15 days of gestation (Figure 33).

After birth, the females were all fed the control diet (AIN-93G) until weaning of the offspring. Every week the mass of the animals was measured (from weaning to the end of the trial. Males and females offspring, at 6 weeks old were randomly assigned to receive a low fat diet (AIN-93M Maintenance Purified Diet) or a high fat diet (DIO Rodent Purified Diet with 45% Energy from Fat) for 8 weeks. At the end of the protocol, after fasted overnight, blood was collected from the mice by cardiac puncture. Serum was isolated by centrifugation at 3000 rpm for 15 min at 4°C and stored at -80°C. The animals were euthanized by cervical dislocation and the various tissues (liver, muscle, spleen, hypothalamus and white adipose tissue deposits) were collected, weighed and stored at -80°C. Eight groups (females and males) were formed: CTRL LF, CTRL HF, VDDLF, VDD HF.

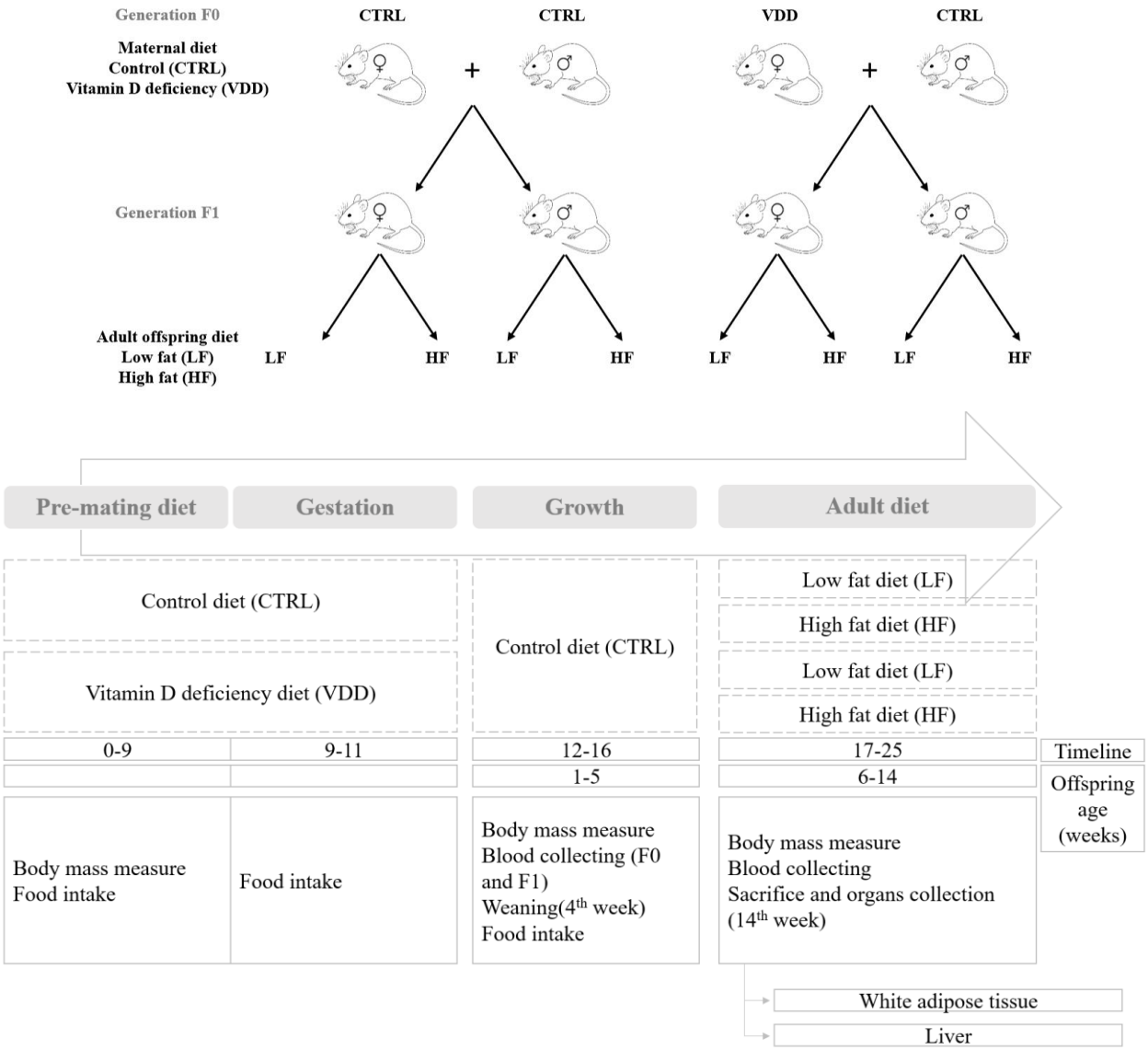

Figure 33: Summary diagram of the experimental protocols.

Total RNA extraction (Article 1 & 2)

For adipose tissue and liver: the extraction was performed by adding 1 mL of TRIzol Reagent (Invitrogen) to each samples. Samples were mixed with 200 µL of chloroform and centrifuged (13000 g, 15 min, 20°C). The upper phase containing RNA was recovered and mixed with 500 µL of isopropanol. After centrifugation (13000 g, 30 min, 4°C), the supernatant was discarded and the RNA pellet was washed with 600 µL of 80% ethanol. The samples were then centrifuged (10 min, 12000 g, 4°C) and the pellets were dried before being taken up in RNA/DNA free. The amount and purity of the extracted RNAs were measured spectrophotometrically (nanodrop).

Reverse transcription (Article 1 & 2)

For mRNA (Article 1& 2): the enzyme used was the M-MLV RT (Moloney Murine Leukemia Virus Reverse Transcriptase, Invitrogen). The reverse transcription was performed in a final volume of 20 µL. 1 µg of total RNA was added to a mixture containing 4 µL of 5X buffer, 2 µL of dithiotreitol (0.1 M), 2 µL of dNTP (5 mM), 1 µL of hexamers (0.3 µg/µL) and 1 µL of M-MLV RT (200 U/µL). The synthesis was performed at 37°C for 60 min then the resulting cDNAs were diluted five times.

For miRs (Article 1): reverse transcription was performed in a final volume of 20 µL. 250 ng of total RNA was added to a mixture containing 4 µL of miscript buffer Hispec Buffer, 2 µL of Nucleics Mix, and 2 µL of miScript reverse transcriptase (Qiagen) (qsp H2O). Synthesis was performed at 37°C for 60 min and followed by inactivation for 5 min at 95°C. The resulting cDNAs were diluted with 200 µL of free RNA/DNA water.

Real-Time Quantitative PCR (Article 1 & 2)

For mRNAs (Article 1 & 2): Amplification was performed with 4 µL of cDNA diluted to a final volume of 10 µL containing 5 µL of SYBR Green, 0.5 µL of each of the two sense and antisense primers. After 2 min at 50°C and then 10 min at 95°C, the amplification reaction was completed in 40 cycles including 2 steps: cDNA denaturation (15 sec at 95°C), primer hybridization (1 min at 60°C). Results were expressed relative to 18S ribosomal RNA. Real-time PCR was performed using the Mx3005P Real-Time PCR System.

For miRs (Article 1): We used the miScript PCR arrays kit (Qiagen, Coutraboeuf, France). Reactions were performed in a volume of 12.5 µL including 6.5 µL of 2X QuantiTect SYBR Green PCR Master Mix (Qiagen, Coutraboeuf, France), 1.25 µL of 10X miScript Universal Primer (Qiagen, Coutraboeuf, France), in the presence of 250 ng of total RNA. After an initial incubation step of 15 min at 95°C, the amplification reaction was performed over 40 cycles, comprising 3 steps (95°C, 15 s; 55°C, 30 s and 70°C, 30 s). For each condition, expression was quantified from 5 biological replicates and SNORD6, RNU6-6P were used as endogenous controls in the comparative threshold cycle (Ct) method (Livak and Schmittgen 2001).

Gene Ontology (GO) And Ingenuity Pathway Analysis (IPA) (Article 1)

Differential expression data were filtered using the following parameters: *padj* <0.01 and fold change (FC) FC>1.5 or FC<-0.66. Those gene lists were implemented in Gene Ontology (GO) and Ingenuity Pathway Analysis (IPA) software. Pathways were manually curated to retain only the inflammatory pathways differentially impacted by maternal diet.

miRNA Target Prediction and Pathway Analysis (Article 1)

Targetscan (gene were selected with an Aggregate PC 90 %) and mirDB (gene were selected with a target score 80 %) were used to identify inflammatory miRNA and create a predicted mRNA target list. To identify biological processes connected with inflammation-related pathways in white adipose tissue, the shared gene list was subjected to analysis method with IPA. Quantitative analysis was carried out.

NF-κB Activation and p38/MAPK Activation (Article 1)

Using the ELISA Instant One kit for adipose tissue homogenates (eBiosciences SAS, Paris, France), the levels of phosphorylation of NFkB p65 (Phospho) [pS536] and NFkB p65 (Total) were determined according to the manufacturer's instructions. The activation of NFkB p65 (Phospho/Total) were used to compare pathway activation in the various groups.

Using the ELISA Instant One kit (eBiosciences SAS, Paris, France), the levels of phosphorylation of p38 MAPK (Phospho) [pT180/pY182 and p38 MAPK (Total) were evaluated according to the manufacturer's instructions. The activation of P38 (Phospho/Total) were used to compare pathway activation in the various groups.

Liver fixation and histology (Article 2)

Liver tissue were fixed with 4% paraformaldehyde overnight at 4°C under overnight at 4°C with agitation, washed in 1X PBS, then dehydrated with 50%, 90%, 100% ethanol (respectively 3 times 1 h) and xylene (2 times 1 h), then embedded in paraffin (3 baths of 1 h at 60°C) in molds. Subsequently, each core was cut at 5µm (Microtome, Leica) and stained in Eosin-Hematoxylin (Harris Hematoxylin Solution Modified, Sigma), according to the following protocol: 2 min hematoxylin, 30 s differentiation solution, H2O rinse, 2 min eosin, 1 min ethanol 70%, 1 min ethanol 95%, 3 baths of 3 min in xylene. Then, the slides are mounted with a mounting medium containing xylene (Eukitt Quick hardening, Sigma). The images were captured by a light microscope (Leica, Germany; 10X magnification). The number of nucleus per area (mm^2) was calculated using (Fiji).

Protein extraction and quantification (Article 2)

First, a small piece of liver is grinded in 2% SDS for one minute at 23Hz.The homogenate is then separated in a new tube and centrifuged for 10 minutes at 4°C at 12700 rpm. In a separate tube, the aqueous phase is collected (tube number 3). Secondly, a detergent-compatible bicinchoninic acid (BCA)-based formulation for colorimetric detection and quantification of total protein is used in the BCA Protein Assay Kit. Working BCA solutions were made by

combining 50 parts of reagent A (BCA, sodium carbonate, sodium bicarbonate, bicinchoninic acid, and sodium tartrate in 0.1 M sodium hydroxide) with 1 part of reagent B (CuSO4, 4 percent) as directed by the manufacturer. As a standard range, 10 µL of 2 µg/µL BCA working solutions were pipetted into the wells of a 96-well plate, followed by 10 liters of samples (the extracted protein stated earlier) and 200 µL of BCA (standard range and samples).

Before reading, the wells were swiftly placed in the reader and gently shaken for 3 minutes in a smooth motion, and the plates were gently incubated for 30 minutes at 37°C. The microplates' absorbance at 562 nm was monitored.

TG extraction and quantification (Article 2)

To extract triglycerides (TG), 1mL 10% NP40 were added to almost 40 mg of liver cut in small pieces, and grinded for one minute at 30Hz. The mixture is incubated in a thermomixer for 2 min at 80°C at maximal speed. TG were quantified in the liver using colorimetric methods (BIOLABO, Maizy, France). Briefly, in a 96 well plate, 10 µL of samples were homogenized with a 200 µL of mixture of enzymes and tampon. Before reading, the wells were left at room temperature for 10 minutes at room temperature. The microplates absorbance at 490 nm was monitored.

Lipids extraction (Article 2)

For high-throughput lipidomics, methyl-tert-butyl ether was used for lipid extraction, as detailed in Journal of Lipid Research Volume 49, 2008 (Matyash et al., 2008). 20 mg of liver were chopped into little pieces before extraction, and 200 µL of PBS and 10 µL of standard solution were mixed together.

The tissue was treated with Avanti Polar lipids, Splash. After 10 min of vortex at 1500 rpm, 1.5 mL of methanol and 5 mL of methyl-terbutyl ether (MTBE) were added to each sample and vortexed for 1 hour.

After homogenization, each tube was filled with 1.25 mL deionized water and incubated for 10 minutes at room temperature. After centrifugation for 10 minutes at 1000 rpm at 10°C, the upper organic phase was collected in another tube.

The bottom phase was treated with 2 mL of a methyl-terbutyl ether/methanol/water combination (10:3:2.5 v/v/v) and centrifuged as stated previously for a second extraction. The upper organic phase was combined with the first one formed in the previous step. After evaporating the solvent with a nitrogen stream, the dry lipid extract was weighted and stored at

-80°C until processing. The lipid extracts were then resuspended in 200 µL of chloroform, methanol, and distilled water (60:30:4.5 v/v/v).

Lipid chromatography (Article 2)

The UHPLC separation was performed on a DionexUltiMate 3000 device (Thermo Fisher Scientific, Courtaboeuf, France) with an Accucore C18 column (1502.1 mm, 2.6 m) on a DionexUltiMate 3000 device (Thermo Fisher Scientific, Courtaboeuf, France).

The temperature in the column was kept at 45°C. 10 mmol/L ammonium formate in 60% acetonitrile with 0.1% formic acid was used in mobile phase A, while 10 mmol/L ammonium formate in acetonitrile: propan-2-ol (1:9, v/v) with 0.1% formic acid was used in mobile phase D. The flow rate was 0.4 milliliters per minute. The elution gradient was as follows: 35% D at the start, 35% to 60% D for 4 minutes, 60% to 85% B for 8 minutes, 85% to 100% B for 9 minutes, 100% B for 3 minutes, and 35% B for 4 minutes.

The injection had a capacity of 2 µL. The samples were randomly arranged on the injection table, and quality control samples made up of a pool of each sample or a solvent for the blank were interspersed (1 of 5) among them.

Mass Spectrometry (Article 2)

The mass spectrometry (MS) spectra of the tested samples were assimilated using a Q-Exactive Plus (Thermo Fisher) spectrometer with electrospray ionization in positive and negative modes and a full scan (m/z 250 to 1200) utilizing Breitkopf et al. methodology (Breitkopf et al., 2017). The drying temperature was set to 285°C and the capillary voltage was set to 3000 V. With a 200 m injection duration, the orbitrap mass analyzer's resolution was set to 4 scans/s at 35 000 resolution. Full MS spectra were obtained for each sample. Data-Driven Analysis The top 15 MS/MS were used to acquire the MS/MS spectra, and performed in one-tenth of the samples. Each sample was captured in both positive and negative ionization modes individually to acquire as much structural information as possible. This method detects the most prevalent ionic species in the primarily obtained complete MS spectra. Increased collision-induced dissociation fragments these 15 ionic species in the next 15 scans, completing the cycle with the last MSMS. A fresh cycle begins with a complete MS scan followed by 15 MS/MS scans for the 15 ionic species that follow.

Lipid data treatment (Article 2)

The generated spectra were visually evaluated to adjust the signal intensity after the data of the molecular ions corresponding to the metabolites were acquired. The three-dimensional raw

data from the liquid chromatography-MS analysis (m/z, retention time, and ion intensity) were deconvolved into a composite matrix of chromatographic peaks aligned in time and a mass-to-charge ratio (m/z) with the intensity of the associated ions. The open-source software XCMS software(Aidoud et al., 2018). was used to process the data. The centWave method was used to detect the peaks. The Obiwarp method was used to align peaks between samples, and the density approach was used to aggregate peaks. After obtaining the raw data matrix, numerous filters were used to remove analytical background and correct analytical drift. Spectra were utilized to establish a database using Lipid Search software (v4.0, Thermofischer scientific): based on the MS/MS spectra obtained in the samples, an in-silico database was built using Lipid Search software. The annotations of various fragmented ions were produced based on the m/z ratio of the parent ions and the fragments created, and this theoretical fragmentation model correlates to the MS/MS spectra of the tissue extracts. The corresponding lipid species could be identified in the majority of the signals.

To identify the lipid species and their intensities in each sample, the m/z and retention time pairs obtained with LipidSearch were compared to those obtained with XCMS using the data inhouse tool of the Galaxy Workflow4Metabolomics online program(Giacomoni et al., 2015). This final database was subjected to a final filter, which included factors such as relative SD, duplication in both modes, and the selection of the most stable and intense peak for each lipid, among others (Che Et Co: Cholesteryl Ester Coenzyme, DG: Diacylglycerol; LPC: Lysophosphatidylcholine, LPE: Lysophosphatidylethanolamine, PC: Phosphatidylcholine, PE: Phosphatidylethanolamine, PG: Phosphatidylglycerol; PI: Phosphatidylinositol, PS: Phosphatidylserine and TG: Triglyceride).

Statistical analyses (Article 1 & 2)

All data are expressed as mean ± SEM. With GraphPad Prism significant differences were determined by Student's t test or ANOVA followed by Fisher's LSD post hoc test. A $p<0.05$ was considered statistically significant (Article 1 & 2).

MetaboAnalyst (http://www.metaboanalyst.ca/) were used to perform principal component analysis, Partial Least Squares Discriminant Analysis (PLS-DA), 2D scores plot, cross validation, important features; the variable importance in projection (VIP) of auto scaled results PLS-DA validation with permutation (2000 repetitions) test $p<0.05$ PLS-DA was chosen to search for factors influencing the separation of two groups, R2 was used the goodness and Q2 for predictive ability. For the validation of the PLSDA model; R2/Q2 needs to be above 0.7 as an indicator of good predictive ability. (Article 2).

RESULTS

Article 1: Maternal vitamin D deficiency in mice increases white adipose tissue inflammation in offspring.

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Published in Cells June 2022

Submitted 2022

VD is an essential micronutrient whose pleiotropic action is exerted in various tissues and is suspected to play a major role in several physiological processes, including the control of AT metabolism. Thus, VDD, defined for a threshold value of 25-hydroxyvitamin (25(OH)D) < 50nmol/L, has become a worldwide public health including women of childbearing age, pregnant and lactating women. Using a mouse model, we wanted to evaluate the impact of maternal VDD associated with a normal nutritional context (LF) and obesogenic (HF, rich in lipids at 45% for 8 weeks) in the adult offspring (12 - 14 weeks).

In order to study the inflammatory parameters, in adult males and females, a transcriptomic (both mRNA and miRNA) approach was conducted on visceral AT together with an analysis of the activation of NFκB and p38 through their phosphorylation level.

Thus, we demonstrated that in males, a high-fat diet combined with maternal VDD increased inflammation-related RNA and miRNA expression, resulting in an over-representation of inflammatory pathways. Interestingly, activation of the NFκB signaling pathway and obesity index were linked by genomic and epigenetic profiles. In contrast to males, there was no significant modification of inflammatory pathways in females born from deficient mothers in vitamin D.

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Maternal Vitamin D Deficiency in Mice Increases White Adipose Tissue Inflammation in Offspring

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Citation: Haroun, N.; Bennour, I.; Seipelt, E.; Astier, J.; Couturier, C.; Mounien, L.; Landrier, J.-F. Maternal Vitamin D Deficiency in Mice Increases White Adipose Tissue Inflammation in Offspring. *Cells* **2022**, *11*, 2024. https://doi.org/10.3390/cells11132024

Academic Editors: Maria Rosa Ciriolo, Fabio Ciccarone and Say Viengchareun

Received: 20 April 2022
Accepted: 23 June 2022
Published: 25 June 2022

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Abstract: Vitamin D is acknowledged to play an important biological and metabolic role in adipose tissue, which is also the main storage site for this vitamin. Its anti-inflammatory effect in adipocytes and adipose tissue has notably been highlighted in adult mice. This vitamin is also crucial during fetal development since maternal vitamin D deficiency is suspected to program future metabolic disorders. Based on these observations, the aim of this study was to evaluate the consequences of maternal vitamin D deficiency (VDD) on white adipose tissue inflammation in adult offspring fed with normal or obesogenic diet (high-fat diet). White adipose tissue morphology, RNA and miRNA expression profiles, and signaling pathways were studied in adult males and females. In males, a HF diet coupled with maternal VDD increased expression of RNA and miRNA linked to inflammation leading to over-representation of inflammatory pathways. Interestingly, genomic and epigenetic profiles were associated with activation of the NF-kB signaling pathway and adiposity index. In females, no major modulation of inflammatory pathways was observed under VDD, contrarily to males. We concluded that maternal VDD coupled with HF diet activated inflammatory pathway in adipose tissue of the offspring, in a sex-dependent manner. Such activation is strongly related to activation of NF-kB signaling and increased adiposity only in males.

Keywords: vitamin D; maternal vitamin D deficiency; offspring; white adipose tissue; inflammation; NF-kB; p38

1. Introduction

Vitamin D (VD) deficiency (VDD), defined by 25-hydroxy vitamin D (25(OH)D) plasma levels below 50 nmol/L has become a worldwide public health problem that affects in particular women of childbearing age, pregnant women, or those breastfeeding [1]. This vitamin, which can be obtained from food or produced endogenously [2], is thought to play an essential function in fetal development but also throughout the life. In pregnant women, VDD is associated with an increased risk of pre-eclampsia [3]. The VDD is associated with a delay of intrauterine growth or SGA (small gestational age) [4], a decrease in head circumference [5] and body size, and low birth weight in the newborn [6]. In 4–6-year-old children born of VDD women, an increase in body fat [7,8], BMI (body Mass Index) and waist circumference has been observed [9]. In children aged 6 and 9.5 years, lean body mass percentages decrease [5], whereas insulin resistance increases [10]. In agreement, we recently showed in mice that maternal VDD induced low body mass in juvenile males, which was related with an increase in energy expenditure, but not in females [11]. In adults submitted to a normal diet, maternal VDD did not massively affect body mass, adiposity index, or insulin resistance in both males and females. Nevertheless, under high fat diet, we observed a rise in adiposity and insulin resistance in males, but not in females. Such bservation strongly, together with others [12–15], supports an impact of VDD on adipose tissue physiology in perinatal context [16].

Of note, the relationship between vitamin D and adipose tissue physiology has been already demonstrated in adulthood, where VD anti-inflammatory effects in adipocyte and adipose tissue have been demonstrated in the context of obesity [16,17]. Indeed, obesity is classically associated with a low-grade inflammatory status in adipose tissue, resulting notably from the activation of several inflammatory signaling pathways including NF-B and p38/mapk [18]. The activation of these pathways contributes to the production of pro-inflammatory cytokines, chemokines, and miRNA synthesis in adipocytes but also in stroma vascular fraction, contributing to leucocyte infiltration in the adipose tissue [18–20]. Interestingly, we and others have demonstrated an anti-inflammatory effect of vitamin D on adipocytes, involving an inhibition of NF-B signaling pathway and p38 protein phosphorylation and leading to a significant decrease in cytokines, chemokines, and miRNA expression and release [21–26].

Altogether these data show a strong inhibitory effect of vitamin D and adipose tissue inflammation in adults, no data are presently available about the programming effect of maternal VDD on adipose tissue inflammation of the offspring. Therefore, the aim of the present study was to determine the impact of maternal VDD in mice (25(OH)D) plasma levels below 10 ng/mL) combined or not with an obesogenic environment on the sex-specific response of inflammation of white adipose tissue in adult offspring.

2. Material and Methods

2.1. Animal Experiments

The protocol was approved from an ethical point of view by the Aix-Marseille University Ethics Committee and the French Ministry of Research (APAFIS#1300-2015072112279135).

Thirty female and ten male C57BL/6JRJ mice were obtained from Janvier Labs (Le Genest-Saint-Isle, France), fed ad libitum during the 1-week acclimation period with control food (chow diet A04 from Safe-diets, Augy, France) and given full access to drinking water. The animals were kept at 22 °C with a 12 h light/12 h dark cycle and a humidity level of 20%. Female mice (15 per group) were mated with males (5 per female group) after being randomly allocated to one of two experimental groups based on the diet: control (AIN-93G with vitamin D3, 1.0 IU/g) or vitamin D-depleted (AIN-93G without vitamin D3, 0.0 IU/g) for 8 weeks, as previously described [11]. After delivery, all females were fed control diet (AIN-93G) until the offspring were weaned. The females' litter size was reduced to six pups. To avoid mother cannibalism and perinatal stress, the offspring's body weight was measured weekly from the time they were weaned until the end of the trial. Males and females of the offspring were randomly assigned to receive either a Low-Fat diet (AIN-93M Maintenance Purified Diet) or a High-Fat diet (DIO Rodent Purified Diet w/45 percent energy from fat) for 8 weeks at 6 weeks of age. The impact of maternal diet (CTRL vs. VDD) and adult diet on offspring mice (males and females) was investigated using eight groups of mice (males and females) (LF vs. HF).

Overnight fasted mice were euthanised by cervical dislocation, and tissue samples were taken, weighed, and kept at −80 °C.

2.2. RNA Extraction Real Time PCR and RNA Sequencing

TRIzol reagent (Thermo Fischer Scientific, Les Ulis, France) was used to extract total RNA from retroperitoneal adipose tissue as previously described [27,28]. Total RNA from three mice per group were used to prepare an RNA-seq library using the Illumina TruSeq Stranded mRNA kit. On the Illumina NextSeq 500 sequencer, libraries were sequenced paired-end. Sickle was used to eliminate reads having a phred score of less than 20 and a length of less than 25 bp (v1.33). MultiQC was used to assess the quality of the trim reads (v1.0). Trim readings were aligned with the STAR aligner (v2.7.0d) with the "outFilterMismatchNoverLmax" and "outFilter-MultimapNmax" options set to 0.08 and 1, respectively. Cufflinks (v2.2.1) was used to find transcripts, with the "library-type" option set to fr-firstrand and a GTF file obtained from GENCODE ("Comprehensive gene annotation", vM1) serving as the genomic annotation. Cuffmerge was used to integrate the GTF files created by Cufflinks for each sample.

Unknown transcripts (class code "u") were identified using the "class code" supplied by Cuffmerge to each transcript. For further analysis, only de novo transcripts with counts greater than 0 in at least one RNA-seq sample were preserved. These newly created transcripts were joined with the GENCODE GTF file to create the final genomic annotation, which was then quantified using FeatureCounts (v1.6.1). DESEQ2 was used to compare gene expression patterns between conditions. Reads from Watson and Crick strands were picked using SAMtools (v1.9) and sent into the RseQC program suite's bam2wig.py script to build bigwig files (v2.6.4). The IGV genome browser was used to visualise RNA-seq profiles. RNA-seq data are available at GEO (accession number: GSE206372).

2.3. Gene Ontology (GO) and Ingenuity Pathway Analysis (IPA)

Differential expression data were filtered using the following parameters: $padj < 0.01$ (*p*-value corrected for multiple tests using Benjamini and Hochberg method) and fold change (FC) FC > 1.5 or <−0.66. Those gene lists were implemented in Gene Ontology (GO) and Ingenuity Pathway Analysis (IPA) software to highlight metabolic pathways differentially impacted by the maternal diet and subsequent LF/HF diet. Pathways were manually curated to retain only those related to inflammation in our study. Finally, quantitative analyses were performed to

characterise the influence of the maternal diet and/or the adult diet in inflammation pathways in the offspring.

2.4. Evaluation of miRNA Expression

The miScript PCR array (Qiagen, Coutraboeuf, France) was used for miRNA expression in white adipose tissue as previously described [25]. Reactions were performed in a volume of 12.5 µL containing 6.5 µL of 2× QuantiTect SYBR Green PCR Master Mix (Qiagen, Coutraboeuf, France), 1.25 µL of 10× miScript Universal Primer (Qiagen, Coutraboeuf, France), in the presence of 250 ng of total RNA. After an initial incubation step of 15 min at 95 °C, the amplification reaction was performed over 40 cycles, comprising 3 steps (95 °C, 15 s; 55 °C, 30 s and 70 °C, 30 s). For each condition, expression was quantified from 5 biological replicates; replicates and SNORD68, RNU6-6P were used as endogenous controls in the comparative threshold cycle (Ct) method [29].

2.5. miRNA Target Prediction and Pathway Analysis

Inflammatory miRNA was selected and predicted mRNA target list was established using Targetscan (gene were selected with an Aggregate PC ≥ 90%) and mirDB (gene were selected with a target score ≥80%). The common gene list was subjected to computational analysis with IPA to identify biological processes associated with the miRNA inflammationrelated pathways in the white adipose tissue. Quantitative analyses were conducted to characterise the influence of the maternal diet and/or the adult diet in inflammation pathways in the offspring.

2.6. NF-κB Activation and p38/MAPK Activation

Retroperitoneal adipose tissue homogenates were prepared as previously described [30]. The levels of phosphorylation of NFkB p65 (Phospho) (pS536) and NFkB p65 (Total) were measured using the ELISA Instant One kit (eBiosciences SAS, Paris, France) according to the manufacturer's instructions. NFkB p65 (Phospho/Total) werwase used to compare pathway activation in the various groups. The levels of phosphorylation of p38 MAPK (Phospho) [pT180/pY182 and p38 MAPK (Total) were measured using the ELISA Instant One kit (eBiosciences SAS, Paris, France) according to the manufacturer's instructions. P38 (Phospho/Total) was used to compare pathway activation in the various groups.

2.7. Statistical Analysis

The data are presented as mean ± SEM. GraphPad Prism (version 9.3.1, GraphPad software LLC, San Diego, CA, USA)) was used to assess significant differences using an ANOVA followed by the Fisher's LSD post hoc test. A statistically significant value of $p < 0.05$ was used.

3. Results

3.1. HF Diet and Maternal VDD Impact Body Weight and Adiposity Index

After euthanasia, CTRL LF and VDD LF males showed similar body weight; similarly, CTRL HF and VDD HF diet had equivalent body weight. A significant difference between the LF (both CRTL and VDD) and HF groups (both CTRL and VDD) (Figure 1A) was observed, with higher body weight in HF-fed groups (Figure 1A). For females, only CTRL HF had higher body weight (Figure 1C) compared with other groups (CTRL LF, VDD LF and VDD HF). Concerning the adiposity index, VDD HF males presented the highest adiposity index (Figure 1B) compared with the three other groups (CTRL LF, CTRL HF, and VDD LF males). The females CTRL HF showed highest adiposity index (Figure 1D) compared with other groups (CTRL LF, VDD LF, VDD HF females).

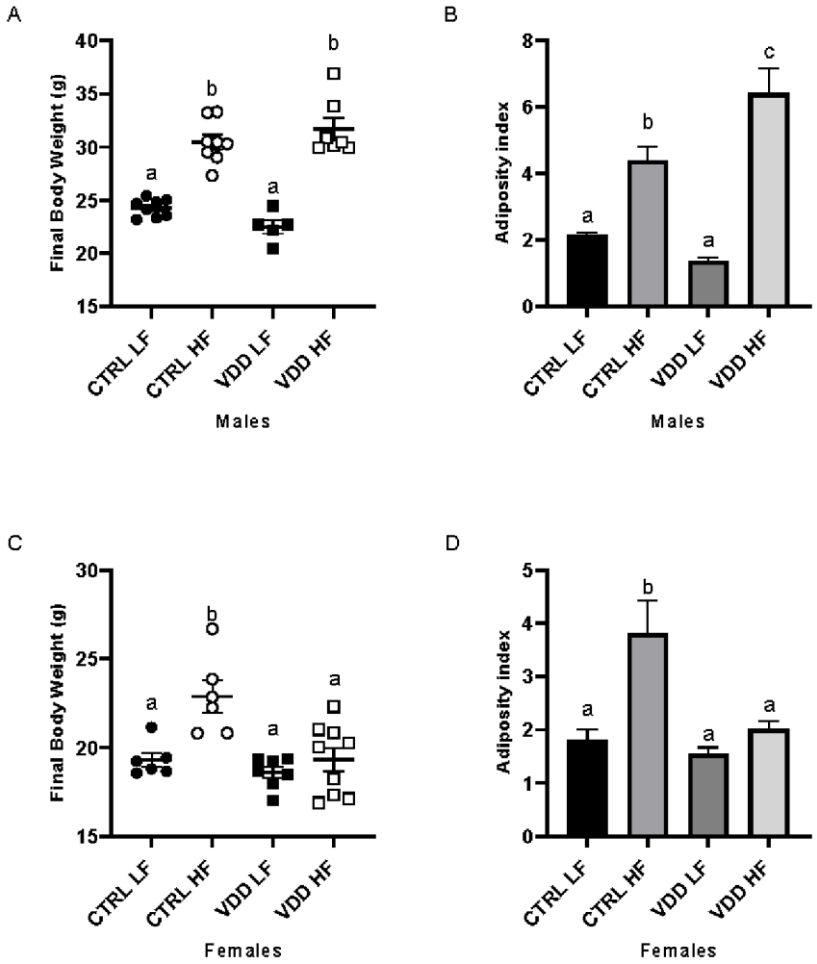

Figure 1. Body weight and adiposity index in males and females offspring. Body weight measured at the protocol end for the males and females offspring (**A**,**C**). Adiposity index of the offspring has been established for males and females (**B**,**D**). Values are presented as mean ± SEM. Bars not sharing the same letter were significantly different in Fisher's LSD post hoc test. $p < 0.05$.

3.2. HF Diet and Maternal VDD Regulate Inflammation Pathways

In order to highlight the inflammatory response in the WAT, RNA-seq analyses were set up in order to study the expression profile of the genes in the WAT. Using the significant regulated genes (Table 1), two sets of data were employed. The first set looked at the effect of maternal VDD in the same diet conditions (i.e., CTRL-LF vs. VDD-LF and CTRL-HF vs. VDD-HF). The second data set evaluated the effects of a HF diet on the same type of maternal diet (CTRL-LF vs. CTRL-HF and VDD-LF vs. VDD-HF).

Table 1. The total number of differentially expressed mRNA genes, including up-regulated and downregulated genes, in both males and females, was determined using data filtering with *padj* < 0.01 and FC > 1.5 or <−0.66.

	Total mRNA Differentially Expressed	Up-Regulated	Down-Regulated
Males			
CTRL LF vs. VDD LF	348	146	20
CTL HF vs. VDD HF	907	650	257
CTRL LF vs. CTRL HF	1065	581	484
VDD LF vs. VDD HF	2406	1315	1091
Females			
CTRL LF vs. VDD LF	1323	820	503
CTL HF vs. VDD HF	1451	719	732
CTRL LF vs. CTRL HF	2234	1250	984
VDD LF vs. VDD HF	363	185	178

In a first set of analysis, we investigated the impact of maternal VDD. No pathway related to inflammation in both conditions, LF and HF diet (CTRL LF vs. VDD LF males and in CTRL HF vs. VDD HF males) were highlighted using GO analysis in males (Figure 2A). Using IPA, we selected inflammatory canonical pathways with determinant z-score. We highlighted 6 differentially expressed canonical pathways between CTRL LF and VDD LF males (Supplementary Table S1), and 15 differentially expressed canonical pathways between CTRL HF and VDD HF males (Supplementary Table S2). Three common pathways were found (Figure 2B). In females, in both conditions CTRL LF vs. VDD LF and in CTRL HF vs. VDD HF, using GO analysis, no pathway that triggers the inflammation in the LF situation and two inflammatory pathways in the HF diet were identified (Figure 2C;Supplementary Table S3). Using IPA, 5 differentially expressed canonical pathways between CTRL LF and VDD LF females (Supplementary Table S4), and 8 differentially expressed canonical pathways between CTRL HF and VDD HF females were identified (Figure 2D, Supplementary Table S5). Five pathways were found in common between the CTRL LF vs.CTRL HF and VDD LF vs. VDD HF.

Then, we analysed the impact of a HF diet on the same maternal vitamin D status background (CTRL or VDD). In males, 2 pathways related to inflammation were observed in CTRL LF vs. CTRL HF (Supplementary Table S6) and 22 pathways in VDD LF vs. VDD HF

(Figure 2A, Supplementary Table S7). In these 2 analyses, the "response to cytokines" pathway was induced, but the upregulation in VDD HF vs. VDD LF condition was higher compared with the CTRL HF vs. CTRL LF condition as revealed by the number of genes involved and the associated *p*-value (120 genes vs. 67 genes, and 6.95×10^{-8} *p*-value vs. 9.48×10^{-4} *p*-value, respectively). The same analyses were performed using IPA software and similar results were obtained. Namely, when comparing CTRL LF vs. CTRL HF, 6 differentially expressed canonical pathways were highlighted (Supplementary Table S8) and 28 differentially expressed canonical pathways between VDD LF and VDD HF males (Supplementary Table S9). Four pathways were found in common between the CTRL LF vs. CTRL HF and VDD LF vs. VDD HF (Figure 2B). In females, GO analysis revealed 11 pathways related to inflammation in CTRL LF vs. CTRL HF (Supplementary Table S10) and none in VDD LF vs. VDD HF females (Figure 2C). Using IPA, 11 differentially expressed canonical pathways between CTRL LF and CTRL HF females (Supplementary Table S11) and 2 canonical pathways between VDD LF and VDD HF females were observed (Supplementary Table S12). No common pathway was found between the two groups (Figure 2D). These gene lists were used in Gene Ontology (GO) and Ingenuity Pathway Analysis (IPA) tools to identify metabolic pathways that were affected differently by the maternal diet and the subsequent LF/HF diet. Pathways were manually filtered to keep those connected to inflammation. Finally, quantitative analyses were performed to determine the impact of the maternal and/or adult diets on inflammation pathways in offspring.

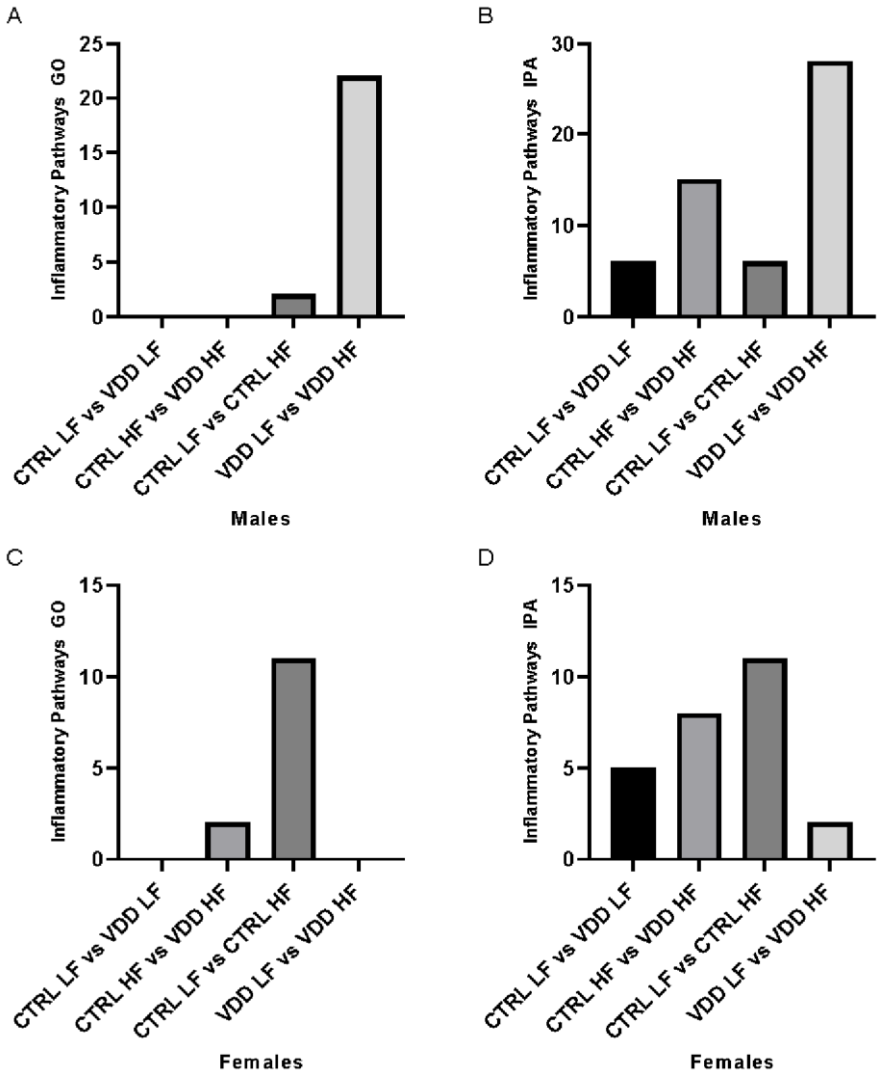

Figure 2. Inflammatory pathways linked to RNA profile in adipose tissue of the offspring. Quantitative representation of inflammatory pathways resulting in differential gene expression in retroperitoneal adipose tissue of the male offspring (**A**,**B**) and female offspring (**C**,**D**) using Gene Ontology (GO) and Ingenuity Pathway Analysis (IPA). Two sets of data were employed. The first is CTRL LF vs. VDD LF and CTRL HF vs. VDD HF. The second data is CTRL LF vs. CTRL HF and VDD LF vs. VDD HF. The genes were originally chosen based on the following criteria: *padj* < 0.01 and fold change FC > 1.5 or −0.66.

3.3. HF Diet and Maternal VDD Regulate miRNA Expression and Related Inflammatory Pathways

To go further in the identification of inflammation process, we investigated the effect of maternal VDD and/or HF diet on inflammation-related miRNA expression (Table 2, Supplementary Table S13) and related inflammatory pathways using IPA. The effect of maternal VDD was analysed in LF and HF conditions. In males, 5

pathways were obtained between CTRL LF and VDD LF (Supplementary Table S14) and no pathways were found in CTRL HF vs. VDD HF (Figure 3A). In females, the comparison CTRL LF vs. VDD LF females identified 16 inflammatory pathways (Supplementary Table S15) and 16 inflammatory pathways in CTRL HF vs. VDD HF (Figure 3B, Supplementary Table S16). Seven of these pathways were discovered to be common. The same methodology was implemented to investigate the effects of HF diet on the same type of maternal regimen. In IPA, the gene list revealed no pathways in CTRL LF vs. CTRL HF and 7 in VDD LF vs. VDD HF in males (Figure 3A, Supplementary Table S17). In females, 19 inflammatory pathway responses in CTRL LF vs. CTRL HF (Supplementary Table S18) and 10 inflammatory pathways responses in VDD LF vs. VDD HF were observed (Figure 3B, Supplementary Table S19), among which 6 were common.

Table 2. The total number of differentially expressed miRNA that enters White adipose tissue inflammatory pathways, including up-regulated and down-regulated miRNA, in both males and females, was determined using data filtering with $p < 0.05$.

	Total miRNA Differentially Expressed	Up-Regulated	Down-Regulated
Males			
CTRL LF vs. VDD LF	1	1	0
CTL HF vs. VDD HF	0	0	0
CTRL LF vs. CTRL HF	0	0	0
VDD LF vs. VDD HF	5	0	5
Females			
CTRL LF vs. VDD LF	4	4	0
CTL HF vs. VDD HF	1	0	1
CTRL LF vs. CTRL HF	1	1	0
VDD LF vs. VDD HF	3	0	3

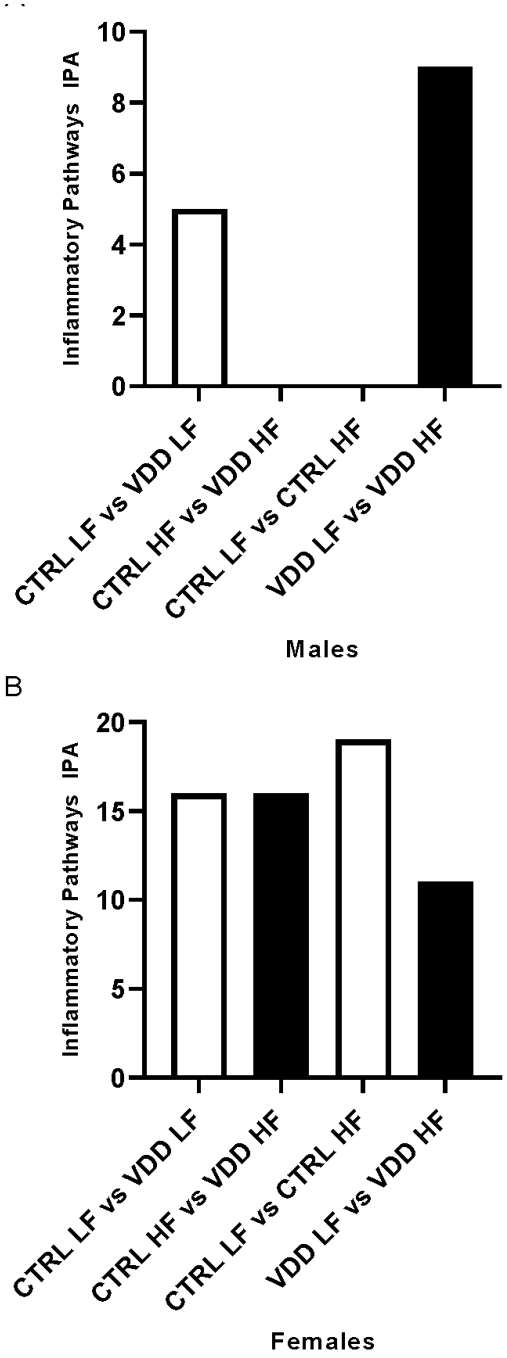

Figure 3. Inflammatory pathway linked to miRNA profile in adipose tissue of the offspring. Quantitative representation of miRNA expression in inflammatory pathways resulting in differential miRNA expression in retroperitoneal adipose tissue of the male offspring (**A**) and female offspring (**B**), using Ingenuity Pathway Analysis (IPA). Two sets of data were employed. The first is CTRL LF vs. VDD LF and CTRL HF vs. VDD HF. The second data set is CTRL LF vs. CTRL HF and VDD LF vs. VDD HF. Bars in white correspond to up-regulated pathways and bars in black for down-regulated pathways. TargetScan (genes were selected with an Aggregate PC 90%) and mirDB (genes were selected with a target score 80%) were used to identify inflammatory miRNA and create a predicted mRNA target list. The miRNA inflammation-related pathways in white adipose tissue were analysed using IPA. To assess the influence of the maternal diet and/or the adult diet on inflammation pathways in the offspring, quantitative analyses were carried out.

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3.4. HF Diet and Maternal VDD Activate NF-kB and p38

To unveil the molecules at the origin of inflammatory response in white adipose tissue, NF-kB and p38 signaling were investigated. Thus, p65 phosphorylation and p38 phosphorylation were quantified. Highest p65 phosphorylation was found in VDD HF males compared with other groups (Figure 4A) whereas p38 phosphorylation was significantly induced in CTRL HF males compared with other groups (Figure 4B). p65 phosphorylation in females was induced only in CTRL HF compared with other groups (Figure 4E), whereas p38 tended to be highly phosphorylated in CTRL HF but did not reach statistical significance (Figure 4F).

In both males (Figure 4C) and females (Figure 4G), a significant correlation was calculated between adiposity index and p65 phosphorylation, whereas no significant correlation was observed between p38 phosphorylation and adiposity index (Figure 4D,H).

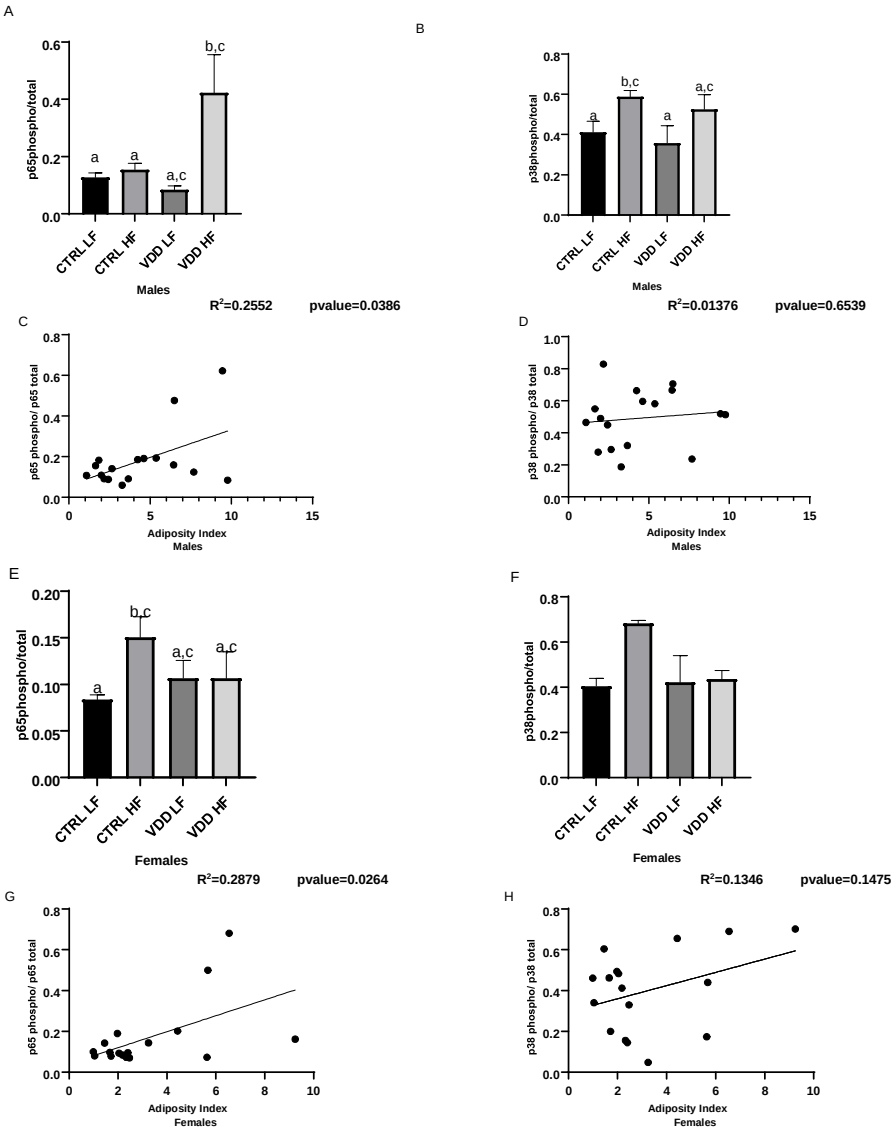

Figure 4. Phosphorylation levels of the NF-kB (p65) and p38 in adipose tissue of the offspring. Phosphorylation levels of p65 and p38 were evaluated using ELISA in males (**A**,**B**) and females (**E**,**F**). The data are expressed as relative expression ratios (p65phosphorylated/p65total and p38 phosphorylated/p38total). Values are presented as mean ± SEM. Bars not sharing the same letter were significantly different in Fisher's LSD post hoc test. $p < 0.05$. Scatter plot of the correlation between adiposity p65 in males (**C**) and females (**G**), and scatter plot of the correlation between adiposity and p38 in males (**D**) and females (**H**), using Pearson r correlation, $p < 0.05$ indicated that positive correlation was significant.

4. Discussion

Maternal diet is now recognised as an important factor in fetal development and offspring metabolic programming [31], in line with the DOHaD concept [32]. This concept states that early life environment may impact the risk of developing chronic diseases, formerly referred to as non-communicable diseases, from childhood to adulthood. In other words, the DOHaD corresponds to fetal or developmental programming. The fact that vitamin D shapes adipose

tissue inflammatory profile in adults prompted us to test its ability to program adipose tissue inflammation in offspring.

C57BL/6J mouse strain has been found to be a *bona fide* interesting model for obesity and associated dysfunctions, including metabolic inflammation. To study the effect of maternal VDD, female mice were fed with a vitamin D-free diet for at least 8 weeks premating and during the gestation period. After delivery, mice were all fed with control diet. At the age of 6 weeks, the offspring were randomised and received a low-fat (7% of total energy from fat) or high-fat diet (45% of total energy from fat) for 8 weeks to study the combined effect of the diet and maternal VDD on retroperitoneal adipose tissue inflammation.

To evaluate the combined effect of VDD and HF, a morphological analysis was established. In agreement with already published data of our team [11], we observed in this model an increase in the body weight under HF diet in both CTRL and VDD males. Adiposity index, which is used as a good marker of fat pads accumulation, independently of total body weight, was not affected by VDD in the LF condition, but was dramatically increased in the HF condition compared with the other group (CTRL LF, CTRL HF, and VDD LF). In females, a higher body weight and adiposity index was observed in CTRL HF compared with the other groups (CTRL LF, VDD LF and VDD HF). Surprisingly, the VDD HF group had a similar body weight and adiposity index to the LF groups (CTRL LF and VDD LF), thus demonstrating a strong sex-specific metabolic response.

Such increase in adiposity index in males and females groups prompted us to evaluate consequences in terms of inflammation, since it is well-established that adiposity or obesity is strongly associated with an increase of adipose tissue inflammation [18]. To study such inflammatory response, an RNA-seq approach was conducted on retroperitoneal adipose tissue and impacted metabolic pathways were estimated using Gene Ontology enrichment analysis (GO) and Ingenuity pathway analysis (IPA). After curation, only pathways related to inflammation were maintained in our quantitative analysis, based on the number of pathways impacted.

When comparing the impact of maternal VDD, under LF diet (CTRL LF vs. VDD LF) in males, only two linked inflammatory pathways were found to be significantly different, including a downregulation of “LPS/IL-1 Mediated Inhibition of RXR Function”, suggesting thus that under normal diet, the effect of VDD in males is very minor or null. However, under

HF diet (CTRL HF vs. VDD HF) in males, we observed that two important pathways for adipose tissue biology [20], i.e., "NF-κB activation by viruses" and "p38 MAPK Signaling" were upregulated, suggesting that VDD exacerbated inflammation mediated by HF diet. To highlight the impact of a HF diet on inflammatory process in both CTRL and VDD situation, we compared the CTRL and the VDD in both LF and HF diets. An induction of the inflammatory response in CTRL LF vs. CRTL HF was observed, but this induction was even more remarkable in VDD males subjected to an HF compared to LF diet. Indeed, in these two conditions, the "response to cytokines" pathway was induced. This is a very important pathway of inflammation that includes 757 genes and recapitulates most on the inflammatory response. In CTRL LF vs. CTRL HF comparison, 67 genes differentially regulated while in VDD LF vs. VDD HF comparison, 120 genes were regulated, suggesting that the response is much more marked in animals born from VDD mice than in animals born from CTRL mice. This qualitative response was confirmed by a statistic *p*-value more important in the VDD animals than in the CTRL animals. Interestingly, such proinflammatory profile of VDD HF males group perfectly fit with adiposity index which is more pronounced in these animals. Altogether theses data suggest an additional effect of VDD and HF in males regarding the mediation of adipose tissue inflammation.

In females, in the response to maternal deficiency, the same response in both CTRL LF vs. VDD LF and CTRL HF vs. VDD HF groups was characterised by an upregulation of "NRF2-mediated Oxidative Stress Response". In CTRL females, under HF diet (CTRL LF vs. CTRL HF) the "chemokine signaling" was upregulated as well as "response to cytokine", in which 106 genes were upregulated in the CTRL HF females. It is noteworthy that that animals also displayed higher adiposity supporting the association between adiposity and inflammation. In agreement, in the VDD LF vs. VDD HF analysis, no inflammatory pathway was overexpressed and no discrepancy in terms of adiposity was observed between these groups.

MicroRNAs(miRNAs)areinvolvedinadiposetissueinflammationduringobesity [19,33–35]. This led us to investigate the miRNA profile and predicted associated pathways. In our study, for the first time, it was demonstrated that maternal VDD deregulated miRNA expression profiles in both male and female offspring. Moreover, this regulation also appeared to be sex-dependent, with a higher number of miRNAs modulated in females. The reason for such a discrepancy will require further investigations. To go further, predicted mRNA targets of the

deregulated miRNAs were collected and enrichment analysis of pathway was conducted using IPA. In males, only one up-regulated miRNA was discovered in the CTRL LF vs. VDD LF, leading to the negative enrichment of five pathways, including "IL6 Signaling" and "ILK Signaling", suggesting a decrease in inflammatory status in VDD LF males compared with CTRL LF males. Such an observation could be explained by the lower (even if not significant) adiposity index between VDD LF and CTRL LF males. In agreement, between VDD LF and VDD HF, five miRNAs were down-regulated, leading to positive enrichment of seven inflammatory-related pathways, including "p38 MAPK" and "Chemokine Signaling", thus demonstrating at the miRNA level, the proinflammatory tone is also observable in male born from VDD mice and fed with HF diet. The comparison CTRL LF vs. VDD LF in females resulted in four up-regulated miRNA and subsequently 16 putative target pathways including "ERBB4 Signaling" and "p38 MAPK Signaling", an important inflammatory pathway, as previously mentioned, supporting at the miRNA level that VDD is associated with a decrease of inflammatory status, even if no morphometric parameters, including adiposity support, are assumed. The origin of such a phenotype will require further investigations. Nevertheless, it is noteworthy that mR-146a-5p and mir-322-5p were both up-regulated within the four up-regulated miRNAs. These miRNAs are known to display anti-inflammatory effect: miR-146a-5p suppresses the inflammatory response in human adipocytes [36] and miR-322-5p targets NFkB1 and suppresses inflammatory cytokine production while promoting cell proliferation in LPSstimulated murine macrophages [37], suggesting that even if pathway analysis revealed an upregulation of inflammatory pathways, miRNA at the origin of these putative enriched pathways display anti-inflammatory response. Similarly, in the CTRL HF vs. VDD HF in females, 16 pathways were predicted to be up-regulated on the basis of the down-regulation of one miRNA. In the VDD LF vs. VDD HF females, ten pathways were predicted to be up-regulated on the basis of the downregulation of three miRNA and in the CTRL LF vs. CTRL HF, 19 pathways were down-regulated (driven but the regulation of one miRNA). Surprisingly, all these data appeared to be inconsistent with morphometric parameters and notably with adiposity index. The origin of such inconsistence is presently unclear but could rely on the predictive approach implemented, which will require further optimisations.

Concerning molecular mechanisms, RNA seq and miRNA analysis as well as bibliography strongly converge to the putative role of NF-kB and p38/MAPK signaling pathways. Indeed,

NF-kB and p38 are known to play a major role during adipose tissue inflammation [38,39]. Furthermore, we and others have demonstrated that the ability of VD to blunt inflammation in adipocytes and adipose tissue was linked to an inhibition of phosphorylation of these two signaling pathways [21,26,40–42]. To test the hypothesis of an NF-kB and/or p38 signaling involvement, we measured the phosphorylation level of p65 and p38 in adipose tissue. No activation of p65 or p38 in the VDD LF compared to CTRL LF males was observed, suggesting that the VDD alone does not activate inflammatory pathways in agreement with pathways analyses. Interestingly, a massive phosphorylation of p65 was observed, suggesting an additional effect of VDD and HF diet in male, as observed for inflammatory pathways and adiposity index. A significant induction of p38 phosphorylation was observed in the CTRL HF group, which is coherent since this pathway is known to be inducted during obesity [39]. Nevertheless, no impact of VDD was observed on the pathway. In females, p65 and p38 were not induced in VDD mice both under LF or HF diet, the only induction of p65 being found in CTRL HF that displayed the higher adiposity and activated inflammatory-related pathways.

The origin of the mechanisms that triggered the effect of vitamin D deficiency on inflammatory process in adipose tissue remains elusive. Nevertheless, we can speculate the epigenetics mechanisms could explain, at least in part, the observed phenotype. It is notably well-established that several Dups proteins are involved in dephosphorylation of stress-activated kinases [43]. It would be of interest to evaluate the epigenetic landscape of genes coding for these proteins. Clearly, further work is mandatory to elucidate the epigenetic mechanisms that may explain the observed regulations.

To summarise, our data established a link between the increased adiposity in males born from VDD mice and fed with a HF diet, which correlates with induction of mRNA and miRNA linked to inflammation and activation of p65 phosphorylation, whereas such a relationship was not observed in females. These data add to our understanding of maternal VDD influence on the offspring, particularly its predisposition to long-term metabolic health issues. It also highlights the sex-specific adipose tissue and inflammatory response that must be considered in terms of public health.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/cells11132024/s1, Supplemental Table S1: CRTL LF vs. VDD LF male, IPA; Supplemental Table S2: CTRL HF vs. VDD HF male, IPA; Supplemental Table S3: CTRL HF vs. VDD HF female, GO; Supplemental Table S4: CTRL LF vs. VDD LF female, IPA; Supplemental Table S5: CTRL HF vs. VDD HF female, IPA; Supplemental Table S6: CTRL LF vs. CTRL HF male, GO; SupplementalTable S7: VDD LF vs. VDD HF male, GO; Supplemental Table S8: CTRL HF vs. CTRL LF male, IPA; Supplemental Table S9: VDD LF and VDD HF male, IPA; Supplemental Table S10: CTRL LF vs. CTRL HF female, GO; Supplemental Table S11: CTRL LF and CTRL HF female, IPA; Supplemental Table S12: VDD LF and VDD HF female, IPA; Supplemental Table S13: miRNA differentially regulated; Supplemental Table S14: CTRL LF vs. VDD LF male, IPA; Supplemental Table S15: CTRL LF vs. VDD LF female, IPA; Supplemental Table S16: CTRL HF vs VDD HF female, IPA; Supplemental Table S17: VDD LF vs. VDD HF male, IPA; Supplemental Table S18: CTRL LF vs. CTRL HF female, IPA; Supplemental Table S19: VDD LF vs. VDD HF female, IPA.

Author Contributions: J.-F.L., L.M. and N.H. designed research; J.-F.L., N.H., I.B. and L.M. analysed data; E.S., N.H., C.C. and J.A. performed research; J.-F.L., N.H. and L.M. wrote the paper. All authors have read and agreed to the published version of the manuscript.

Funding: This study has been supported by INRAE, INSERM, AMU and the Fondation de France.

Institutional Review Board Statement: The animal study protocol was approved by the Institutional Review Board (or Ethics Committee) of Aix Marseille University and by the French Ministry of research (protocol code APAFIS#1300-2015072112279135).

Informed Consent Statement: Not applicable.

Data Availability Statement: RNA seq data are available on GEO (accession number: GSE206372).

Conflicts of Interest: The authors declare no conflict of interest.

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Supplemental tables

Supplemental table 1: CRTL LF vs VDD LF male, IPA

Ingenuity Canonical Pathways	p value	z-score	number of genes
LXR/RXR Activation	1,38E-05	0,707	9
HIF1α Signaling	0,0030903	0,707	8
LPS/IL-1 Mediated Inhibition of RXR Function	0,0281838	-1	7
AMPK Signaling	0,0075858	-0,447	8
cAMP-mediated signaling	0,0177828	-0,378	7
White Adipose Tissue Browning Pathway	0,0052481	0,816	6

Supplemental table 2: CTRL HF vs VDD HF male, IPA

Ingenuity Canonical Pathways	p value	z-score	number of genes
IL-8 Signaling	0,0371535	-0,277	15
LPS/IL-1 Mediated Inhibition of RXR Function	0,042658	0	17
HIF1α Signaling	1,995E-08	0,186	46
ErbB4 Signaling	0,0081283	0,816	8
ILK Signaling	9,772E-08	1,043	27
IL-6 Signaling	0,0467735	1,134	10
ERK/MAPK Signaling	0,0001288	1,213	22
PI3K Signaling in B Lymphocytes	0,0081283	1,265	13
NF-κB Activation by Viruses	0,047863	1,342	7
p38 MAPK Signaling	0,0295121	1,414	10
PPARα/RXRα Activation	0,0002291	1,604	20
ERK5 Signaling	0,0112202	1,633	8
IL-3 Signaling	0,0063096	1,89	9
Integrin Signaling	0,0003236	2,065	21
White Adipose Tissue Browning Pathway	1,95E-05	2,357	18

Supplemental table 3: CTRL HF vs VDD HF female, GO

GO biological process complete	Mus musculus - REFLIST (21988)	upload_1 (1935)	upload_1 (raw P-value)	upload_1 (FDR)
positive regulation of MAPK cascade	516	51	7,28E-04	2,43E-02
regulation of MAPK cascade	716	66	5,86E-04	2,06E-02

Supplemental table 4: CTRL LF vs VDD LF female, IPA

Ingenuity Canonical Pathways	p value	z-score	number of genes
LPS/IL-1 Mediated Inhibition of RXR Function	0,0070795	-1,414	21
LXR/RXR Activation	0,0046774	2,309	13
NRF2-mediated Oxidative Stress Response	0,0144544	2	19
Apelin Adipocyte Signaling Pathway	0,018197	1	9
VDR/RXR Activation	0,0031623	0,816	10

Supplemental table 5: CTRL HF vs VDD HF female, IPA

Ingenuity Canonical Pathways	p value	z-score	number of genes
LPS/IL-1 Mediated Inhibition of RXR Function	1,023E-09	-1,528	55
NRF2-mediated Oxidative Stress Response	0,0177828	2,714	32
IL-15 Production	0,0323594	-2,828	18
LXR/RXR Activation	0,0018197	0,535	22
p53 Signaling	0,0077625	0	17
White Adipose Tissue Browning Pathway	1,38E-05	-0,756	29
VDR/RXR Activation	0,0017378	0	16
Apelin Adipocyte Signaling Pathway	0,0019055	1,5	17

Supplemental table 6: CTRL LF vs CTRL HF male, GO

GO biological process complete	Mus musculus - REFLIST (21988)	upload_1 (1260)	upload_1 (raw P-value)	upload_1 (FDR)
response to cytokine	757	67	9,48E-04	3,76E-02
brown fat cell differentiation	39	12	1,35E-05	1,09E-03

Supplemental table 7: VDD LF vs VDD HF male, GO

GO biological process complete	Mus musculus - REFLIST (21988)	upload_1 (1260)	upload_1 (raw P-value)	upload_1 (FDR)
regulation of cytokine production involved in inflammatory response	51	13	2,13E-03	4,95E-02
negative regulation of response to cytokine stimulus	57	14	1,97E-03	4,66E-02
positive regulation of tumor necrosis factor superfamily cytokine production	102	25	5,92E-05	2,71E-03
positive regulation of tumor necrosis factor production	101	24	1,12E-04	4,54E-03
regulation of tumor necrosis factor superfamily cytokine production	169	37	1,00E-05	5,88E-04
regulation of tumor necrosis factor production	167	36	1,62E-05	8,81E-04
regulation of cytokine-mediated signaling pathway	112	24	4,49E-04	1,46E-02
regulation of response to cytokine stimulus	122	26	2,58E-04	9,17E-03
inflammatory response	464	87	7,72E-09	1,03E-06
cytokine-mediated signaling pathway	278	48	1,10E-04	4,48E-03
positive regulation of cytokine production	448	77	1,29E-06	9,73E-05
regulation of cytokine production	714	121	2,16E-09	3,34E-07
negative regulation of cytokine production	268	45	4,06E-04	1,35E-02
response to cytokine	757	120	6,95E-08	7,45E-06
cellular response to cytokine stimulus	658	101	2,64E-06	1,85E-04
regulation of ERK1 and ERK2 cascade	313	47	2,04E-03	4,79E-02
positive regulation of MAP kinase activity	209	37	4,41E-04	1,45E-02
regulation of MAP kinase activity	285	50	5,71E-05	2,64E-03
positive regulation of MAPK cascade	518	74	5,02E-04	1,60E-02
regulation of MAPK cascade	704	100	5,38E-05	2,51E-03
response to leptin	20	8	1,58E-03	3,98E-02
brown fat cell differentiation	39	14	8,11E-05	3,53E-03

Supplemental table 8: CTRL HF vs CTRL LF male, IPA

Ingenuity Canonical Pathways	p value	z-score	number of genes
IL-15 Production	0,0019498	3	9
Gαi Signaling	0,0041687	-0,447	9
Integrin Signaling	0,0093325	3	11
ILK Signaling	0,0147911	2,121	10
HGF Signaling	0,0301995	2	7
White Adipose Tissue Browning Pathway	0,0346737	2,646	7

Supplemental table 9: VDD LF and VDD HF male, IPA

Ingenuity Canonical Pathways	p value	z-score	number of genes
IL-8 Signaling	3,09E-05	4,796	25
CXCR4 Signaling	0,0001622	2,673	20
IL-15 Production	0,0008511	3,873	15
IL-7 Signaling Pathway	0,0012303	2,53	11
Inflammasome pathway	0,002138	2,236	5
p53 Signaling	0,0025704	0,302	12
Apelin Adipocyte Signaling Pathway	0,0083176	0,333	10
IL-2 Signaling	0,0085114	2,121	8
IL-9 Signaling	0,0251189	2	5
CCR3 Signaling in Eosinophils	0,0295121	2	12
HIF1α Signaling	0,0436516	3,5	16
Th2 Pathway	0,0001047	1,732	18
Fcγ Receptor-mediated Phagocytosis in Macrophages and Monocytes	0,0001585	3,742	14
PI3K/AKT Signaling	0,000631	2,646	21
Th1 Pathway	0,0022387	2,714	14
STAT3 Pathway	0,0056234	1,265	14
FcγRIIB Signaling in B Lymphocytes	0,0075858	2,646	10
ERK/MAPK Signaling	0,0077625	2,309	18
PI3K Signaling in B Lymphocytes	0,0091201	3,464	14
Gαs Signaling	0,0138038	2,828	12
Gαi Signaling	0,0158489	1,667	13
Gαq Signaling	0,017378	3,317	15
Noradrenaline and Adrenaline Degradation	0,0251189	2,236	5
CREB Signaling in Neurons	0,0251189	5,667	41
Gα12/13 Signaling	0,0269153	3,464	12
Integrin Signaling	3,802E-08	5,292	31
ILK Signaling	1,072E-06	3,4	27
Neuroinflammation Signaling Pathway	0,0014125	4,796	30

Supplemental table 10: CTRL LF vs CTRL HF female, GO

GO biological process complete	Mus musculus - REFLIST (21988)	upload_1 (1935)	upload_1 (raw P-value)	upload_1 (FDR)
regulation of interleukin-2 production	59	15	7,45E-04	2,24E-02
positive regulation of cytokine production	448	68	7,83E-05	3,36E-03
regulation of ERK1 and ERK2 cascade	313	47	1,20E-03	3,31E-02
response to cytokine	757	106	1,40E-05	7,82E-04
cellular response to cytokine stimulus	658	90	1,66E-04	6,35E-03
regulation of cytokine production	714	96	1,74E-04	6,58E-03
positive regulation of MAP kinase activity	209	39	5,53E-05	2,53E-03
positive regulation of MAPK cascade	518	87	1,38E-07	1,43E-05
regulation of MAP kinase activity	285	47	1,56E-04	6,02E-03
regulation of MAPK cascade	704	105	1,41E-06	1,05E-04

Supplemental table 11: CTRL LF and CTRL HF female, IPA

Ingenuity Canonical Pathways	p value	z-score	number of genes
Gαi Signaling	0,0066069	1,265	14
α-Adrenergic Signaling	0,030903	2,236	10
CCR3 Signaling in Eosinophils	0,0288403	2,646	12
Th1 Pathway	0,0144544	2,714	12
Chemokine Signaling	0,0380189	2,828	8
ERK/MAPK Signaling	0,0288403	2,887	17
White Adipose Tissue Browning Pathway	0,030903	2,887	12
Neuroinflammation Signaling Pathway	0,0301995	2,982	23
HIF1α Signaling	0,0229087	3,5	17
IL-15 Production	0,0008128	3,873	15
IL-8 Signaling	0,0029512	4,123	20

Supplemental table 12: VDD LF and VDD HF female, IPA

Ingenuity Canonical Pathways	p value	z-score	number of genes
Apelin Liver Signaling Pathway	2,239E-05	2	4
Hepatic Fibrosis Signaling Pathway	0,0004169	2,646	10

Supplemental table 13: miRNA differentially regulated

miR CTRL LF VDD LF males

mature ID	pval	Fold Change
mmu-miR-142a-5p	0,033762	0,7445
mmu-let-7d-5p	0,008579	1,685
mmu-miR-141-3p	0,031802	0,0419
mmu-let-7f-5p	0,009719	1,7523
mmu-miR-29a-3p	0,035893	1,4357

miR VDD LF VDD HF males

mature ID	pval	Fold Change
mmu-miR-29b-3p	0,005735	1,8142
mmu-miR-30e-5p	0,045846	1,4028
mmu-miR-30a-5p	0,04772	1,398
mmu-miR-29a-3p	0,008446	1,9846
mmu-miR-101a-3p	0,001021	2,8915

miR CTRL LF CTRL HF females

mature ID	pval	Fold Change
mmu-miR-30d-5p	0,01473	0,5886

miR CTRL LF VDD LF females

mature ID	pval	Fold Change
mmu-miR-29b-3p	0,032927	1,6249
mmu-miR-30e-5p	0,039019	1,6521
mmu-miR-322-5p	0,044697	2,2197
mmu-miR-146a-5p	0,019032	2,3236

miR VDD LF VDD HF females

mature ID	pval	Fold Change
mmu-miR-29b-3p	0,002694	3,9986

miR CTRL HF VDD HF females

mature ID	pval	Fold Change
mmu-miR-29a-3p	0,037866	0,5728

Supplemental table 14: CTRL LF vs VDD LF male, IPA

Ingenuity Canonical Pathways	PVALUE	number of genes
IL-6 Signaling	1,95E-05	7
IL-10 Signaling	0,0112202	3
IL-7 Signaling Pathway	0,0141254	3
ILK Signaling	0,040738	4
HIF1α Signaling	0,0457088	4

Supplemental table 15: CTRL LF vs VDD LF female, IPA

Ingenuity Canonical Pathways	PVALUE	number of genes
ILK Signaling	0,002884	8
IL-4 Signaling	0,0051286	5
STAT3 Pathway	0,0057544	6
ERK5 Signaling	0,0120226	4
IL-8 Signaling	0,0134896	7
IL-7 Signaling Pathway	0,0141254	4
p38 MAPK Signaling	0,0147911	5
JAK/STAT Signaling	0,0169824	4
Th1 and Th2 Activation Pathway	0,017378	6
Role of IL-17F in Allergic Inflammatory Airway Diseases	0,0186209	3
IGF-1 Signaling	0,0363078	4
IL-2 Signaling	0,0363078	3

HIF1α Signaling	0,0389045	6
IL-17A Signaling in Airway Cells	0,0457088	3
TGF-β Signaling	1,995E-05	8
ERBB4 Signaling	0,047863	3

Supplemental table 16: CTRL HF vs VDD HF female, IPA

Ingenuity Canonical Pathways	PVALUE	number of genes
IL-6 Signaling	6,026E-05	10
STAT3 Pathway	0,0004571	9
p38 MAPK Signaling	0,0009333	8
Role of MAPK Signaling in the Pathogenesis of Influenza	0,0025119	6
ERK/MAPK Signaling	0,0036308	10
ILK Signaling	0,0067608	9
IGF-1 Signaling	0,0081283	6
IL-8 Signaling	0,0091201	9
Chemokine Signaling	0,0109648	5
TNFR1 Signaling	0,0112202	4
IL-2 Signaling	0,0190546	4
CCR3 Signaling in Eosinophils	0,0263027	6
IL-10 Signaling	0,0323594	4
ERK5 Signaling	0,0354813	4
IL-17 Signaling	0,0380189	7
NF-κB Activation by Viruses	0,0416869	4

Supplemental table 17: VDD LF vs VDD HF male, IPA

Ingenuity Canonical Pathways	PVALUE	number of genes
TGF-β Signaling	8,913E-06	13
p38 MAPK Signaling	0,001349	11
Th1 and Th2 Activation Pathway	0,0029512	13
ERK/MAPK Signaling	0,0033113	15
STAT3 Pathway	0,0097724	10
Role of IL-17F in Allergic Inflammatory Airway Diseases	0,0151356	5
ILK Signaling	0,0234423	12
CCR3 Signaling in Eosinophils	0,025704	9
Chemokine Signaling	0,0389045	6

Supplemental table 18: CTRL LF vs CTRL HF female, IPA

Ingenuity Canonical Pathways	PVALUE	number of genes
IGF-1 Signaling	0,0002884	8
Integrin Signaling	0,0007244	11
ERK5 Signaling	0,0012589	6
Role of JAK2 in Hormone-like Cytokine Signaling	0,0020893	4
ERK/MAPK Signaling	0,0028184	10
IL-4 Signaling	0,0039811	6
TGF-β Signaling	0,0046774	6
Role of JAK1 and JAK3 in γc Cytokine Signaling	0,0051286	5
IL-23 Signaling Pathway	0,0064565	5
Role of JAK family kinases in IL-6-type Cytokine Signaling	0,0074131	3
IL-3 Signaling	0,0091201	5
Chemokine Signaling	0,0095499	5
JAK/STAT Signaling	0,0107152	5
ILK Signaling	0,0162181	8
IL-6 Signaling	0,0177828	6
CCR3 Signaling in Eosinophils	0,0223872	6
STAT3 Pathway	0,0223872	6
Role of MAPK Signaling in Promoting the Pathogenesis of Influenza	0,0363078	5
IL-7 Signaling Pathway	0,0371535	4

Supplemental table 19: VDD LF vs VDD HF female, IPA

Ingenuity Canonical Pathways	PVALUE	number of genes
Th1 and Th2 Activation Pathway	0,0001698	16
TGF-β Signaling	2,754E-06	14
STAT3 Pathway	0,0015849	12
p38 MAPK Signaling	0,042658	8
IL-8 Signaling	0,0436516	12
IL-3 Signaling	0,0446684	6
IL-23 Signaling Pathway	0,0165959	5
IL-17 Signaling	0,0199526	12
IL-15 Production	0,0070795	10
ERK/MAPK Signaling	0,011749	14
CCR3 Signaling in Eosinophils	0,0331131	9

Article 2: Maternal vitamin D deficiency in mice sex-dependently affects hepatic lipid accumulation in the offspring.

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To be submitted

In a societal and maternal context similar to the one previously described, our interest impact of maternal VDD on the development of HS in the offspring.

In this model, we collected liver tissue from VDD and control mice, to assess how maternal VDD affected hepatic lipid accumulation in adult offspring who were fed a normal or obesogenic (HF) diet.

In both males and females, several methods were used, including histology and lipidomics on the liver. At the histological level, neither males nor females showed any significant effects from HF or VDD. Nonetheless, a rise in total lipids and alteration of the relative lipid species distribution, defined by a decrease in TG and an increase in phospholipids, was found in males born from VDD mice and fed an HF diet. There was no significant lipid profile in females.

Therefore, we concluded that maternal VDD paired with a HF diet may predispose males to hepatic lipid accumulation with a particular lipid profile. Such findings add to our understanding of the effect of maternal VDD on offspring hepatic programming.

Maternal vitamin D deficiency in mice sex-dependently affects hepatic lipid accumulation in the offspring.

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Short running title: maternal vitamin D deficiency programs offspring liver lipid accumulation.

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Abstract

During the perinatal period, maternal nutrition plays a critical role in maintaining a steady supply of vital nutrients to ensure the foetus's growth and development. Among these nutrients, vitamin D is now considered to display an important role in this development. Vitamin D deficiency (VDD) is becoming a global issue and low 25-hydroxyvitamin D (25(OH)D) plasma levels have been linked to hepatic steatosis in adulthood. Nevertheless, the impact of maternal VDD on lipid metabolism and hepatic steatosis remains poorly documented, especially under obesogenic condition. The goal of this study was to assess the effects of maternal vitamin D deficiency (VDD) on hepatic lipid accumulation in adult offspring fed a normal or obesogenic (high fat) diet. Several approaches were implemented including histology and lipidomics on the liver in both males and females. No major impact of HF or VDD was observed at histological level in both males and females. Nevertheless, in males born from VDD mice and fed a HF diet, an increase of total lipids and modulation of the relative lipid species distribution, characterized by a decrease of triglycerides and increase of phospholipids was observed. In female no major lipid profile was noticed.

We concluded that maternal VDD combined with a high-fat diet in male may predispose to hepatic lipid accumulation, with a specific lipid profile. Such observations reinforce our knowledge of the impact of maternal VDD on hepatic programming in the offspring.

Keywords: Vitamin D, Maternal vitamin D deficiency, offspring, hepatic steatosis, liver, lipids.

Abbreviation list: Che Et Co: Cholesteryl Ester Coenzyme, DG: Diacylglycerol; LPC: Lysophosphatidylcholine, LPE: Lysophosphatidylethanolamine, PC: Phosphatidylcholine, PE: Phosphatidylethanolamine, PG: Phosphatidylglycerol; PI: Phosphatidylinositol, PS: Phosphatidylserine and TG: Triglyceride.

Introduction

Vitamin D deficiency (VDD), defined as a plasma level of 25 hydroxyvitamin D (25(OH)D) below 50 nmol/L and obesity are two global public health issues that are closely associated (1, 2). Indeed, a large number of cross-sectional studies have highlighted the inverse correlation between low serum 25(OH)D and obesity (3-5). Obesity is primarily due to an imbalance between energy intake and energy expenditure, leading to an excessive fat storage in adipocytes. When the capacities of storage of adipose are overwhelmed by the overload of dietary fatty acid, it leads to adipose tissue dysfunction, which participates to a deterioration of lipid metabolism (6). It results in increased lipid flux to the liver that contributes to hepatic steatosis (7).

Remarkably, several epidemiologic studies reported that low 25(OH)D plasma levels are frequently associated with hepatic steatosis (8-10). Preclinical studies also suggested a relationship between plasma VD concentration and hepatic steatosis (8). Vitamin D deficiency for 10 weeks in mice resulted in hepatic steatosis, whereas vitamin D supplementation proved beneficial in reducing steatosis (11). Similarly, in high fat diet, it has been demonstrated that VD supplementation reduced the expression of genes coding for lipogenic actors and reduced steatosis (12-14).

In addition, a study conducted in rats reported that maternal VDD altered lipid and liver metabolism, promoting the development of hepatic steatosis in the offspring (15). However, because this VDD maintained till the weaning, the specific role of intrauterine programming remains questionable and the overall observed effect could be priming during lactation.

Based on the role of the maternal life environment on the risk of developing chronic diseases; according to the DoHad concept (16), and based on the role of VDD together with high fat diet on adiposity and insulin resistance in males, it is tempting to speculate that such embryonic environment may have a programming role of hepatic lipid metabolism and susceptibility to hepatic steatosis.

Consequently, the question that arises in this study is the consequence of maternal VDD in combination with a high-fat diet, on the sex-specific response during adulthood, on the hepatic steatosis development.

Material and Methods

Animal Experiments

The Aix-Marseille University Ethics Committee and the French Ministry of Research both gave their approval to the protocol (APAFIS#1300-2015072112279135). Female and male C57BL/6JRJ mice were obtained from Janvier Labs (Le Genest-Saint-Isle, France) and fed ad libitum with control food (chow diet A04 from Safe-diets, Augy, France) and full access to drinking water during the 1-week acclimation period. The animals were kept at a constant temperature of 22°C, with a 12-hour light/12-hour dark cycle and a humidity of 20%. Female mice (15 per group) were mated with males after being assigned to one of two experimental groups based on diet: control (AIN-93G with vitamin D3, 1.0 IU/g) or vitamin D-depleted (AIN-93G without vitamin D3, 0.0 IU/g) for eight weeks, as previously described (17). All females were fed a control diet (AIN-93G) after delivery until the offspring were weaned. The litter size of the females was lowered to six pups. The offspring's body weight was measured weekly from the time they were weaned until the end of the trial to avoid mother cannibalism and prenatal stress. At six weeks of age, males and females of the offspring were randomly assigned to either a Low-Fat (AIN-93M Maintenance Purified Diet) or a High-Fat (DIO Rodent Purified Diet w/45% Energy from Fat) diet for eight weeks. Using eight groups of mice (males and females), the impact of maternal diet (CTRL versus VDD) and adult diet on offspring mice (males and females) was studied (LF vs HF). Overnight fasted mice were euthanized by cervical dislocation, and tissue samples were collected, weighed, and stored at -80°C.

Histological Analysis

Liver tissue samples were fixed in 10% buffered formalin, embedded in paraffin, and sliced to prepare 5 µm tissue sections whose were stained with hematoxylin and eosin (H&E) as previously reported (18). The images were captured by a light microscope (Leica, Germany; 10X magnification). The number of nucleus per area (mm^2) was calculated using Image J software).

Protein extraction and quantification

The protein extraction was made on ice where a small piece of liver is grinded in 2% SDS for one minute at 23Hz. Then the homogenate is separated in another tube, and centrifuged at 4°C at 12700 rpm for 10min. The protein dosage using Pierce BCA Protein Assay Kit according to the manufacturer’s instructions (ThermoFischer, France), as previously described (19).

TG extraction and dosage

To extract triglycerides (TG), 1mL 10% NP40 was added to about 40 mg of liver chopped into small pieces and the mixture was ground for one minute at 30Hz. The mixture was incubated in a thermomixer for 2 minutes at 80°C at maximum speed. Colorimetric methods were used to measure TG in the liver (BIOLABO, Maizy, France). Briefly, in a 96 well plate, 10 µL of samples were homogenised with a 200 µL of mixture of enzymes and tampon. Before reading, the wells were left at room temperature for 10 minutes at room temperature. The absorbance of the microplates at 490 nm was measured.

Lipid Extraction

For lipid extraction by methyl-tert-butyl ether was used for high-throughput lipidomics; as described (20). Before extraction, 20 mg of liver were cut in small pieces, and 200 µL of PBS and 10 µL of standard solution mix. Avanti Polar lipids, Splash were added to the tissue. After 10 min of vortex at 1500 rpm, 1.5 mL of methanol and 5 mL of methyl-terbutyl ether (MTBE) were added to each sample and vortexed for 1 hour.

After homogenization, 1.25 mL deionized water was added to each tube and incubated for 10 minutes at room temperature. The upper organic phase was separated in another tube after centrifugation for 10 minutes at 1000 rpm at 10°C.

For a second extraction, the bottom phase was washed with 2 mL of a methyl-terbutyl ether/methanol/water mixture (10:3:2.5 v/v/v) and centrifuged as previously described.

The upper organic phase and the first one obtained in the previous step were combined. The solvent was then evaporated using a nitrogen stream, and the dry lipid extract was weighted and kept at -80°C until processing. The lipid extracts were then resuspended in 200 µL of (60:30:4.5 v/v/v) mixture of chloroform, methanol, and distilled water.

Lipid chromatography

The UHPLC separation was carried out using a DionexUltiMate 3000 device (Thermo Fisher Scientific, Courtaboeuf, France) with an Accucore C18 column (1502.1 mm, 2.6 m) on a DionexUltiMate 3000 device (Thermo Fisher Scientific, Courtaboeuf, France). The temperature in the column was controlled at 45°C. Mobile phase A had 10 mmol/L ammonium formate in 60%acetonitrile with 0.1%formic acid, while mobile phase D had 10 mmol/L ammonium formate in acetonitrile: propan-2-ol (1:9, v/v) with 0.1%formic acid. The flow rate

was 0.4 milliliters per minute. The elution gradient was as follows: 35% D at the start, 35% to 60% D for 4 minutes, 60% to 85% B for 8 minutes, 85% to 100% B for 9 minutes, 100% B for 3 minutes, and 35% B for 4 minutes. The injection was 2 µL in volume. The samples were placed on the injection table at random and were interspersed (1 of 5) with quality control samples made up of a pool of each sample or a solvent for the blank.

Mass Spectrometry

The analyzed samples' mass spectrometry (MS) spectra were obtained using a Q-Exactive Plus (Thermo Fisher) spectrometer with electrospray ionization in positive and negative modes and a full scan (m/z 250 to 1200) according to Breitkopf et al. methodology (21). The capillary voltage was set to 3000 V and the drying temperature was set at 285°C. The orbitrap mass analyzer's resolution was set to 4 scans/s at 35 000 resolution, with a 200 m injection time. For each sample, full MS spectra were acquired. Data-Driven Analysis The top 15 MS/MS were used to acquire the MS/MS spectra, and performed in one-tenth of the samples. To collect as much structural information as possible, each sample was captured in both positive and negative ionization modes separately. The most common ionic species in the principally collected full MS spectra are detected using this approach in the next 15 scans, increased collision-induced dissociation fragments these 15 ionic species, completing the cycle with the last MSMS. A new cycle begins with a full MS scan and 15 MS/MS scans for the following 15 ionic species.

Data treatment

The generated spectra were visually evaluated to adjust the signal intensity after the data of the molecular ions corresponding to the metabolites were acquired. The spectra were separated into two groups: The following spectra were utilized to create a data matrix for use in the XCMS software: The three-dimensional raw data from the liquid chromatography-MS analysis (m/z, retention time, and ion intensity) were deconvolved into a composite matrix of chromatographic peaks aligned in time and a mass-to-charge ratio (m/z) with the intensity of the associated ions. The original data files (*.raw) were converted into more interchangeable formats and centroid mode (*. mzXML) for both studied positive and negative spectra using Proteo Wizard MS Convert program. The open-source software XCMS software. was used to process the data (22). The centWave method was used to detect the peaks. The maximum and lowest peak widths were 2 and 60 seconds, respectively, and the S/N threshold was maintained at 3, with noise levels of 12000 for positive and 6000 for negative ionization modes, a m/z difference of 0.00005, and a maximum deviation of 5 ppm in two consecutive scans. A true peak was defined

as the detection of a peak in four successive scans with a minimum intensity of 60 000. The Obiwarp method was used to align peaks between samples, and the density approach was used to aggregate peaks. After obtaining the raw data matrix, numerous filters were used to remove analytical background and correct analytical drift. All electronic noise signals, as well as null samples, were manually eliminated. The coefficients of variation of the peaks applied to the control samples were used to eliminate unstable peaks and redundant information (fragments, adducts, and isotopes). Spectra were utilized to establish a database using LipidSearch software (v4.0, Thermofischer scientific): based on the MS/MS spectra obtained in the samples, an in-silico database was built using LipidSearch software. This program offers an in-silico database of several lipid families, organized by their specific fragmentation mode and FA chain length. The annotations of various fragmented ions were produced based on the m/z ratio of the parent ions and the fragments created, and this theoretical fragmentation model correlates to the MS/MS spectra of the tissue extracts. The corresponding lipid species could be identified in the majority of the signals.

To identify the lipid species and their intensities in each sample, the m/z and retention time pairs obtained with Lipid Search were compared to those obtained with XCMS using the data inhouse tool of the Galaxy Workflow4Metabolomics online program (23). This final database was subjected to a final filter, which included factors such as relative SD, duplication in both modes, and the selection of the most stable and intense peak for each lipid, among others. For statistical analysis, a curated and annotated matrix comprising semi-quantitative values for each lipid per sample was used.

Statistical Analysis

The data is presented as mean ± SEM. GraphPad Prism was used to assess significant differences using an unpaired Student's t test or an ANOVA followed by the Fisher's LSD post hoc test. A statistically significant value of $p<0.05$ was used.

MetaboAnalyst (24) were used to perform principal component analysis, Partial Least Squares Discriminant Analysis (PLS-DA), 2D scores plot, cross validation, important features; the variable importance in projection (VIP) of auto scaled results PLS-DA validation with permutation (2000 repetitions) test $p<0.05$.

Results

Impact of Maternal VDD on liver absolute and relative mass

Maternal VDD diet intake led to a similar liver mass in both LF and HF males (CTRL LF similar to VDD LF and CTRL HF similar to VDD HF) (Fig. 1A). A significant higher relative liver mass was observed in VDD LF compared to CTRL LF and CTRL HF. Under HF diet, no difference was noticed in relative liver mass between CTLF HF and VDD HF males (Fig. 1B). In female, only the CTRL HF mice displayed higher liver mass, compared to other groups (Fig. 1C). Relative liver masses were similar in the 4 groups (Fig. 1D).

Impact of Maternal VDD on liver histology

The effect of the deficiency was assessed on liver histology and hepatocyte cellularity. No major histological difference was observed between VDD LF and CTRL LF males. Similarly, no significant difference was observed between the VDD HF and CTRL HF males (Fig. 2A, B). In females, no morphological impact of the maternal deficiency was noticed in both conditions LF (CTRL LF and VDD LF) and HF (CTRL HF and VDD HF) (Fig. 2E, F).

In the males, both VDD LF and VDD HF compared respectively to CTRL LF and CTL HF showed a not significant decrease in the number of nucleus over the surface were measured. (Fig. 2C, D). In the VDD females LF the number of nucleus/mm^2 tend to decreased compared to the CTRL LF and in VDD HF the number of nucleus/mm^2 tend to increased compared to CTRL HF (Fig. 2 G, H) but not reached statistical significance.

Impact of Maternal VDD on total lipid mass and TG in the liver

The total lipid mass did not significantly vary between the CTRL and VDD males fed LF diet, whereas in the HF situation the total lipid mass increased in VDD males compared to the CTRL male mice (Fig. 3A). In the females in both situation LF and HF (Fig. 3B) no difference in total lipid mass was observed. The quantity of TG was expressed as a ratio between TG and total lipids. In this case in VDD HF, a decrease was observed compared to other male groups (Fig. 3C), whereas no difference was noticed in females (Fig.3D).

Impact of Maternal VDD on lipidome in the liver

An analysis of the percentage of each lipid classes among the total quantity of lipids was conducted. In total, 11 lipid classes were identified in the various groups. Comparing LF fed CTRL and VDD males, lysophosphatidylcholine (LPC) and lysophosphatidylethanolamine

(LPE) increased significantly from 4.9% to 9.6% and from 0.06% to 0.09% respectively in the VDD condition compared to CTRL condition (*p*-value <0.05; Fig 4 A). In CTRL and VDD males fed HF comparison the triglycerides (TG) decreased from 58.5 to 38.7%, while the phosphatidylserine (PS; 3% to 7%), phosphatidylglycerol (PG; 0.17 % to 0.26%), phoshatidylethanolamine (PE; 12% to 16%), LPE (0.08% to 0.12%), cholesterol esters and co-enzyme (ChE and Co; 2.2% to 2.7%) and diglycerides (DG; 5.2% to 9.3%) increased in VDD compared to CTRL (*p*-value <0.05; Fig 4 B). LF female showed a decrease in the LPE (0.08% to 0.07%), ChE and Co (5% to 2.5%) and DG (5/8% to 3.2%) in the VDD compared to the CTRL (*p*-value <0.05; Fig 4 C), while in the VDD HF group only the ChE and Co (5% to 2.5%) decreased compared to the CTRL HF (*p*-value <0.05; Fig 4 D).

To go further, a supervised PLS-DA was calculated and revealed that the two groups (CTRL HF compared to VDD HF) were well-clustered, with distinct metabolic profiles for each (Fig. 5A). The 95 percent confidence ellipses calculated from PLS-DA scores showed group membership (VDD HF vs CTRL HF). The permutation test demonstrated that the classification of global metabolite profiles was significantly different, with a *p* value of 0.021. By assessing the importance of each maternal VD status, we used the variable importance in projection (VIP) learning algorithm to identify relevant lipids for maternal status categorization with 30 lipid species differing in expression (Fig. 5B), where most of the lipids were higher in the VDD HF situation. 27 out of 30 were higher in VDD HF and only 2 TG and 1 PG are higher in CTRL HF males with a good ratio of R2/Q2 of 1,081 (Table 2). In the LF situation the PLS-DA also revealed that the two groups were divided in terms of maternal VD status with a *p*-value of 0.016 (Fig. 5C), the VIP between the two groups, showed 8 lipid species were significantly different (Fig. 5D) with a R2/Q2 ration of 1,028 (Table 2).

In females a very well separation between CTRL LF compared to VDD LF with a *p*-value of 0.013 (Fig. 6A), indicated a variation of 28 lipid species (Fig. 6B), with a R2/Q2 ration of 1,183 (Table 2), without any clear pattern, some lipids being higher in the CTRL group, others in the VDD group. Similarly, in HF diet, the PLS-DA also revealed that the two groups CTRL HF and VDD HF were divided (Fig. 6C) with a *p*-value of 0.008. This difference showed a variation in 29 lipid species that varied in both groups (Fig. 6D), with a R2/Q2 ration of 1,15 (Table 2).

Discussion

Adult diseases have been linked to foetal origins (concept of DOHaD). It was thought that maternal or foetal environment, including nutrition, during specific stages of pregnancy caused the foetus to adapt in order to survive in the uterus. In line, it has been reported that VD deficiency during gestation in mice is associated to metabolic disruptions in adult offspring (4, 5).

Based on the ability of VD to blunt hepatic steatosis (8-10), we hypothesised that maternal vitamin D deficiency impacted the liver lipid metabolism in the offspring, and possibly potentialized the effect of HF diet on hepatic steatosis. To this aim, female mice were provided a control or a VD-free diet for at least 8 weeks previous mating and during the gestation period. After delivery, all mice were fed a control diet. The offspring (from control and VDD mice) were randomly assigned to either a low fat (7% total energy from fat) or a high fat (45%total energy from fat) diet for 8 weeks at the age of 6 weeks to evaluate the combined effect of the diet and maternal VDD on liver steatosis. Such HF diet conditions (45% HF diet for six weeks) were chosen to mediate a challenging environment, suitable but without clear induction of hepatic steatosis, since we wanted to test the combined effect of HD diet and VDD.

A first analysis was conducted at the morphological level. Whereas liver mass appeared to be increased in HF diet in both CTRL and VDD groups, the relative liver mass increased only in males born from VDD fed a LF diet, suggesting that the observed increased liver mass under HF diet was only due to an overall mass gain, except in VDD LF group where a specific liver mass gain occurred. In female, an increase was observed in the liver mass under HF diet in the CTRL group, but was lost after normalization to body mass, suggesting no effect of VDD or diet on liver mass. Such original observation suggested that VDD combined with diet differentially programmed liver metabolism in a sex-specific way. To go further, we conducted a histological study in both groups, where we did not find major significant changes in the liver histology in both, which correspond males and females (CTRL LFVDD LF and CTRL HF VDD HF). No sign of hepatic steatosis was observed in the different groups according to the classification the grades of the disease (25). Altogether these data demonstrated that the VDD together with diet had a modest impact on liver morphology and histology at a macroscopic level. Such observation are clearly not in agreement with a previous report related to the impact of maternal VDD on lipid hepatic metabolism of the offspring and showing that female born from VDD rats were more prone to steatosis (15). Several explanations could be emitted to explain such discrepancy. First, our study was performed in mice and not rats which could

influence VDD associated phenotype. Second, the VDD diet was maintained in the offspring till the weaning, whereas in our case, mice were given a normal diet after delivery, to limit the impact of VDD to the intrauterine period (no impact through lactation). Third, the observed steatosis in animals was observed at 30 weeks of age, suggesting that our study was too short in time to observed such phenotype.

To go further on the combined impact of VDD and diet on liver lipid metabolism, we implemented experiments to characterized lipids in the liver of animals of the different groups. First, we basically quantified total amount of lipids in the liver. Such quantification revealed that males of the VDD HF groups presented a significant accumulation of total lipids in the liver, suggesting that even in absence of steatosis in this group, the combination of maternal VDD together with HF diet led to an accumulation of lipids in males only. Such state could be transient and may be considered as hepatic pre-steatosis, which could degenerate into hepatic steatosis. This last assumption will require further investigations to be confirmed.

TG represents the main lipid class within the liver (around 75% of total lipids). We thus quantified TG and observed that in males only the VDD HF group displayed a lower ratio of TG / total lipids. Since total lipids were increased in this group, it was not clear if this reduced ratio was only due to the increased total lipids of to a concomitant reduced TG level.

Thanks to lipidomic analysis, we quantified several classes of lipids including TG. We observed in VDD LF higher LPE and LPC percentage compared to CTRL LF. The impact of such modest modifications in the liver physiology remains questionable. Interestingly, the most contrasted condition, in agreement with other measurements is the VDD HF compared to VDD LF in males. Indeed, the TG percentage decreased in VDD HF in males compared to CTRL HF. Such observation is not in agreement with studies reporting that hepatic steatosis is associated with higher levels of TG (26). In parallel with this decrease of TG, most of the lipid membrane such as PE, PG, PS were increased in the VDD HF compared to VDD LF, which could be explained by a reduction of TG accumulation in hepatocyte and a proportionally increase of lipid membranes.

In female groups only very modest modification of lipid classes were observed suggesting once again a very limited impact of VDD with LF or HF diet in females offspring in our conditions, and contrarily to previous published data (15). The origin of such lack of effect in female will require investigations. Nevertheless, the role of sexual hormones including estrogens could be hypothesised to protect female from hepatic steatosis contrarily to males (27).

PLS-DA was conducted in the liver to identify lipids that discriminated groups of mice. A first analysis was conducted in VDD LF and CTRL LF, where only 8 lipids where found to be different in this comparison. The impact of such minor alterations in liver metabolism is still unknown. The samples were not well separated with low discrimination. In these small number of lipids, no specific pattern was observed, suggesting a low physiological relevance of such discrimination.

Interestingly, between CTRL HF and VDD HF males the samples were well separated with 30-lipid species that were differentially expressed with 27 higher in the VDD HF group. 8 out of these 27 lipids belong to the PE family. In addition, several other membrane lipids including PS, PG and LPC were found to be higher in VDD HF. These amphipathic lipids which are the most abundant phospholipids in mammalian are necessary components of cellular membranes and, as such, display a structural role (28). The fact that these lipids are proportionally increased may suggest that the ratio between membranes and storage lipids (TG and DG) is modified. Such assumption agrees with data related to the quantification of this class of lipids. Nevertheless, it has been demonstrated in a 14 weeks mice study fed 45% HF, that PE tended to be reduced in the liver (29). It is noteworthy that in our conditions, we mixed the effect of HF diet with maternal VDD, suggesting that this maternal deficiency may drive different lipid accumulation programming compared to HF diet alone. Together with the increase of phospholipids, we observed that 8 DG were discriminants between groups and were induced in the VDD HF group compared to VDD LF. This observation reinforces the fact that phospholipids are induced in VDD HF since DG can be used as precursors of phospholipids. In addition, DG are also precursors of TG, thus if DG are engaged in the way of phospholipids they are less available to participate to the production of TG, supporting at least in part there decrease in VDD HF group.

The result that we obtained from females regarding discrimination of groups, showed that the lipids species varies between the 2 groups CTRL vs VDD in both diet LF and HF do not display particular pattern, implying again the very restricted effect on female offspring in our conditions. Indeed, in both comparisons, we observed that the same types of lipids (TG for instance) were both up or down-regulated but without clear tendency. In addition, it is important to note that the ability of our models to predict groups was relatively low, even if statistically significant. Finally, the relative quantity of discriminants lipids between groups was extremely low, suggesting a very limited biological impact.

To summarize, our data established a link between the increased total lipid mass in males born from VDD mice and fed with a HF diet, with a reduction of TG number and increase of lipid membranes resulting in a programming of hepatic lipid metabolism, whereas such relationship was not observed in females. The origin and mechanisms of such maternal programming remain elusive and will require further investigations. Nevertheless, we can speculate that epigenetic mechanisms associated to VDD impact liver biology.

These data reinforce our knowledge regarding the impact of maternal VDD on offspring, and the combine effect of obesogenic situation. It also illustrates the sex-specific hepatic response that must be considered in terms of public health

Funding. The work has been supported by funds from AMU, INRAE, INSERM and La Fondation de France

Conflict of interest. No conflict of interest as to be disclose.

Author Contributions: JF Landrier and N Haroun designed research; JF Landrier, N Haroun, I Bennour, L Mounien, JC Martin analysed data; N Haroun, E. Seipelt, L Svilar, C. Tardivel, C Couturier, J Astier, performed research; JF Landrier, N Haroun wrote the paper.

Figures legends

Figure 1. Liver absolute and relative weight in male and female offspring.

Liver weight and Relative liver weight (liver weight/total body weight) measured at the protocol end respectively for the males (A, B) and females offspring (C, D). Values are presented as mean ± SEM. Bars not sharing the same letter were significantly different in Fisher's LSD post hoc test. $p < 0.05$

Figure 2. **Liver histology of male and female offspring**.

Representative histological images of liver tissue of males (A) and females (D) adult offspring after eosin-hematoxylin coloration (10X magnification). The number of nucleus per area (mm2) was calculated using Fiji software for CTRL LF VDD LF males (B) CTRL HD VDD HF males (C) and CTRL LF VDD LF females (E) CTRL HD VDD HF females (F). Values are presented as mean. SEM. Bars not sharing the same letter were significantly different in Fisher' s LSD post hoc test. $p < 0.05$

Figure 3. **Total lipid and TG quantification in male and female offspring.**

Total lipid mass weighted after extraction from liver tissue for the males (A) and females offspring (B). The relative expression ratio TG/Total lipid mass in males (C) and females (D). Values are presented as mean ± SEM. Bars not sharing the same letter were significantly different in Fisher's LSD post hoc test. $p < 0.05$.

Figure 4. **Lipid quantification in male and female offspring.**

Histograms displaying the quantification of the lipid classes in CTRL and VDD LF males (A), CTRL HF and VDD HF Males (B), CTRL and VDD LF females (C) and CTRL and VDD HF females (D).

Figure 5. Partial Least Squares Discriminant Analysis (PLS-DA) graph and Important features (VIP) identified by PLS-DA in males and percentage contribution of lipid species in CTRL and VDD males.

In the Cross-validated PLS-DA score plot the red spots represent samples from CTRL and green spots represent samples from VDD male Model Permutation test validation (1000 repetitions) $p < 0.05$ shows the separation achieve according to maternal deficiency with a

LF(A) and HF(C) diet. VIP demonstrating the effect of liver on lipidomic profiles. The coloured boxes on the right indicate the relative concentrations of the corresponding metabolite in each group under study LF (B) and HF (D).

Figure 6. **Partial Least Squares Discriminant Analysis (PLS-DA) graph and Important features (VIP) identified by PLS-DA in females and percentage contribution of lipid species in CTRL VDD females.**

In the Cross-validated PLS-DA score plot the red spots represent samples from CTRL and green spots represent samples from VDD female Model Permutation test validation (1000 repetitions) $p < 0.05$ shows the separation achieve according to maternal deficiency with a LF(A) diet and HF(B). VIP demonstrating the effect of liver on lipidomic profiles. The coloured boxes on the right indicate the relative concentrations of the corresponding metabolite in each group under study LF (C)and HF(D).

R2/Q2	CTRL LF and VDD LF	CTRL HF and VDD HF
Males	1,02824323	1,08092672
Females	1,18290739	1,1495463

Table 1. The ratio of R2/Q2, in both males and females, was determined using data from Metaboanalyst.

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Figure 1

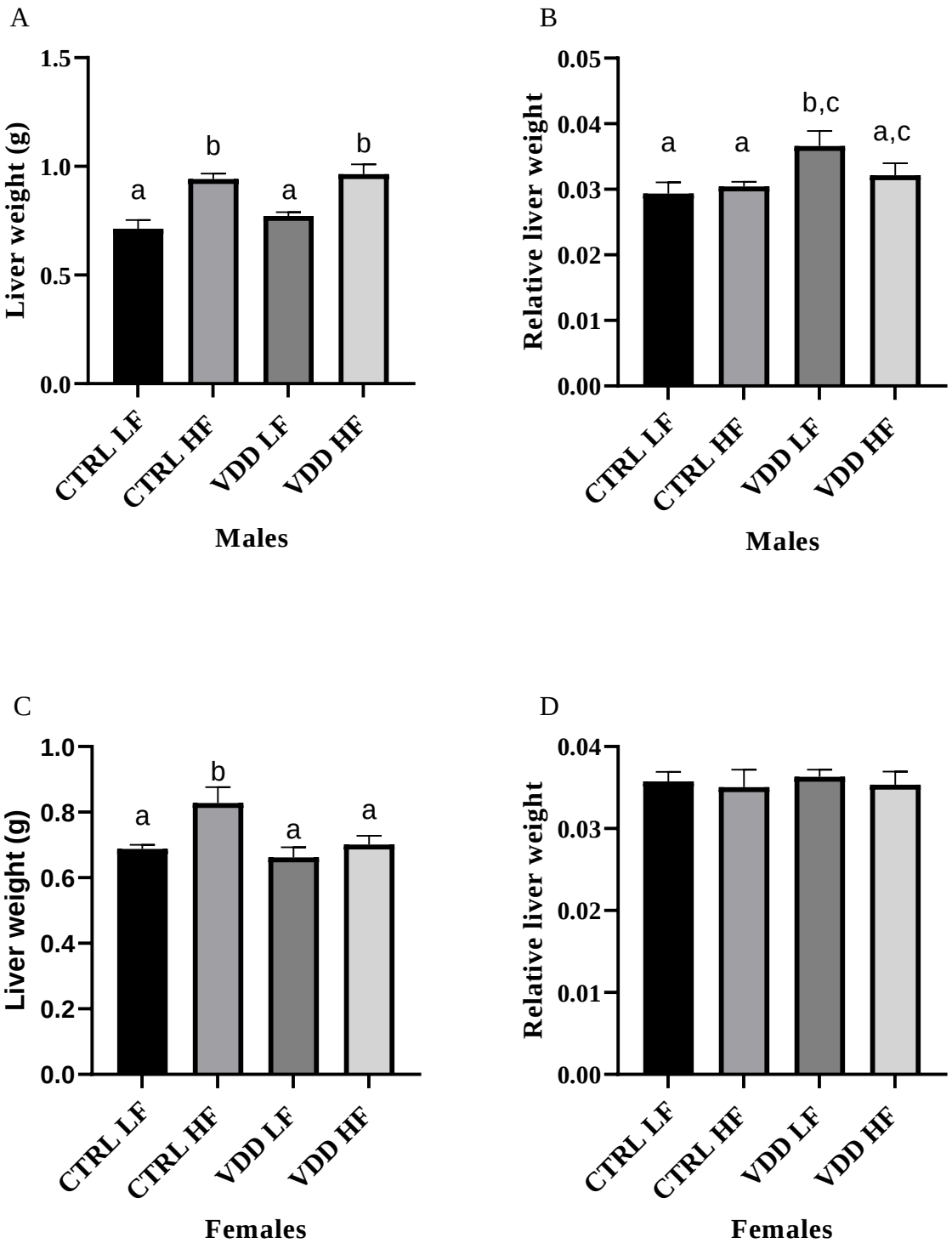

A
Liver weight (g)
1.5
1.0
0.5
0.0
a
b
a
b
CTRL LF
CTRL HF
VDD LF
VDD HF
Males
B
Relative liver weight
0.05
0.04
0.03
0.02
0.01
0.00
a
a
b,c
a,c
CTRL LF
CTRL HF
VDD LF
VDD HF
Males
C
Liver weight (g)
1.0
0.8
0.6
0.4
0.2
0.0
a
b
a
a
CTRL LF
CTRL HF
VDD LF
VDD HF
Females
D
Relative liver weight
0.04
0.03
0.02
0.01
0.00
CTRL LF
CTRL HF
VDD LF
VDD HF
Females

Figure 2

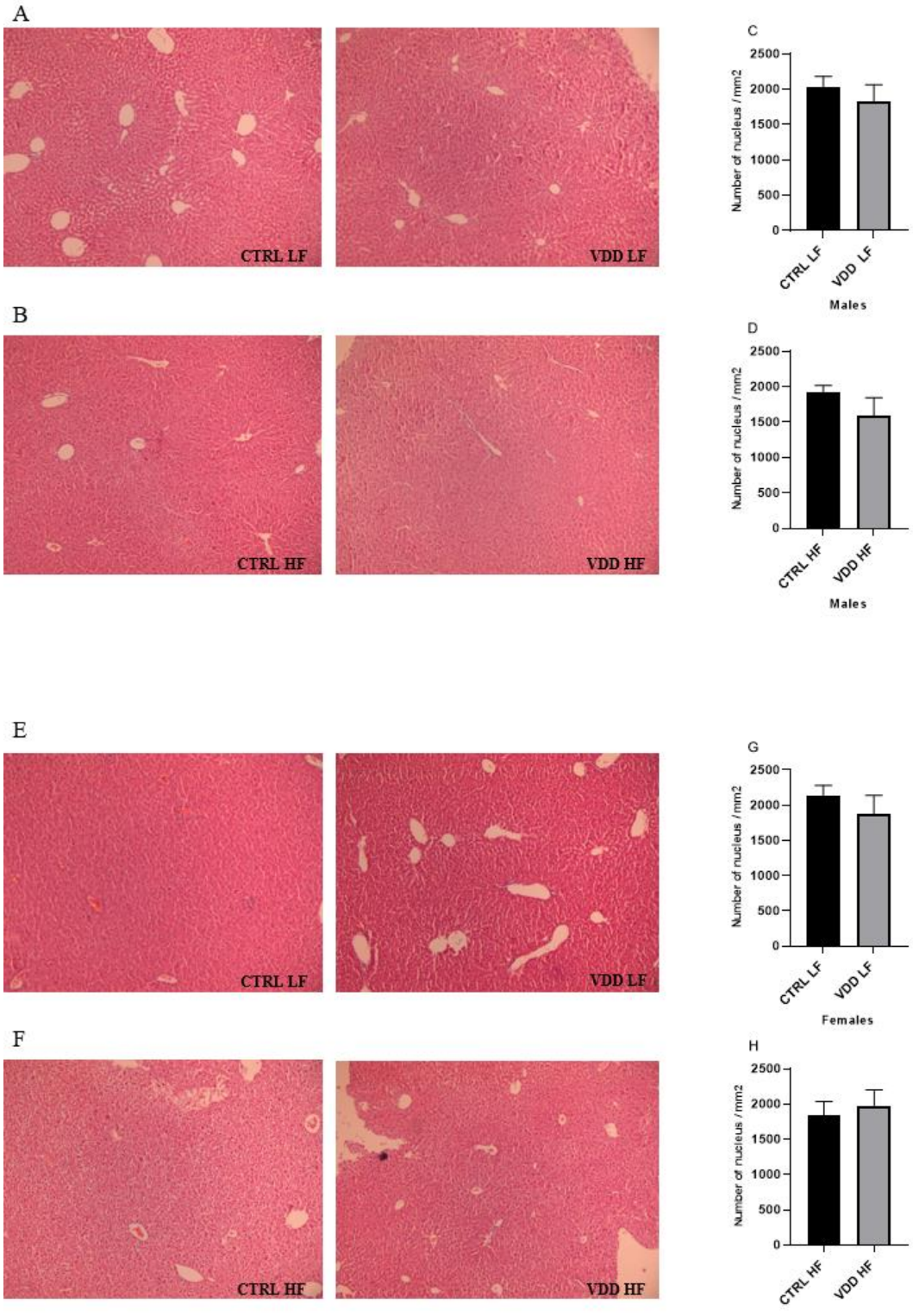

A
CTRL LF
VDD LF
B
CTRL HF
VDD HF
C
Number of nucleus / mm2
2500
2000
1500
1000
500
0
CTRL LF
VDD LF
Males
D
Number of nucleus / mm2
2500
2000
1500
1000
500
0
CTRL HF
VDD HF
Males
E
CTRL LF
VDD LF
F
CTRL HF
VDD HF
G
Number of nucleus / mm2
2500
2000
1500
1000
500
0
CTRL LF
VDD LF
Females
H
Number of nucleus / mm2
2500
2000
1500
1000
500
0
CTRL HF
VDD HF

Figure 3

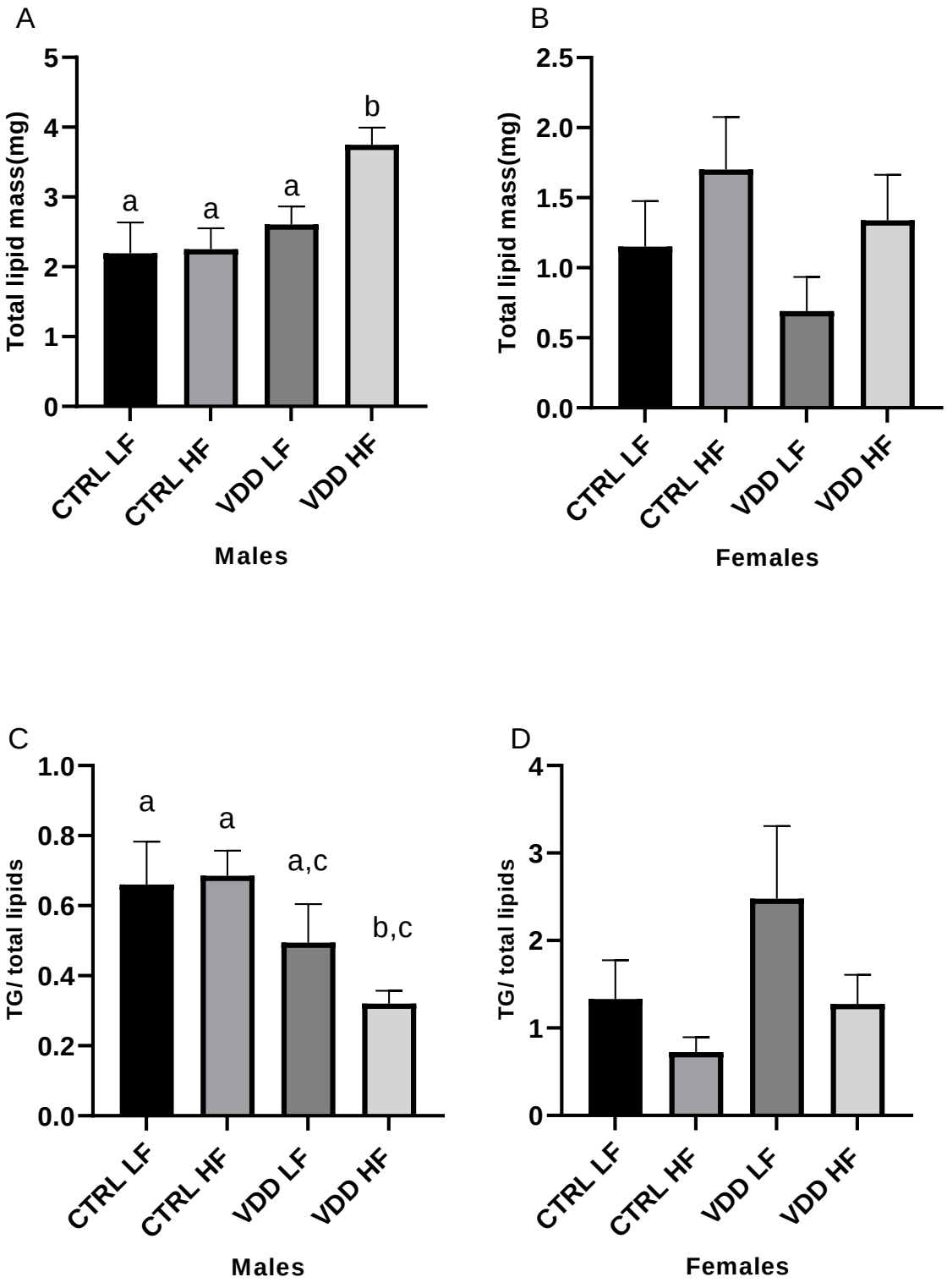

A
Total lipid mass(mg)
5
4
3
2
1
0
a
a
a
b
CTRL LF
CTRL HF
VDD LF
VDD HF
Males
B
Total lipid mass(mg)
2.5
2.0
1.5
1.0
0.5
0.0
CTRL LF
CTRL HF
VDD LF
VDD HF
Females
C
TG/ total lipids
1.0
0.8
0.6
0.4
0.2
0.0
a
a
a,c
b,c
CTRL LF
CTRL HF
VDD LF
VDD HF
Males
D
TG/ total lipids
4
3
2
1
0
CTRL LF
CTRL HF
VDD LF
VDD HF
Females

Figure 4

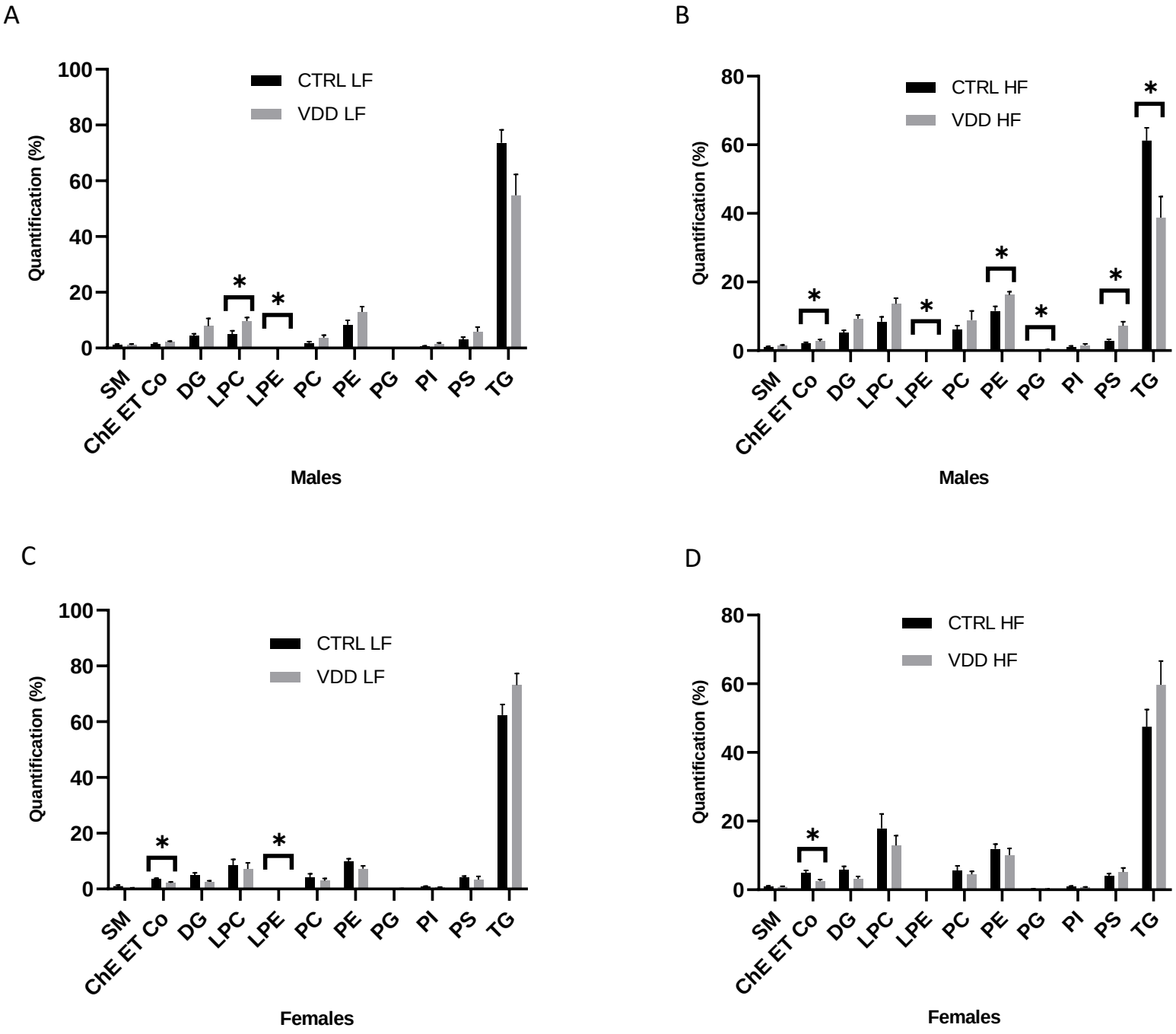

A
B
C
D
CTRL LF
VDD LF
CTRL HF
VDD HF
Quantification (%)
SM
ChE ET Co
DG
LPC
LPE
PC
PE
PG
PI
PS
TG
Males
Females

Figure 5

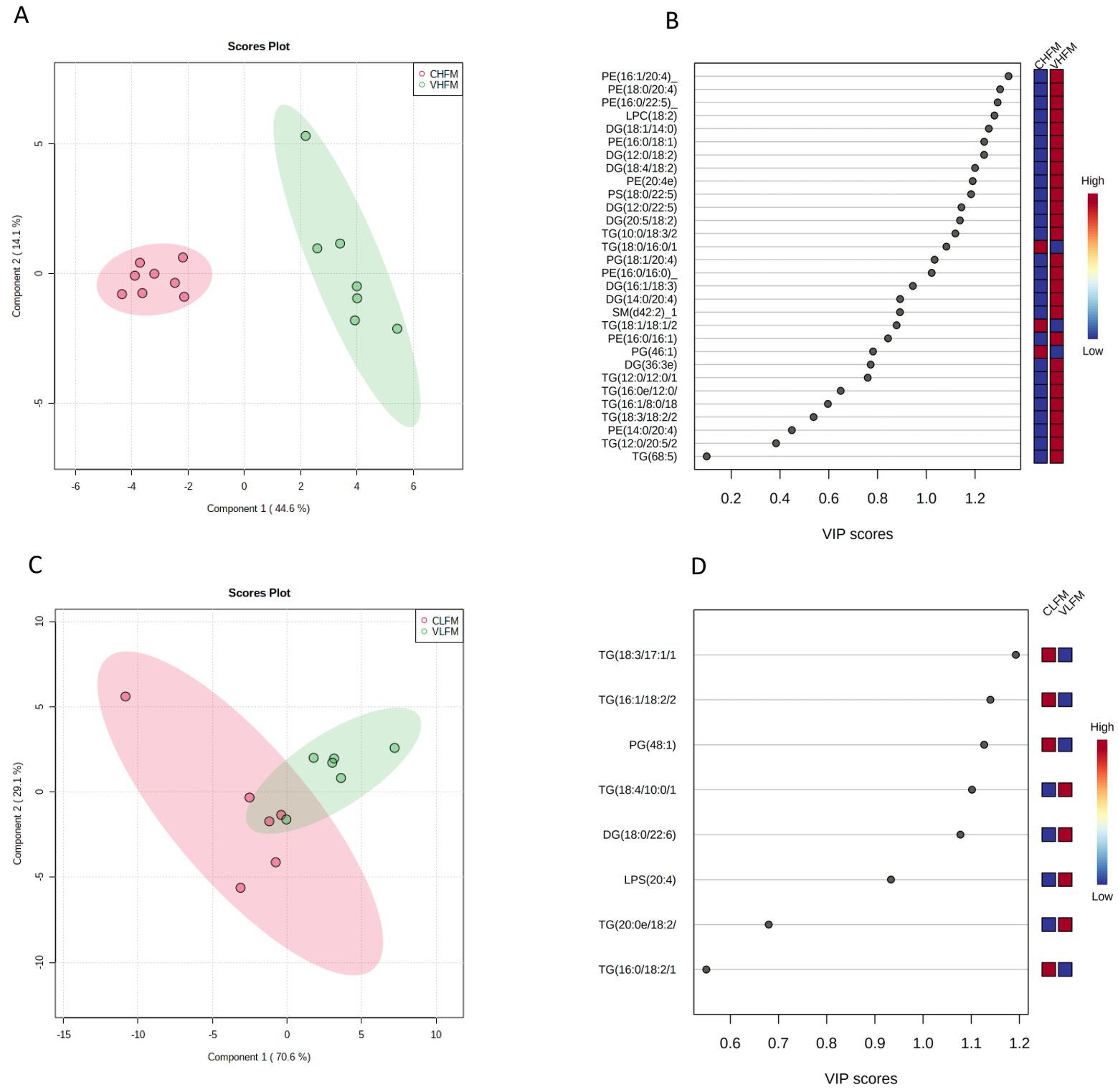
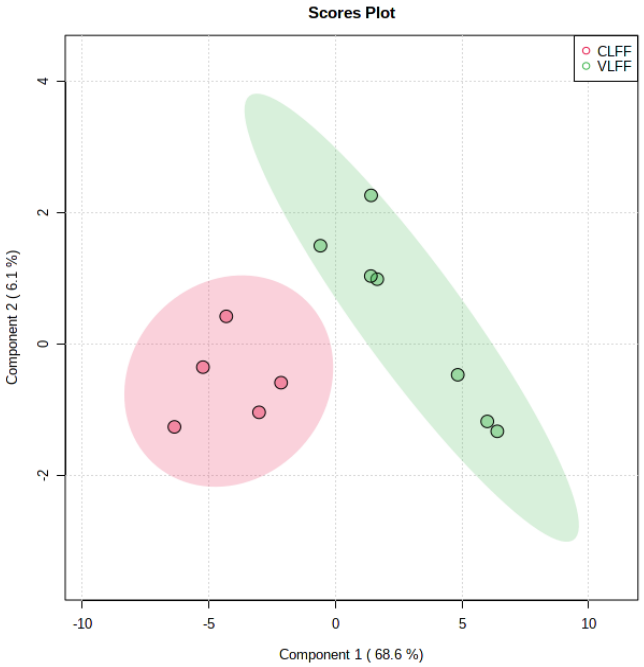
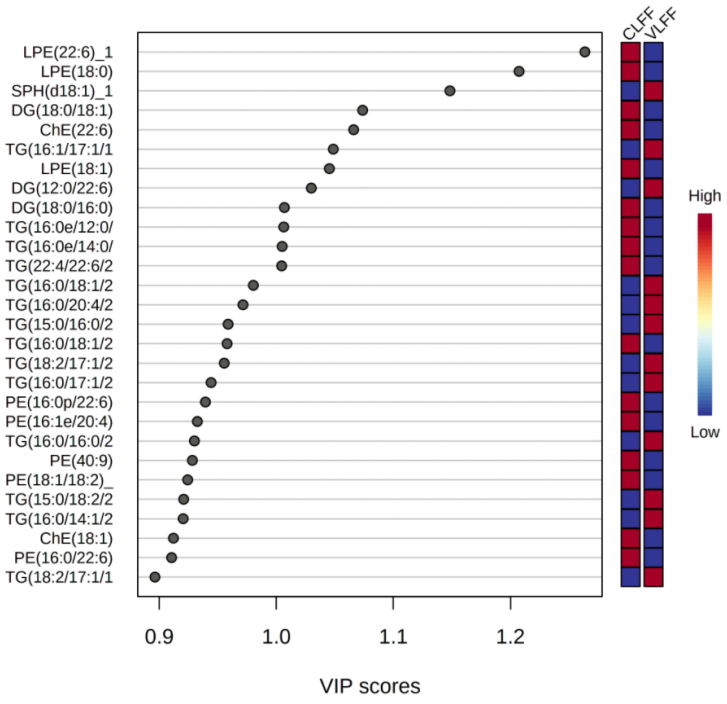

A
Scores Plot
CHFM
VHFM
Component 2 (14.1 %)
Component 1 (44.6 %)
B
PE(16:1/20:4)_
PE(18:0/20:4)
PE(16:0/22:5)_
LPC(18:2)
DG(18:1/14:0)
PE(16:0/18:1)
DG(12:0/18:2)
DG(18:4/18:2)
PE(20:4e)
PS(18:0/22:5)
DG(12:0/22:5)
DG(20:5/18:2)
TG(10:0/18:3/2
TG(18:0/16:0/1
PG(18:1/20:4)
PE(16:0/16:0)_
DG(16:1/18:3)
DG(14:0/20:4)
SM(d42:2)_1
TG(18:1/18:1/2
PE(16:0/16:1)
PG(46:1)
DG(36:3e)
TG(12:0/12:0/1
TG(16:0e/12:0/
TG(16:1/8:0/18
TG(18:3/18:2/2
PE(14:0/20:4)
TG(12:0/20:5/2
TG(68:5)
CHFM
VHFM
High
Low
VIP scores
C
Scores Plot
CLFM
VLFM
Component 2 (29.1 %)
Component 1 (70.6 %)
D
TG(18:3/17:1/1
TG(16:1/18:2/2
PG(48:1)
TG(18:4/10:0/1
DG(18:0/22:6)
LPS(20:4)
TG(20:0e/18:2/
TG(16:0/18:2/1
CLFM
VLFM
High
Low
VIP scores

Figure 6

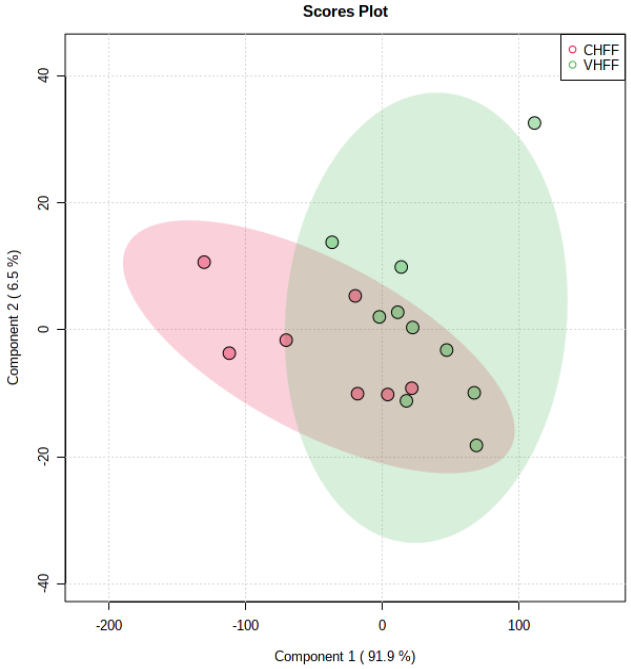
A



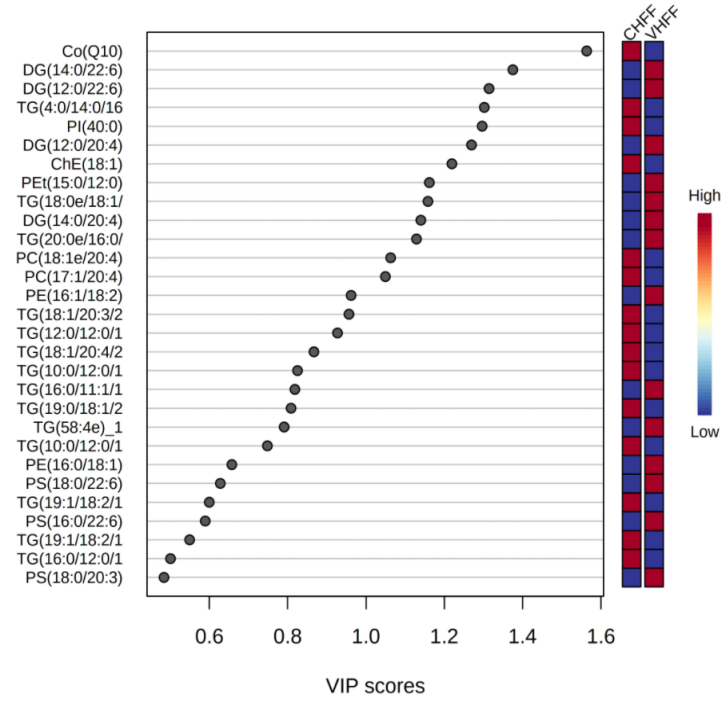
B



C



D



GENERAL DISCUSSION

Maternal diet is now recognized to play a significant role in foetal development and offspring metabolic programming according to the hypothesis of DOHAD (Barker & Osmond, 1986; Hales et al., 1991). In the seminal work of Barker and colleagues, it has been demonstrated that undernutrition during gestation was associated with an increased risk for cardiovascular disease in adulthood (Hales & Barker, 2001). This DOHaD paradigm has expanded and several studies showed that a wide range of nutrients can influence offspring development (Heindel & Vandenberg, 2015). It's worth noting that VDD is becoming a worldwide problem. In particular, it has been shown that VDD is associated with several obesity-related parameters, such as body composition percentage of lean mass and fat mass (Boyle et al., 2017; Crozier et al., 2012; Miliku et al., 2018), body weight (Daraki et al., 2018; Morales et al., 2015), adiposity index (Seipelt et al., 2020). Obesity is defined by excessive fat deposit in the WAT leading to inflammation and when the body is overwhelmed the fat accumulation will occur in ectopic deposit such as liver, where HS will occur (Figure 34). VD ability to program AT inflammation in offspring was motivated by the fact that it affects the inflammatory profile of AT in adults. In addition, low 25(OH)D plasma levels have been linked to obesity (Bonnet et al., 2019, 2021; Walsh et al., 2016) and HS (Barchetta et al., 2011). In this context, we wondered about the existing interactions between maternal VDD and HF diet as well as involved mechanisms involved in the programming of the WAT inflammation and hepatic fate of the offspring, in long term.

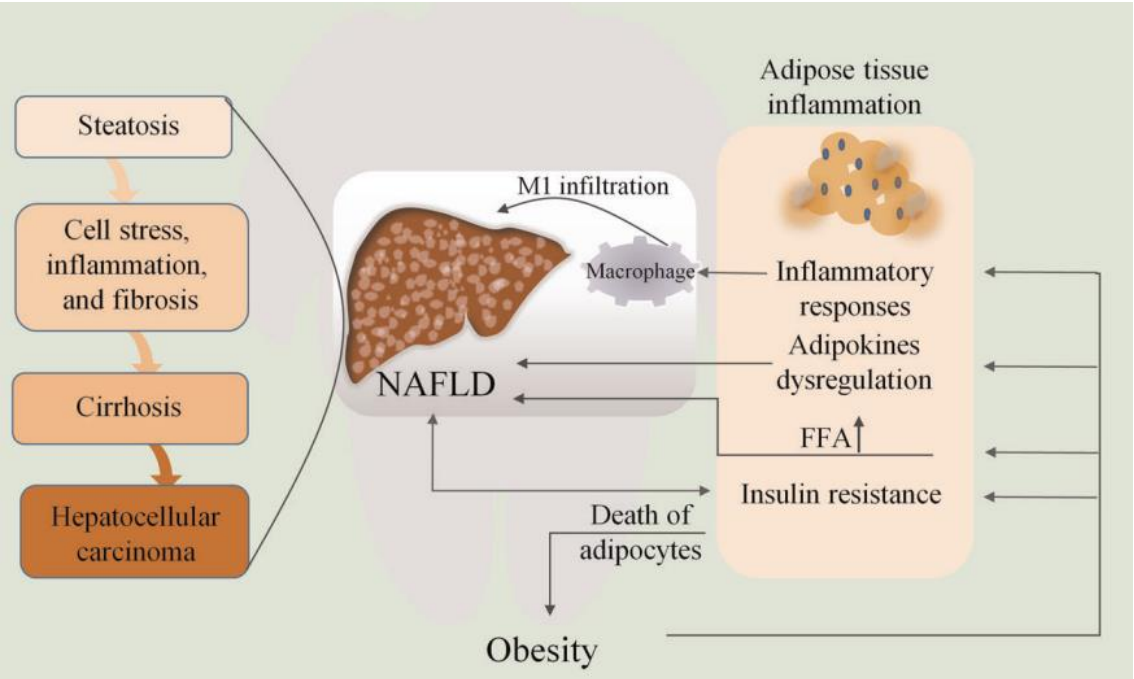


Figure 34: The relationship between obesity-induced AT inflammation and NAFLD (Y. Luo & Lin, 2021).

To test theses different hypotheses, we have set up a mouse model of mice fed a VD free diet for at least 8 weeks prior to coupling and during the gestation period. After delivering, all the mice were fed a control diet. At the age of six weeks, the offspring were randomly assigned to a LF or HF diet for eight weeks to study the combined effect of the maternal VDD and diet on the AT inflammation and the hepatic lipid accumulation.

In a first step, a morphological analysis was performed to assess the combined effect of VDD and HF. In line with our team's previously published data (Seipelt et al., 2020), we observed an increase in body weight in both CTRL and VDD males under HF diet. The adiposity index, which is a good marker of fat pads accumulation independent of total body weight, was unaffected by VDD in the LF condition, but was dramatically increased in the HF condition when compared to the other groups (CTRL LF, CTRL HF, and VDD LF). Females of the CTRL HF group had a higher body weight and adiposity index than the other groups (CTRL LF, VDD LF and VDD HF). Surprisingly, the VDD HF group had a body weight and adiposity index that were similar to the LF groups (CTRL LF and VDD LF) indicating a strong sex-specific metabolic response.

Since that adiposity or obesity is strongly associated with an increase in AT inflammation, we decided to assess the consequences in terms of inflammation in both males and females groups (Gregor & Hotamisligil, 2011). In this context, an RNA-seq approach was conducted on retroperitoneal AT to study the inflammatory response and impacted metabolic pathways were estimated using Gene Ontology enrichment analysis (GO) and Ingenuity pathway analysis (IPA). Based on the high number of pathways impacted in the different conditions, only pathways related to inflammation were implemented in our quantitative analysis.

When comparing the impact of maternal VDD in males on inflammatory pathways under LF diet (CTRL LF *vs* VDD LF), only two pathways were found to be significantly different, including a downregulation of "LPS/IL-1 Mediated Inhibition of RXR Function," implying that the effect of VDD in males is minor or null under normal diet. However, we found that two important pathways for AT biology (Tourniaire et al., 2013), *i.e.* "NF-κB activation by viruses" and "p38 MAPK Signaling" were upregulated in males on the HF diet (CTRL HF *vs* VDD HF), suggesting that VDD amplified inflammation mediated by HF. We compared the CTRL and the VDD in both LF and HF diets to highlight the impact of an HF diet on the inflammatory process in both CTRL and VDD situation. The inflammatory response was induced in CTRL LF vs CRTL HF, but this induction was even more remarkable in VDD males fed an HF diet compared to an LF diet. The "response to cytokines" pathway was activated in both of these conditions. This is a critical inflammatory pathway that contains 757 genes and encloses the

majority of the inflammatory response. 67 genes were differentially regulated in the CTRL LF *vs* CTRL HF comparison, whereas 120 genes were differentially regulated in the VDD LF *vs* VDD HF comparison, implying that the response is much more marked in animals born from VDD mice than in animals born from CTRL mice. A statistical *p*-value that was higher in the VDD animals than in the CTRL animals confirmed this qualitative response. Interestingly, the proinflammatory profile of the VDD HF males group matched with the adiposity index, which was higher in these animals. Overall, these findings suggest that VDD and HF have an additional effect in males when it comes to mediating AT inflammation.

In female group, the response to maternal deficiency was characterized by an upregulation of the "NRF2-mediated Oxidative Stress Response" which was the same in both CTRL LF *vs* VDD LF and CTRL HF *vs* VDD HF groups. Under the HF diet (CTRL LF *vs* CTRL HF), "chemokine signaling" and "response to cytokine" were upregulated in CTRL females, with 106 genes upregulated in the CTRL HF females. It's worth noting that those animals also had higher adiposity, implying that adiposity and inflammation are associated. In the VDD LF *vs* VDD HF analysis, no inflammatory pathway was overexpressed and there was no difference in adiposity between the two groups.

The miRNAs play a role in obesity-related AT inflammation (P. Arner & Kulyté, 2015; Ge et al., 2014; Karkeni et al., 2016; Landrier et al., 2019). In accordance with these observations, we investigated the miRNA profile and the predicted associated pathways. For the first time, it has been shown that maternal VDD altered miRNA expression profiles in both male and female offspring. In addition, this regulation appeared to be sex-dependent, with a higher number of miRNAs modulated in females. Further research will be required to determine the cause of this discrepancy. To go further, predicted mRNA targets of deregulated miRNAs were gathered and pathway enrichment analysis was performed using IPA. In males, only one up-regulated miRNA was found in the CTRL LF *vs* VDD LF, resulting in the negative enrichment of five pathways, including "IL6 Signaling" and "ILK Signaling" involving a decrease in inflammatory status in VDD LF males compared to CTRL LF males. The lower adiposity index (even if not significant) in VDD LF compared to CTRL LF males could explain this observation. Five miRNAs were down-regulated in VDD LF *vs* VDD HF mice, leading to positive enrichment of 7 inflammatory-related pathways, including "p38 MAPK" and "Chemokine Signaling" demonstrating that the pro-inflammatory tone is also observable at the miRNA level in male VDD mice fed with HF diet. The comparison of CTRL LF *vs* VDD LF in females resulted in 4 up-regulated miRNA and 16 putative target pathways, including "ERBB4 Signaling" and "p38 MAPK Signaling" an essential inflammatory pathway as previously mentioned, indicating that

VDD is associated with a decrease in inflammatory status at the miRNA level, even though no morphometric parameters, such as adiposity, support such assumption. Further research into the origins of this phenotype is required. Nevertheless, it is noteworthy that mR-146a-5p and mir-322-5p were both up-regulated within the 4 up-regulated miRNA. MiR-146a-5p suppresses the inflammatory response in human adipocytes (Roos et al., 2016) and miR-322-5p targets NF-κB1 and suppresses inflammatory cytokine production while promoting cell proliferation in LPS-stimulated murine macrophages (K. Zhang et al., 2017), suggesting that even if pathway analysis revealed an upregulation of inflammatory pathways, miRNA at the origin of these putative enriched pathways have anti-inflammatory properties. Similarly, 16 pathways were predicted to be up-regulated in the CTRL HF vs VDD HF in females based on the down-regulation of one miRNA. Ten pathways were predicted to be up-regulated in the VDD LF *vs* VDD HF females based on the downregulation of three miRNA while 19 pathways were predicted to be down-regulated in the CTRL LF *vs* CTRL HF females (driven by the regulation of one miRNA). Surprisingly, all of these findings appeared to contradict morphometric parameters, particularly the adiposity index. The cause of such inconsistency is currently unknown, but it could rely on predictive approach that will necessitate further optimizations.

Regarding molecular mechanisms, RNA sequencing and miRNA analysis, as well as bibliography, strongly point to the putative roles of NF-κB and p38/MAPK signaling pathways. Indeed, NF-κB and p38 are known to play a key role during AT inflammation (Hernandez & Zhou, 2021; Leiva et al., 2020). In addition, we and others have showed that the ability of VD to reduce inflammation in adipocytes and AT is linked to phosphorylation of these two signaling pathways (Gao et al., 2013; Karkeni et al., 2015; Lorente-Cebrián et al., 2012; Marcotorchino et al., 2012; Mutt et al., 2012). We measured the phosphorylation levels of p65 and p38 in AT to see if NF-κB and/or p38 signaling were involved. In comparison to CTRL LF males, no activation of p65 or p38 was observed in the VDD LF, suggesting that the VDD alone does not activate inflammatory pathways, which is consistent with pathway analyses. Interestingly, a massive phosphorylation of p65 was observed, indicating that VDD and HF diet have additional effects on inflammatory pathways and adiposity index in males. The CTRL HF group showed a significant increase in p38 phosphorylation, which is consistent with the fact that this pathway is known to be activated during obesity (Mutt et al., 2012). Despite this, there was no evidence of VDD having an effect on the pathway. In Female VDD mice under LF or HF diets, there is no induction of p65 or p38. The only induction of p65 has been found in CTRL HF which had the higher adiposity and activated inflammatory-related pathways.

The mechanisms that caused VDD to affect the inflammatory process in AT are still unknown. Nevertheless, epigenetic mechanisms may be able to explain at least some of the observed phenotype. Several Dups proteins have been shown to be involved in the dephosphorylation of stress-activated kinases (Lang & Raffi, 2019). It would be interesting to look into the epigenetic landscape of genes encoding these proteins.

To summarize, our findings establish a link between the increased adiposity in males born from VDD mice and fed with a HF diet, that correlates with induction of mRNA and miRNA linked to inflammation and activation of p65 phosphorylation, whereas such relationship was not observed in females. These data add to our understanding of maternal VDD influence on the offspring, particularly its predisposition to long-term metabolic health issues. It also highlights the sex-specific AT and inflammatory response that must be considered in terms of public health.

Since VD is able to blunt hepatic steatosis (Marziou et al., 2020), we hypothesized that maternal VDD impacted the liver lipid metabolism in the offspring and possibly potentialized the effect of HF diet on HS. In our maternal VDD mice model, the HF diet conditions (45% HF diet for six weeks) were chosen to mediate a challenging environment, suitable to mediate steatosis but without clear induction of HS, since we wanted to test the combined effect of HD diet and VDD.

A first analysis was conducted at the morphological level. While liver mass appeared to increase in both the CTRL and VDD groups on the HF diet, relative liver mass increased only in males born from VDD who were fed an LF diet, implying that the observed increased liver mass on the HF diet was due to an overall mass gain, with the exception of the VDD LF group, where a specific liver mass gain occurred. In the CTRL group, females had an increase in liver mass while on the HF diet, but this was lost after normalization to body mass, suggesting that VDD or diet have no effect on liver mass. This original finding suggested that VDD combined with diet differentially programmed liver metabolism in a sex-specific manner. To go further, we performed a histological study in both groups and found no significant differences in liver histology in both groups of males and females (CTRL LFVDD LF and CTRL HF VDD HF). There is no sign of HS in any of the groups according to the classification grade of the disease. Overall, these findings showed that the VDD in combination with diet had a minor effect on liver morphology and histology at the macroscopic level. This finding contradicts a previous study on the effect of maternal VDD on the lipid hepatic metabolism of the offspring, which found that females born to VDD rats were more prone to steatosis (Sharma et al., 2017). A variety of explanations could explain this discrepancy. First, our study was conducted in mice

rather than rats, which may have influenced the VDD-related phenotype. Second, the VDD diet was maintained in the offspring until weaning, whereas in our study, mice were given a normal diet after delivery to limit the impact of VDD to the intrauterine period (no impact through lactation). Third, the animals with steatosis were observed at 30 weeks of age, suggesting that our study was too short to detect such phenotype.

To go further on the combined effects of VDD and diet on liver lipid metabolism, we conducted experiments to characterize lipids in the liver of the different groups. To start, we quantified the total amount of lipids in the liver. This analysis revealed that males in the VDD HF groups had a significant accumulation of total lipids in the liver, indicating that even in the absence of steatosis, the combination of maternal VDD and HF diet resulted in lipid accumulation in males only. This condition could be temporary and be classified as hepatic pre-steatosis, which could progress to hepatic steatosis. This last assumption will need to be confirmed through further investigation.

The main lipid class found in the liver is TG (around 75% of total lipids). Thus, we measured TG and discovered that only the VDD HF group had a lower TG/total lipids ratio in males. Since total lipids were higher in this group, it was unclear whether the lower ratio was due to the higher total lipids or to a lower TG level. By means of lipidomic analysis, we quantified several classes of lipids including TG. In comparison to CTRL LF, VDD LF had a higher percentage of LPE and LPC. The impact of such minor alterations in liver physiology remain still unknown. Interestingly, the most contrasted condition, in agreement with other measurements is the VDD HF compared to VDD LF in males. Indeed, the TG percentage decreased in VDD HF in males compared to CTRL HF. Such observation is not in agreement with studies reporting that HS is associated with higher levels of TG (Kartsoli et al., 2020). Most of the lipids that participate to membrane composition, such as PE, PG, PS, were increased in the VDD HF compared to the VDD LF, which could be explained by a decrease in TG accumulation in hepatocytes and a proportional increase in lipid membranes.

In female groups, very modest changes in lipid classes were identified, indicating that VDD with an LF or HF diet has a very limited influence on female offspring in our conditions, contrarily to previous published results (Sharma et al., 2017). Investigations on the cause of such a lack of effect in females will be required. Nevertheless, the sexual hormones such as estrogens could explained the protection of females to hepatic steatosis (Hart-Unger et al., 2017).

PLS-DA was used in the liver to find lipids that differentiated across groups of mice. Only 8 lipids were discovered to be different in the comparison between VDD LF and CTRL LF.

The consequences of the small changes in liver metabolism are unknown. With low discrimination, the samples were not properly separated. In these small number of lipids, no specific pattern was observed, suggesting a low physiological relevance of such discrimination. The samples were well differentiated between CTRL HF and VDD HF males since 30 lipid species differently expressed with 27 greater in the VDD HF group. The PE family accounts for 8 of the 27 lipids. Several additional membrane lipids, such as PS, PG, and LPC, were also found to be greater in VDD HF. These amphipathic lipids, which make up the majority of phospholipids in mammals, are essential components of cellular membranes and perform a structural role. Because these lipids have increased in proportion, it's possible that the ratio between membranes and storage lipids (TG and DG) has changed. This hypothesis is supported by data on the measurement of this class of lipids. Nevertheless, it has been demonstrated in a 14 weeks mice study fed 45% HF, that PE tended to be reduced in the liver (Eisinger et al., 2014).

It is noteworthy that we combined the effects of the HF diet with maternal VDD in our study, suggesting that maternal deficiency may cause distinct lipid accumulation programming than the HF diet alone. We discovered that 8 DG were discriminants between groups and were induced in the VDD HF group compared to the VDD LF group associated with an increase in phospholipids. This observation supports the idea that phospholipids are induced in VDD HF since DG can be used as precursors of phospholipids. In addition, DG are also precursors of TG, thus if DG are engaged in the way of phospholipids they are less available to participate to the production of TG, supporting at least in part their decrease in VDD HF group.

The results obtained from females in terms of group discrimination revealed that the lipid species differs between the two groups CTRL *vs* VDD in both diet LF and HF, suggesting that the influence on female offspring in our conditions is relatively limited. Indeed, we found that the same types of lipids (for instance TG) were up- or down-regulated in both comparisons, but with no clear tendency. In addition, it is important to note that the ability of our models to predict groups was relatively low, even if statistically significant. Finally, the relative quantity of discriminants lipids between groups was extremely low, suggesting a very limited biological impact.

To summarize, our findings show a link between increased total lipid mass in males born from VDD mice and fed an HF diet, as well as a reduction in TG quantity and an increase in lipid membranes, resulting in hepatic lipid metabolism programming, but no such correlation was found in females. The origins and modalities of maternal programming are yet unknown and more research is needed. Nonetheless, we can assume that VDD related epigenetic

pathways have an impact on liver biology. These findings add to our understanding of the effects of maternal VDD on offspring, as well as the combined effect of an obesogenic environment. It also shows the sex-specific hepatic response that must be considered in terms of public health.

Altogether, these data related to the combined effect of maternal VDD and HF diet on AT inflammation and liver lipid accumulation are fully in agreement with the relationship previously described in the beginning of the discussion, between AT inflammation and the pathogenesis of NAFLD. Indeed, in males born of VDD mice and fed a HF diet, we observed an impact on both tissues, i.e. an induction of inflammation in AT and in parallel an impact on the lipid accumulation in the liver. The major point which remains to be demonstrate is the link and the causality between these two evens. Based on the literature related to obesity, it is highly probable that a causality occurs, nevertheless in the context of VDD it remains to be established. In addition, it also shed light on the putative sex-specific effect in this relationship, since males and females clearly do not response in the same way.

CONCLUSION AND PERSPECTIVES

In this thesis, we have established the combined impact of maternal VDD and HF diet in mice on WAT inflammation and hepatic lipid accumulation in the offspring.

To summarize, our data established a link between the increased adiposity in males born from VDD mice and fed with a HF diet, that correlates with induction of mRNA and miRNA expression linked to inflammation and activation of p65 phosphorylation, whereas such relationship was not observed in females. These data add to our understanding of maternal VDD influence on the offspring, particularly its predisposition to long-term metabolic health issues.

Moreover, our findings shed light on the sex-specific hepatic lipid response, which must be considered in the public health implications of maternal VDD in offspring. Our results showed a link between increased total lipid mass in males born from VDD mice and fed an HF diet, as well as a reduction in TG quantity and an increase in lipid membranes, resulting in hepatic lipid metabolism programming, but no such correlation was found in females.

The origin of the mechanisms that triggered the effect of VDD on both tissues remains vague. Nonetheless, we can speculate the epigenetics mechanisms may be able to explain at least some of the observed phenotype. Epigenetics is a term that refers to a group of processes that alter DNA or chromatin architectural proteins (histones). Gene expression, chromatin compaction, and DNA repair are all regulated by these processes. Epigenetics refers then to the mechanisms by which a cell can adapt its response to changing environmental conditions. As a result, the epigenetic landscape functions are a fine-tune regulator of cellular responses in response to the cell energetic, metabolic and physiological conditions.

In one-part, epigenetic mechanisms may be able to explain at least some of the observed phenotype in AT. Thus, the mechanisms that caused VDD to affect the inflammatory process in AT and the discrepancy in the miRNA results in both males and females. Along with several Dups proteins that have been shown to be involved in the dephosphorylation of stress-activated kinases (Lang & Raffi, 2019), which it would be interesting to look into the epigenetic landscape of these proteins genes. In another part it would be important to look at the epigenetic processes of VDD influence on liver biology and especially lipid metabolism.

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ANNEXES

Received: 23 February 2022 | Accepted: 18 March 2022

DOI: 10.1111/obr.1345

REVIEW

OBESITY Reviews WILEY

Recent insights into vitamin D, adipocyte, and adipose tissue biology

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Summary

Several studies bring strong evidence for an active role of vitamin D and its metabolites in physiological adipocyte and adipose tissue processes in adulthood. This role includes effects of vitamin D on key adipose tissue and adipocyte biology parameters, including adipogenesis, energy metabolism, and inflammation. Interestingly, recent data also point to a role of maternal vitamin D deficiency in adipocyte and adipose tissue metabolic programming in offspring. This review summarizes the current state of knowledge on the biological effect of vitamin D on adipocyte/adipose tissue physiology.

KEYWORDS

maternal programming, micronutrients, obesity, preventive nutrition

1 | INTRODUCTION

Vitamin D (VD, used here as a generic term) is particularly well known for its key role as a regulator of phosphate and calcium homeostasis and is essential for bone and muscle health.[1] There are also regular reports of several extraskeletal benefits of VD, including for cardiometabolic health.[1,2] Results from a huge number of human, animal, and in vitro studies on cardiometabolism all point to a relationship between VD and obesity/adiposity. Even if these results are contrasted, most of these studies bring strong evidence for a key role of VD in adipose tissue physiology. Here, we review the current knowledge on the effect of VD on adipocyte and adipose tissue biology in adulthood and the role of maternal VD deficiency on adipose tissue biology programming in offspring.

2 | METABOLISM OF VD IN ADIPOCYTES AND ADIPOSE TISSUE

2.1 | Storage, uptake, and release of VD and metabolites by adipocytes and adipose tissue

Adipose tissue is considered a major reservoir for VD, where it is mostly found as cholecalciferol, 25(OH)D, and the 1,25$(OH)_2$D form[3–8] (Figure 1). Indeed, more than 65% of total-body VD is present in the form of vitamin D_3. Adipose tissue pools 73% of this total-body vitamin D_3 and 34% of 25(OH)D.[9] In humans, visceral fat contains 20% more VD than subcutaneous fat,[10] but subcutaneous adipose tissue also contains 25(OH)D.[11] The distribution of VD between visceral and subcutaneous fat is similar between subjects with or without obesity, whereas subjects with obesity have larger VD stores in adipose tissue.[12] Cholecalciferol accumulation in adipose tissue varies widely between individuals and is not correlated to plasma 25(OH)D levels,[6] whereas 25(OH)D content in adipose tissue seems to correlate to

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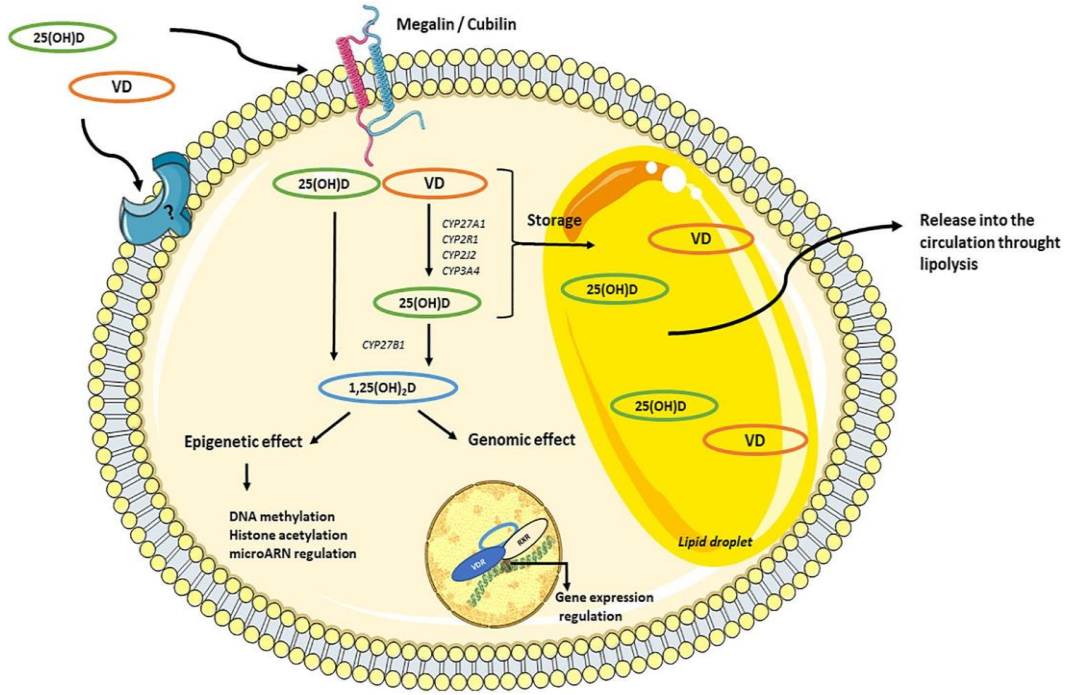

FIGURE 1 Vitamin D metabolism in adipocytes. The uptake of 25(OH)D and VD in adipose tissue is mediated by the megalin/cubilin pathway. All the enzymes necessary for 25-hydroxylation (CYP27A1, CYP2R1, CYP2J2, and CYP3A4) and 1α-hydroxylation (CYP27B1) are found in the adipose tissue. In addition to its genomic effect, $1,25(OH)_2D$ can also have epigenetic effects in adipose tissue. VD and 25(OH)D can be stored in lipid droplets and could be released into circulation during lipolysis

plasma 25(OH)D levels.[11] In adipose tissue, VD and its metabolites are located in adipocyte lipid droplets.[7]

The molecular mechanism governing cholecalciferol uptake in adipose tissue and adipocytes is still unclear, but evidence suggests 25(OH)D uptake may be mediated by the megalin/cubilin pathway.[13] We recently published data pointing to cubilin, which is responsible for sequestration of the VDBP-25(OH)D complex before internalization by megalin, as a major protein in 25(OH)D uptake in adipocytes and in adipose tissue of mice.[14] However, other mechanisms involving cholesterol transporters in the intestine[15] or other phytochemicals in adipose tissue[16] cannot be ruled out.

VD is known to release slowly from adipose tissue into circulation,[3] but the molecular mechanisms involved have not been fully unraveled. Lipolysis appears to be involved in this process, and dysfunctional adipose tissue of subjects with obesity presented a reduced, catecholamine-induced release of VD and 25(OH)D compared with lean subjects.[17] In addition, the β-adrenergic-stimulated release of $1,25(OH)_2D$ by abdominal subcutaneous adipose tissue is blunted in men with obesity.[18]

2.2 | Hydroxylations of VD and its metabolites in adipose tissue and adipocytes

Interestingly, all hydroxylations and associated enzymes involved in VD metabolism are expressed in adipocytes and in adipose tissue. Zoico et al[19] demonstrated that the 25-hydroxylation was functional in 3T3-L1 adipocytes and that CYP27A1 was expressed in these cells. This enzyme, together with CYP2R1, considered as the key enzyme for 25-hydroxylation,[20–22] CYP2C11, CYP3A4, and CYP2J2 in humans[23–25] displays 25-hydroxylation activity for VD. The expression of CYP27A1, CYP2R1, and CYP2J2 was confirmed in human adipose tissue, suggesting its ability to convert VD to 25(OH)D.[26] The 1α-hydroxylation also occurs in adipocytes. This step was initially demonstrated through the identification of radiolabeled 1,25(OH)2D derived from labeled 25(OH)D in 3T3-L1[27] and then further confirmed later on.[28,29] The expression of CYP27B1, the key enzyme for 1α-hydroxylation,[30] was found in 3T3-L1 adipocytes in basal conditions and demonstrated to be induced by cholecalciferol.[19] CYP27B1 expression has also been confirmed in human adipose tissue biopsies.[26] Finally, CYP24A1 expression, which mediates 24-hydroxylation,[31] has been detected in both mouse and human adipocytes[26,27,29] and is induced by $1,25\ (OH)_2D$ incubation.[27] Taken together, these lines of evidence suggest that adipose tissue and adipocytes are involved in VD metabolism and act as a target tissue able to synthesize 25(OH)D and $1,25\ (OH)_2D$ that may be locally active via paracrine, autocrine, or intracrine processes.

2.3 | Regulation of VD metabolism in adipose tissue and adipocytes

2.3.1 | Effect of VD supplementation on its own metabolism

The impact of VD or its active metabolites on regulation of its own metabolism has been under-researched, but a handful of reports have shown that $1,25(OH)_2D$ regulates some enzymes in cell culture models.[19,27] To address this gap, we recently evaluated the effect of short-term (4 days) cholecalciferol supplementation on VD metabolism in adipose tissue in mice. We observed a decrease in Cyp27a1, Cyp24a1, and cubilin mRNA in epididymal adipose tissue of supplemented mice,[14] suggesting that VD regulates in own metabolism by reducing 25(OH)D uptake and 25-hydroxylation to avoid overaccumulation of 25(OH)D in adipose tissue.

2.3.2 | Effect of obesity on VD metabolism in adipose tissue and adipocyte

The impact of obesity on VD metabolism in adipose tissue has been studied in mice. Several modulations of gene expression were reported, including increased Cyp27a1, Cyp2j3, and Vdr in visceral adipose tissue.[32] We previously reported that obesity in mice induced Cyp2r1 expression and accumulation of 25(OH)D in adipose tissue,[33] suggesting that diet-induced obesity could transcriptionally—and not just passively as widely assumed—mediate VD capture and conversion to 25(OH)D. Interestingly, we showed that this induction of Cyp2r1 was observed under a short (4 days) high-fat diet. This short-term induction of Cyp2r1, together with the reported induction of Cyp27a1 and decrease of Cyp27b1, are supportive of an early active mechanism leading to increased capacity for 25(OH)D storage in adipose tissue.[34] Note that a similar process of Cyp2r1 induction was recently described in adipose tissue of obese mice.[35] Conversely, in humans, Wamberg et al[26] reported that obesity was characterized by a decrease of CYP2J2 and CYP27B1 expression in subcutaneous adipose tissue. The source of this discrepancy remains unknown.

2.4 | Mechanisms of action of VD in adipocytes and adipose tissue

The nuclear receptor vitamin D receptor (VDR) is known to mediate the bulk of biological effects of $1,25(OH)_2D$.[36] Its ubiquitous distribution explains why $1,25(OH)_2D$ directly or indirectly regulates over a thousand genes.[36] VDR heterodimerizes with retinoid X receptor (RXR) and binds to DNA at sites called vitamin D response elements (VDRE), which are located in the promoter regions of regulated genes. In absence of ligand, this heterodimer complexes with corepressors and histone deacetylases, whereas in presence of $1,25(OH)_2D$, it recruits co-activators and histone acetyltransferases leading to transcriptional activation.[37] Interestingly, the VDR is widely found in adipocytes,[38,39] which

definitively supports the genomic effect of VD metabolites in adipocytes. Moreover, VDR expression is increased in adipose tissue biopsies of subjects with obesity compared with biopsies from lean subjects.[26,40,41]

Non-genomic effects of VD have been described in several cell types and are characterized by very fast (seconds to minutes) activation of signaling pathways, such as phospholipase C and phospholipase A2, phosphoinositide 3-kinase, protein kinase A, and mitogenactivated protein kinases. These effects also include the opening of Ca^{2+} and Cl channels. These non-genomic effects of VD are dependent on protein disulfide-isomerase family A, member 3 (Pdia3, also known as ERp57, GRP58 and 1,25-MARRS).[42–46] Pdia3 is expressed in adipocytes, but a non-genomic effect of VD involving this protein in adipocytes has not yet been reported,[47] and further investigation is warranted.

Evidence of epigenetic effects of VD or its active metabolites are beginning to emerge.[48] These effects include DNA methylation, possibly through the modulation of DNA methyltransferases and/or DNA demethylase expression.[49] VD can also regulate histone acetylation via activation of histone acetyltransferases and histone deacetylases, but also histone methylation and demethylation, thus enabling it to modulate chromatin accessibility for transcription factors.[50] There is also evidence that VD is involved in regulating the expression of micro-RNA (miRNA),[50] and we recently found that $1,25(OH)_2D$ downregulated inflammation-linked miRNA expression in adipocytes in vitro and in vivo.[51] Effects of VD or its metabolites on the other epigenetic mechanisms remain to be studied.

3 | BIOLOGICAL EFFECTS OF VD ON ADIPOCYTES AND ADIPOSE TISSUE

3.1 | Impact of VD or its metabolites on energy metabolism-related gene expression in adipose tissue and adipocytes

The impact of VD or its active metabolite ($1,25(OH)_2D$) on the regulation of several genes linked to energy metabolism has already been studied (Figure 2). $1,25(OH)_2D$ was found to induce leptin expression and secretion by adipose tissue[52] whereas it repressed adiponectin expression.[53,54] $1,25(OH)_2D$ also improved glucose metabolism via an upregulation of Glut4 expression and translocation to the cell surface,[55] possibly via an insulin-independent signaling pathway involving Sirt1 and AMPK,[56] and leading to an increase in glucose uptake by adipocytes.[57] $1,25(OH)_2D$ also has effects on lipid metabolism. $1,25(OH)_2D$ upregulates several genes related to β-oxidation and lipolytic enzymes and thus reduces intracellular fat accumulation in adipocytes.[58,59] Note that the ability of $1,25(OH)_2D$ to induce lipid oxidation in adipose tissue has been confirmed in a cyp2r1-deficient zebrafish model.[60,61] Furthermore, even though the molecular mechanism has not been not fully elucidated, VD supplementation in mice plays a role in the induction of lipid catabolism, notably in brown adipose tissue.[62] Furthermore, $1,25(OH)_2D$ reduced the utilization of glucose as a substrate for fatty acid synthesis, possibly through a downregulation of pyruvate carboxylase.[59] $1,25(OH)_2D$ was also found to down-regulate Elovl3 expression, suggesting it can potentially impact fatty acid composition in adipocytes.[63]

Effects of 1,25(OH)$_2$D were recently demonstrated in brown adipocytes, where it inhibited differentiation and mitochondrial respiration, possibly through a suppression of PPARγ transactivation.[64] Furthermore, 1,25(OH)$_2$D repressed UCP1, 2, and 3 expression levels in brown adipocytes via a mechanism involving VDR and the induction of the corepressor hairless protein.[65] Note that the role of 1,25 (OH)$_2$D in regulation of UCP1 remains controversial, since it has been shown that unliganded VDR can down-regulate UCP1 by itself, independently of its ligand.[66] This observation is fully consistent with studies showing UCP1 expression was increased in VDR$^{/}$ mice,[67,68] whereas overexpression of human VDR in mice adipose tissue reduced UCP1 expression.[69]

VDR$^{/}$ mice have been studied to gain insight into the role of VD metabolism in body weight management and adipose tissue biology.[67,68,70–72] VDR$^{/}$ mice are lean and resistant to diet-induced obesity. This phenotype is strongly linked to induction of fatty acid oxidation and uncoupling proteins (including UCP1, 2, and 3) in adipose tissue leading to increased energy expenditure. Even if these data strongly suggest that VDR deletion improves energy homeostasis, it is must be kept in mind that there are several caveats, notably the fact that the mice were fed a rescue diet containing large amounts of calcium, which is suspected to regulate energy homeostasis.[73] In addition, VDR expression was knocked out from the entire mouse, making it impossible to attribute the overall phenotype to a specific tissue.[30,74] Finally, VDR$^{/}$ mice develop alopecia, which could increase energy expenditure to maintain body temperature due to reduced insulation.[75]

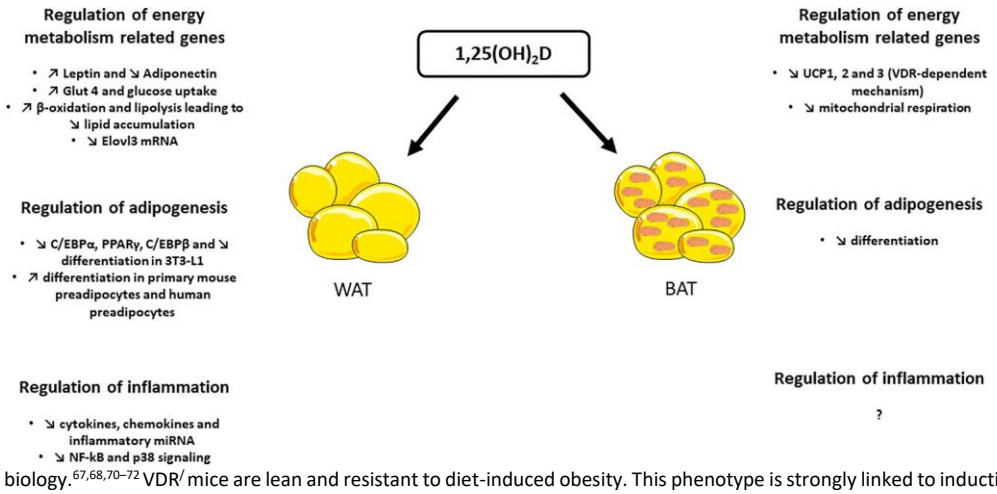

FIGURE 2 Metabolic effects of 1,25(OH)$_2$D in adipocytes. 1,25(OH)$_2$D has various biological effects in white adipose tissue (WAT) or adipocytes and in brown adipose tissue (BAT) or adipocytes

Other studies have been conducted using targeted overexpression or knockout of VDR in adipose tissue. Overexpression of human VDR in mouse adipose tissue was found to induce increased weight and fat pad mass, associated with a decrease in energy expenditure and fatty acid oxidation.[69,72] Conversely, adipose-specific VDR knockout (Cre recombinase under FABP4 promoter control) increased visceral fat pad weight compared with wild-type mice but in females only,[74] whereas it did not modify adiposity and body weight in another model (Cre recombinase under adiponectin promoter control).[76] Taken together, these observations do not clearly converge towards a specific role of VDR in adipose tissue biology.

3.2 | Effect of VD or its metabolites on the control of adipogenesis

The relationship between VD and adipogenesis has been recently reviewed in detail.[77] Briefly, it is well established that 1,25(OH)$_2$D displays anti-adipogenic effect in 3T3-L1 preadipocyte cells and reduces lipid accumulation.[38,78–80] Molecular mechanisms have been studied, and it was found that 1,25(OH)$_2$D may block 3T3-L1 cell differentiation by acting on multiple targets in a dose-dependent and timedependent manner and only inhibit the early events of adipogenic programming. 1,25(OH)$_2$D suppresses the expression of the master regulators of adipogenesis, including CCATT enhancer binding protein (C/EBP) α, peroxisome proliferator-activated receptor (PAR) γ, C/EBPβ and adipocyte protein 2 (aP2),[79,80] thus blocking the adipogenic program. Antagonization of PPARγ activity and stabilization of the VDR protein are also components of this complex regulation process.[79] In addition, 1,25(OH)$_2$D in 3T3-L1 can increase the expression of the eight-twenty-one protein (ETO/MTG8), which inhibits adipogenesis through repression of C/EBPβ and inhibition of PPARγ.[80] Inhibition of adipogenesis has also been reported in mouse brown adipocytes[64] and in bone marrow cell lines.[81,82] Nevertheless, adipogenic stimulatory effect of 1,25(OH)$_2$D has also been shown in 3T3-L1 cells[83] and in mouse primary preadipocytes,[29] and unliganded VDR seems to be required for adipogenic programming.[80] Note that the studies with VDR$^{/}$ mice reported a reduction of adipose tissue,[67,68] suggesting that adipogenesis could be impacted. However, as these VDR$^{/}$ mice also showed increased energy metabolism, it is still not clear where adipogenesis per se is really involved in the resulting reduced adipose tissue mass.

1,25(OH)$_2$D was found to promote adipogenesis in primary human preadipocytes, as revealed by an increase in lipid accumulation and expression of adipogenic markers.[29] During the late phase of differentiation, 1,25(OH)$_2$D increased the gene expression of C/EBPα and PPAR-γ but not C/EBPβ.[29] A similar impact was observed in adult stem cells derived from adipose tissue.[84] Nevertheless, this effect seems to be dose-dependent, since a low dose (10^{10} M) inhibited adipogenesis in mesenchymal stem cells, whereas a higher dose (10^{8} M) stimulated the adipogenic program.[85]

3.3 | Impact of VD and its metabolites on adipocytes and adipose tissue inflammation

Although initial studies suggested that 1,25(OH)$_2$D induced the expression of several proinflammatory cytokines and decreased the expression of anti-inflammatory cytokines in human and mouse adipocytes,[86–88] further studies failed to confirm these conclusions. Indeed, several groups consistently reported anti-inflammatory effects (Figure 3). 1,25(OH)$_2$D significantly reduced the basal release of monocyte chemoattractant protein 1 (MCP-1), IL-8, and IL-6 from human preadipocytes, monocyte recruitment by the same type of cells,[89] and MCP-1 by human adipocytes.[53] Our team reported that 1,25(OH)$_2$D inhibited the expression of inflammatory markers (IL-6, MCP-1, and IL-1β) under basal and TNF-stimulated conditions in human adipocytes and in 3T3-L1 adipocytes.[57] We observed that NFkB signaling was involved in the anti-inflammatory effect of 1,25 (OH)$_2$D but also p38 dephosphorylation resulting from 1,25(OH)$_2$Ddriven induction of Dusp10 in 3T3-L1 adipocytes.[57] A similar antiinflammatory effect was reported in bone marrow-derived human mesenchymal stromal cells (hMSCs) differentiated into adipocytes and in adipocytes isolated from biopsies stimulated with lipopolysaccharide (LPS), where 1,25-(OH)$_2$D interfered with NF-κB signaling leading to an inhibition of interleukin-6 secretion.[90] The ability of 1,25(OH)$_2$D to blunt the LPS-mediated induction of proinflammatory response has been further confirmed in murine adipocytes.[19,91] 1,25(OH)$_2$D was also able to reverse Il-1β-mediated proinflammatory response in human adipocytes[92] and human preadipocytes.[93] Macrophageconditioned medium (MCM)-induced cytokine and chemokine release was also attenuated by 1,25(OH)$_2$D in human adipocytes and resulted in inhibition of MCM-induced monocyte chemotaxis.[94] To go further, we implemented a microarray approach and found that 1,25(OH)$_2$D was able to down-regulate a substantial set of chemokines induced by inflammatory stimuli in both human and murine adipocytes. Consequently, there was a reduction in macrophage migration mediated by an adipocyte-conditioned medium.[95] In addition to the classical impact of 1,25(OH)$_2$D on cytokines and chemokines, we recently described a new anti-inflammatory effect mediated by the inhibition of the several inflammatory-linked miRNA, including miR-146a, miR150, and miR-155,[51] which are known to be related to inflammatory processes in adipocytes.[96,97]

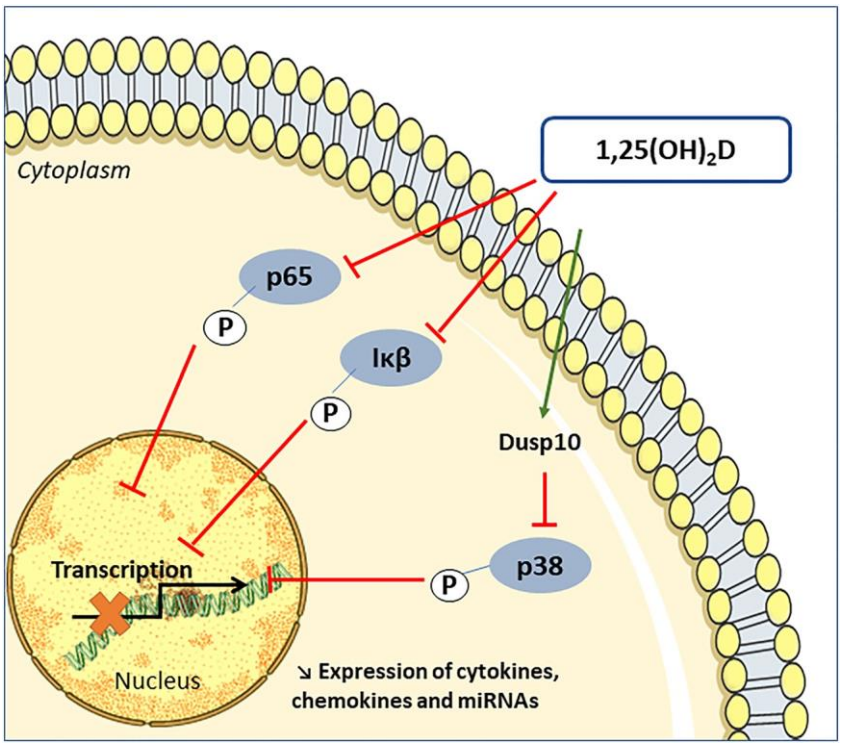

FIGURE 3 Anti-inflammatory effects of 1,25 (OH)$_2$D. 1,25(OH)$_2$D has an anti-inflammatory effect. This effect involves interference with NFkB signaling through inhibition of p65 and Iκβ, as well as p38 dephosphorylation resulting from the induction of Dusp10, leading to down-regulated expression of inflammatory markers (cytokines, chemokines, and miRNA)

Note too that the anti-inflammatory activity of 1,25(OH)$_2$D has been established in several immune cell types found in adipose tissue, including lymphocytes, macrophages, and T-cells.[98,99]

The anti-inflammatory effect of VD has also been investigated in vivo. An important finding was that, mRNA levels several cytokines were upregulated in epidydimal adipose tissue of adipocyte-specific VDR knockout mice.[76] Concerning the effect of VD supplementation, Palaniswamy et al[100] recently reported that VD supplementation has no benefit in obesity-related inflammation at systemic level, and Wamberg et al[92] reported that VD-supplemented subjects with obesity showed no decrease in adipose tissue expression of MCP-1, IL-6, or IL-8. Nevertheless, several other studies argue for an antiinflammatory effect of VD in vivo. A recent study showed that VD repletion in subjects with overweight or obesity and a characterized 25(OH)D deficiency reduced TNF, IL-6, iNOS, and PAI-1 expression in subcutaneous adipose tissue and in adipose tissue macrophages.[76] Several experiments in rodents have confirmed the ability of VD or 1,25$(OH)_2$D to blunt inflammatory processes in adipose tissue. IL-6 protein levels were reduced in epididymal adipose tissue of high-fatfed mice supplemented with 1,25$(OH)_2$D.[91] Furthermore, we previously published data showing that VD supplementation in a heart failure (HF) diet model leading to metabolic inflammation reduced the expression and protein levels of proinflammatory cytokines and chemokines, but also inhibited macrophage infiltration in adipose tissue of obese mice.[95] These effects were confirmed in an acute model of inflammation mediated by intraperitoneal injection of LPS.[95] Similarly, VD supplementation down-regulated epididymal fat CCL2 and CCL5 and reduced the number of macrophages and natural killer cells found in adipose tissue in HF-fed mice.[101] In rats, VD supplementation was also found to limit TNF-α and MCP-1 concentrations in rat adipose tissue.[102] We recently tested the curative effect of a VD supplementation regimen and found that it reduced the expression of chemokines in obese mice.[103,104]

4| MATERNAL VD DEFICIENCY LINKED TO ADIPOSE TISSUE BIOLOGY PROGRAMMING

Research is starting to understandthe role of VD during pregnancy.[105–108] Adequate VD intake in pregnancy is essential for both maternal and fetal health. Epidemiological studies have described a wide range of adverse maternal, fetal, and neonatal outcomes associated with VD deficiency,[109] such as increased risk for preeclampsia,[110] gestational diabetes mellitus,[111] higher risk of smallfor-gestational-age, reduced term birthweight, and lower head circumference.[112–115]

Several recent studies support the role of VD deficiency in adipose tissue metabolism programming. In rats, it was reported that maternal VD deficiency induced before and during gestation appeared to promote the differentiation and proliferation of adipocytes and preadipocytes in VD-deficient male offspring. This phenomenon seems to be associated with epigenetic changes (differential methylation of promoters and CpG islets), leading to an obese phenotype (increased body mass and adiposity) in offspring of VD-deficient females.[48] In mice, maternal VD deficiency is also associated with an obese phenotype in male offspring, characterized by higher body mass, adiposity, and glucose intolerance.[114,116]

In a transgenerational study, maternal VD deficiency induced by a VD-deficient diet (5 weeks before mating until weaning) led to disrupted DNA methylation in somatic liver and germ cells (sperm) over two successive generations. These epigenetic changes were associated with differences in body weight and lean mass-to-fat mass ratio over the two generations.[117]

However, this association between maternal VD deficiency and a putative impact on adipose tissue in the offspring is not systematically observed. A study on male Sprague–Dawley rats from dams deficient in VD during gestation found no difference in body mass.[118] However, these animals showed insulin resistance (high HOMA-IR and lowered glucose tolerance) associated with persistent inflammation (in particular with high plasma and liver concentrations of IL-1β, IL-6, IL-8, and TNFα). Interestingly, the persistently increased inflammation was explained by the continuously increased IκBα expression related to methylation modifications in the liver[118] but could also occur in adipose tissue. Likewise, male mice from deficient mothers exhibiting intrauterine growth retardation and accelerated growth early in life did not have a higher mass in adulthood.[119] Nevertheless, these animals were predisposed to develop adipocyte hypertrophy following a HF diet.

We recently reported that juvenile males born to VD-deficient dams had lower body weight and higher energy expenditure compared with controls, whereas littermate females showed no difference in body weight.[120] This highlights thus a strong metabolic sex-specific response, which has never previously been pointed out. Furthermore, we showed that challenging offspring with a HF diet strongly increased adiposity index and insulin resistance in males born to VD-deficient mice, which correlated with insulin resistance, whereas females born to VD-deficient mice and fed HF diet had a similar adiposity index and insulin sensitivity to females fed control diet. Note that these phenotypes (adiposity and insulin sensitivity) were associated with different transcriptomic profiles in white adipose tissue (related to lipid metabolism),

leading us to posit that the specific phenotypic response in females was related to 17β-estradiol concentrations increased by maternal VD deficiency.

Taken together, these observations support a detrimental role of VD deficiency in terms of adipose tissue biology programming, which appears to be sex-dependent and further exacerbated by HF diet in rodents. However, the molecular mechanisms mediating this phenotype remain elusive.

5| CONCLUSION AND PERSPECTIVES

Recent data from several research groups strongly support numerous impacts of VD on several aspects of adipose tissue/adipocyte biology, including adipogenesis, energy metabolism, and inflammation. However, there are several key points that need to be addressed regarding VD metabolism in adipocytes, especially its uptake which has only partly been unraveled, and the respective contributions of the different hydroxylation enzymes and mechanisms of secretion of VD and metabolites from adipocytes. In addition, the role of obesity on adipocyte and adipose-tissue VD metabolism still needs to be clarified, as it is now obvious that obesity strongly impacts overall VD metabolism.

In the past decade, research has generated very interesting data in transgenic mice and rodents subjected to VD supplementation or restriction, but still without a cohesive set of findings. There is an urgent need to identify the origin of this non-convergence, but also to keep in mind that VD deficiency during adulthood is totally different from embryonic all-animal VDR knockout (due to VDR ligandindependent activities and non-genomic effects of VD, among other factors).

Rodent models have recently been implemented and will enable in-depth exploration of the metabolic and adipose phenotype of VDdeficient female mice offspring. Such models will also provide opportunities to uncover the molecular and epigenetic mechanisms involved in adipose metabolic programming.

To conclude, several lines of evidence support the key role of VD in adipocyte and adipose tissue physiology. This active role strongly argues for maintaining adequate VD intake to blunt the risk of adipose tissue dysfunction and negative consequences of VD deficiency on energy homeostasis, both in adults and during pregnancy. Welldesigned clinical studies and fundamental research are now urgently needed to confirm these assumptions.

ACKNOWLEDGMENTS

AMU, INRAE, and INSERM are acknowledged for their financial support.

CONFLICT OF INTEREST

No conflict of interest statement.

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How to cite this article: Bennour I, Haroun N, Sicard F, Mounien L, Landrier J-F. Recent insights into vitamin D, adipocyte, and adipose tissue biology. Obesity Reviews. e13453. doi:10.1111/obr.13453

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Review

Vitamin D and Obesity/Adiposity—A Brief Overview of Recent Studies

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Citation: Bennour, I.; Haroun, N.; Sicard, F.; Mounien, L.; Landrier, J.-F. Vitamin D and Obesity/Adiposity— A Brief Overview of Recent Studies. *Nutrients* **2022**, *14*, 2049. https://doi.org/10.3390/nu14102049

Academic Editor: Luigi Barrea

Received: 15 March 2022 Accepted: 11 May 2022

Published: 13 May 2022

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

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Abstract: Observational studies classically find an inverse relationship between human plasma 25hydroxyvitamin D concentration and obesity. However, interventional and genetic studies have failed to provide clear conclusions on the causal effect of vitamin D on obesity/adiposity. Likewise, vitamin D supplementation in obese rodents has mostly failed to improve obesity parameters, whereas several lines of evidence in rodents and prospective studies in humans point to a preventive effect of vitamin D supplementation on the onset of obesity. Recent studies investigating the impact of maternal vitamin D deficiency in women and in rodent models on adipose tissue biology programming in offspring further support a preventive metabolically driven effect of vitamin D sufficiency. The aim of this review is to summarize the state of the knowledge on the relationship between vitamin D and obesity/adiposity in humans and in rodents and the impact of maternal vitamin D deficiency on the metabolic trajectory of the offspring.

Keywords: preventive nutrition; maternal programming; micronutrients; obesity

Nutrients **2022**, *14*, 2049

1 . Introduction

Vitamin D (VD, here used as a generic term) has a well-established role in the maintenance of phosphate and calcium homeostasis and a clearly essential function in bone and muscle health [1]. For some years now, VD has attracted increasing attention due to the resurgence of VD deficiency in children and adults worldwide [2–4] and its suspected multiple extraskeletal benefits, including for cardiometabolic health [1,5]. In the cardiometabolic context, a huge number of in vitro experiments have clearly mapped out the actions of vitamin D on key parameters of adipose tissue and adipocyte biology including adipogenesis and the regulation of gene expression in response to energy homeostasis and inflammation (see [6–8] for reviews). These lines of evidence all converge towards a beneficial role of vitamin D in the physiology of adipose tissue, prompting a massive body of research attempting to unravel the relationship between vitamin D and obesity/adiposity in humans and in animals. This review set out to summarize the state of knowledge on this VD–obesity/adiposity relationship and point towards its grey areas and its more robust conclusions.

2 . Sources and Absorption of Vitamin D

Vitamin D is unique in that it can be both sourced by food and produced endogenously, which means that it has the properties of both a vitamin and a hormone. VD is mainly produced in skin exposed to UVB light, which converts 7-dehydrocholesterol to pre-vitamin D3, which is then further isomerized to vitamin D3 by the action of heat before being released into the circulation [9], where it binds to vitamin D-binding protein (VDBP). The real contribution of endogenous VD production is still under debate: some papers assert that endogenous VD synthesis accounts for up to 70–90% of VD supply whereas others suggest it is just 10–25% of VD supply [10]. The amount of vitamin D3 that is endogenously produced remains highly variable and depends on several factors including latitude, pollution, season, use of sunscreen, clothing, sedentary lifestyle, skin color, and age, among others [11].

Natural exogenous sources of VD are relatively scarce. VD is found in animal-source products (mainly fish liver oil, fatty fish such as salmon, sardines, herring and mackerel, and egg yolk) providing vitamin D3 or 'cholecalciferol' [12,13]. Plants and mushrooms are sources of vitamin D2 or 'ergocalciferol'. Vitamin D (mainly as D3) can also be added in small amounts in fortified milk, margarine or butter, orange juice, and bread or cereals.

After ingestion and emulsion with bile acids [14,15], dietary VD is absorbed in the median part of the small intestine [16]. This process, initially assumed to be passive [17], also requires three apical membrane transporters: Scavenger receptor class B type I (SR-BI), Cluster of differentiation 36 (CD36) and Niemann–Pick C1-like 1 protein (NPC1L1) [18]. A part of the absorbed VD is effluxed by enterocytes via ATP-binding cassette subfamily B member 1 (ABCB1) [19]. The intracellular transport of VD in enterocytes remains unclear, but it ultimately is incorporated in chylomicrons to be secreted in the lymph and delivered to the liver and/or storage organs.

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3. Metabolism of the Vitamin D

3.1. Hydroxylation of Vitamin D and Metabolites

In the liver, endogenous or dietary VD is enzymatically hydroxylated to 25-hydroxyvitamin D (calcifediol, 25(OH)D) (Figure 1). This reaction is catalyzed by microsomal cytochrome P450 enzyme CYP2R1, which is considered the key enzyme of 25 hydroxylation [20–22]. However, several other enzymes display a 25-hydroxylation activity on VD, including CYP27A1, CYP2C11, CYP3A4, and CYP2J2 in humans [23–25].

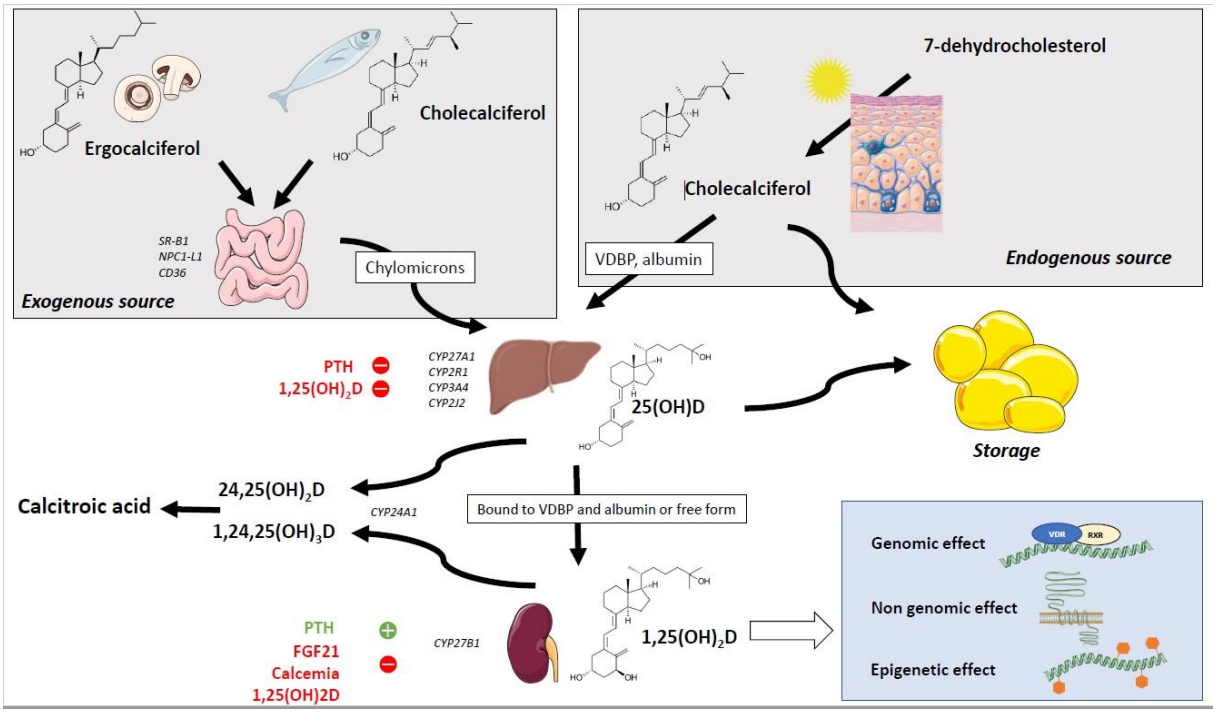

Figure 1. Vitamin D metabolism.

Although the 25-hydroxylation is classically thought to be poorly regulated, Bell et al. reported that hepatic 25-hydroxylation was inhibited by $1,25(OH)_2D$ and parathyroid hormone (PTH) in humans [26]. Furthermore, several recent studies have described the inhibition of *Cyp2r1* mRNA levels and/or 25-hydroxylation activity in the liver of obese/diabetic mice [27–31] via mechanisms involving several transcription factors including PGC1α and GR [31]. The 25(OH)D thus produced is the major circulating form of VD, with a half-life of about 15 days, and is classically used as a biomarker of VD status [32]. Circulating 25(OH)D is bound to VDBP [33], a serum α2-globulin encoded by the *Gc* gene and synthesized by the liver that is considered as the major plasma protein carrier of VD and its metabolites [34,35]. Indeed, around 80% of plasma 25(OH)D is bound to VDBP, while 19% of 25(OH)D is linked to albumin and the remainder is a free fraction that is thought to be biologically active [36]. The fact that VDBP-null mice remained normal under a VD-sufficient diet suggests that free 25(OH)D levels cover body requirements for physiological functions [37].

The VDBP-bound 25(OH)D is then transported to the kidneys and various other organs and tissues, where it is used for 1α-hydroxylation. In renal proximal tubule cells, the VDBP–25(OH)D complex enters by endocytosis and thus escapes urinary loss [38,39]. This step requires the presence of megalin and cubilin [40]. Cubilin is responsible for sequestration of the VDBP–25(OH)D complex before internalization by megalin. After internalization into vesicles, the VDBP is degraded by lysosomes and the 25(OH)D is handled by intracellular VDBP [41]. The 25(OH)D is then either secreted into circulation or delivered to mitochondria to be metabolized into $1,25(OH)_2D$, the biologically active form of VD. This reaction is catalyzed by 1α-hydroxylase CYP27B1 and stimulated by PTH and low calcium and phosphorus concentrations, inhibited by FGF23, and self-regulated by $1,25(OH)_2D$ via a negative feedback mechanism [42].

VD metabolism is ultimately self-regulated via an inactivation pathway that involves CYP24A1-mediated 24-hydroxylation leading to the conversion of 25(OH)D and $1,25(OH)_2D$ into 24,25(OH)D and $1,24,25(OH)_3D$, catabolized into inactive calcitroic acid [43].

This inactivation is induced by $1,25(OH)_2D$ itself via an induction of CYP24A1.

3.2. Mechanisms of Action of Vitamin D

The nuclear receptor-family vitamin D receptor (VDR) is known to mediate the bulk of the biological effects of $1,25(OH)_2D_3$ [44]. Its ubiquitous distribution explains why a large number of genes (more than 1000) are either directly or indirectly regulated by $1,25(OH)_2D$ [44]. VDR heterodimerizes with retinoid X receptor (RXR) and binds to DNA at sites called vitamin D response elements (VDRE) that are located in the promoter regions of VD-regulated genes. In the absence of a ligand, this heterodimer complexes with corepressors and histone deacetylases, whereas in the presence of $1,25(OH)_2D$, it recruits co-activators and histone acetyltransferases, leading to transcriptional activation [45]. This genomic effect of VD through VDR is strongly suspected to drive a large share of the biological effects of VD in health and disease, notably in the context of obesity, based on evidence from several studies that point to a correlation between VDR polymorphism and pathological issues [46].

The non-genomic effects of VD are characterized by very fast (seconds to minutes) activation of signaling pathways involving phospholipases C and A2, phosphoinositide 3-kinase, protein kinase A, and mitogen-activated protein kinases. It also includes the opening of Ca^{2+} and Cl^- channels. These non-genomic effects of VD are dependent on protein disulfide isomerase family A member3 (PDIA3, also known as ERp57, GRP58 and 1,25-MARRS) [47,48], a membrane receptor found in enterocytes, osteoblasts and hepatocytes [49–51].

Several epigenetic effects of VD have been described in a number of models and pathophysiological contexts. These effects include DNA methylation, possibly through modulation of DNA methyltransferase and/or DNA demethylase expression [52]. VD can also regulate histone acetylation via activation of histone acetyltransferases and histone deacetylases, but also histone methylation and demethylation, thus modulating chromatin accessibility to transcription factors [53]. Finally, VD is also reported to play a role in regulating the expression of some micro-RNAs (miRNAs) [53], and we recently published data showing that $1,25(OH)_2D$ downregulates inflammation-related miRNAs expression in adipocytes both in vitro and in vivo [54].

4. Relationship between Obesity and Vitamin D in Rodents

The decrease in plasma 25(OH)D concentrations with obesity is clear in humans, but is less clear in mice (Table 1). Indeed, the decrease in total 25(OH)D is not always obvious, since authors report no modification of 25(OH)D plasma concentration [28,29,55–58], whereas other studies depict a decrease [27,59–61]. The origin of these divergences in mouse studies is still unclear, but could be due to the composition of the high-fat (HF) diet used to induce obesity and/or the methods used to quantify 25(OH)D, i.e., immunoassay tests vs. mass

spectrometry. For instance, we initially reported a decrease in 25(OH)D plasma concentrations under HF diet based on ELISA quantification [60], but recent protocols, performed with the same type of diet but using mass spectrometry analysis for 25(OH)D quantification, found no such decrease [29,55].

Table 1. Relationship between VD and obesity in rodents.

	In Rodents	References
25(OH)D plasma levels	Lack of clear-cut results (decrease or no effect reported)	[27–29,55–61]
Free 25(OH)D plasma levels	Reduced in obese mice	[29,55]
1,25(OH)2D plasma levels	Lack of clear-cut results (increase, decrease or no effect reported)	[28,29,55,56,59]
$VDR^{-/-}$ mice	Reduced obesity/adiposity	[62–66]
Adipose tissue human VDR overexpression	Increase obesity/adiposity	[66,67]
Adipose tissue $VDR^{-/-}$ mice	Increase obesity/adiposity	[68,69]
	No effect on obesity/adiposity	[70]
Curative effect of VD supplementation on obesity	No	[58,71]
Curative effect of $1,25(OH)_2D$ supplementation on obesity	Yes	[72]
Preventive effect of VD supplementation on obesity	Reduction of obesity/adiposity	[60,73–76]
	No effect on obesity/adiposity	[56,57,59,61,77–79]
	Decrease in obesity/adiposity	[80–82]
Effect of VD supplementation on 25(OH)D plasma levels in obese rodents	Increase 25(OH) plasma levels	[58–60,83,84]
	No effect	[56,57]

VDR: vitamin D receptor; VD: vitamin D; 25(OH)D: 25-hydrixyvitamin D; 1,25(OH)2D: 1,25-dihydroxyvitamin D.

Other VD metabolites have been measured in obese rodents. We recently reported lower free 25(OH)D rates in obese mice [29,55], which is in agreement with human data [85]. Studies investigating the relationship between obesity and plasma 1,25(OH)2D concentrations in mice have found no clear pattern, as some report decreased plasma 1,25(OH)2D [56,59] whereas others find no change [55] and others even find increased plasma 1,25(OH)2D [28,29]. The pattern probably depends on composition of the HF diet and/or duration of the diet administration.

As is the case in humans, several rodent studies using wild-type or transgenic mice models have attempted to elucidate the impact of diet supplementation with cholecalciferol or active metabolites of VD on obesity. $VDR^{-/-}$ mice were studied to gain insight into the role of VD metabolism on body weight management and adipose tissue biology [62–66]. These mice remain lean and resistant to diet-induced obesity, probably due to the induction of fatty acid oxidation and uncoupling proteins (including UCP1, 2 and 3) in adipose tissue leading to increased energy expenditure. Even if these data strongly suggest that VDR deletion improves energy homeostasis, there are several caveats: the mice were fed a rescue diet containing large amounts of calcium, which is suspected to regulate energy homeostasis [86]; the VDR gene was ablated from the entire mouse, making it impossible to attribute the overall phenotype to a specific tissue [42,68]; $VDR^{-/-}$ mice develop alopecia, and the resulting reduced insulation could increase energy expenditure to maintain body temperature [87].

Other studies have using targeted overexpression or invalidation of VDR in adipose tissue. The overexpression of human VDR in mouse adipose tissue induced an increase in weight and fat pad mass associated with a decrease in energy expenditure and fatty acid oxidation [66,67]. Conversely, adipose tissue-specific invalidation of VDR (Cre recombinase under control of the FABP4 promoter) increased visceral fat pad weight compared to wildtype mice in females only [68] but had no effect on adiposity and body weight in another model (Cre recombinase under control of the adiponectin promoter) [70]. Interestingly, a recent article also reported that adipocyte-specific VDR ablation (Cre recombinase under control of the mouse adiponectin promoter) led to a slight increase in weight gain and an increase in visceral white adipose tissue [69]. Taken together, these observations still give no clear picture of the specific role of VDR in adipose tissue biology and weight management. Furthermore, they also point to a sex-specific effect of VDR that warrants further investigation.

Few studies have been conducted in rodents to evaluate the value or benefit of VD supplementation as a curative strategy. We recently tested VD supplementation in obese mice and found that despite several phenotypical improvements, notably in terms of adipose tissue inflammation, hepatic steatosis and cardiac function, there was no observable improvement in body weight or adiposity [58,74,75], which is in agreement with another study [71]. However, obese mice subjected to $1,25(OH)_2D$ injections showed improved body weight and adiposity [72], suggesting that $1,25(OH)_2D$ could be more efficient than VD, probably due to the effect of obesity on VD metabolization.

The effect of VD or its metabolites has been more amply described as a preventive strategy against obesity. Several studies have reported a reduction in body weight and/or adiposity in rodents under VD [60,73–75] or $1,25(OH)_2D$ supplementation [76]. Additionally, it should be noted that VD insufficiency exacerbated adiposity and body weight gain [88–90]. The molecular mechanisms are not fully elucidated but may involve an action of VD in the induction of lipid catabolism, notably in the liver and in brown adipose tissue [60]. Interestingly, the ability of $1,25(OH)_2D$ to reduce adiposity and induce lipid oxidation in adipose tissue has been confirmed in a *cyp2r1*-deficient zebrafish model [91,92].

Conversely, some studies have reported no effect of VD supplementation or VD deficiency on body weight, adiposity, or adipose tissue fat pad mass [56,57,59,61,77–79], whereas other studies reported that VD deficiency decreased HF diet-induced obesity in rodents [80–82]. The lack of clear-cut results is problematic, and the origin of these discrepancies remains partly unexplained. Of course, several key parameters are highly variable between studies and could explain the divergent results. Among them are the different quantities of dietary and/or supplemented VD, the quantity and type of lipids incorporated in the HF regimen used, the duration of the regimen and supplementation, and the age of the animals at the beginning of the supplementation are regularly cited, but would require a deeper investigation. Another important issue is the lack of reproducible effect of VD supplementation on plasma 25(OH)D, especially against a background of obesity. Indeed, some authors reported an increase in plasma 25(OH)D [58–60,83,84], whereas others reported no effect under VD supplementation [56,57]. It is, therefore, conceivable that the lack of effect of VD on obesity parameters coincides with an absence of detectable increase in 25(OH)D. Note that the difference in concentrations of VD used for supplementation could partly explain these discrepancies, but is clearly not the sole factor. Diet composition and level of 25(OH)D at baseline could also play a major role, as recently highlighted in the case of Mediterranean diet [93], but further research is needed.

5. Relationship between VD and Obesity in Human Observational Studies

A large set of cross-sectional studies recently reviewed in deep [94,95] have pointed out the inverse relationship between low serum 25(OH)D and obesity [96] (Table 2). Indeed, it is assumed that plasma 25(OH)D is inversely correlated with most parameters of obesity, such as body mass index (BMI), total fat mass, subcutaneous and visceral adiposity, and waist circumference [97]. These observations have been found in adults but also in children [98] and in aging people [99]. Furthermore, a recent study found that free plasma 25(OH)D and 1,25$(OH)_2$D level are lower in obese subjects than normal-weight subjects (85), as also suggested elsewhere [100,101]. This could be due to the reduced release of 1,25$(OH)_2$D from subcutaneous adipose tissue under the isoprenaline-mediated lipolysis that occurs during obesity [102].

Many hypotheses have been put forward to explain the low levels of 25(OH)D (total and/or free) associated with obesity. Differences in lifestyle patterns between people with or without obesity cannot be ruled out, including dietary habits, sedentary lifestyle or clothing habits, and there may also be reduced hepatic synthesis of 25(OH)D due to obesity-associated secondary hyperparathyroidism [26], but the key mechanism appears to be VD sequestration. Indeed, the excess adipose tissue observed during obesity could offer an extended storage site for VD and/or 25(OH)D, leading to low plasma 25(OH)D concentrations [103], but this assumption was challenged by Drincic et al. who reported that 25(OH)D was simply diluted in a higher volume in people

with obesity, in line with the volumetric dilution hypothesis [104]. Alternative hypotheses have been put forward that suggest modifications in VD metabolism, notably in adipose tissue, where *CYP2J2* mRNA levels were found to be lower in obese women compared to lean women [105]. A recent review argued for a similar set of possible factors affecting vitamin D levels in aging people with obesity (i.e., sequestration or dilution of VD in adipose tissue, increased catabolism of VD in adipose tissue, reduced 25-hydroxylation and reduced sun exposure) [99].

In accordance with the hypothesis of VD sequestration n adipose tissue, a recent meta-analysis highlighted that obesity reduces the effect of VD supplementation in patients with obesity [106]: serum VD concentrations were found to be −38.17 nmol/L lower in obese subjects compared to the normal-weight group, and increasing VD doses did not significantly increase 25(OH)D plasma concentrations, underlining the urgent need to develop strategies for optimal VD supplementation for people with obesity.

Prospective studies suggest that low plasma 25(OH)D levels are associated with a strong prevalence of obesity in children [107], adults [108,109], and elderly women [110], and low VD intake may predict later obesity and metabolic syndrome [111] and even onset of obesity [112].

Some studies have found relationships between VDR polymorphisms and BMI, adiposity markers, or obesity [113–116] and other research has found a relationship between BMI and polymorphisms of VDBP and CYP27b1 [117]. However, larger studies failed to demonstrate correlations between polymorphisms in genes coding for key drivers of VD metabolism [118,119]. Interestingly, it has been reported that polymorphisms in VDR may influence changes in visceral fat and waist circumference in people supplemented with VD [46]. Mendelian randomization analysis using genes involved in VD metabolism as instrumental variables (VDBP, DHCR7, CYP2R1 and CYP24A1) suggested that low 25(OH)D has little or no impact on BMI [120], that obesity promoted the reduction in plasma 25(OH)D and that a 1 kg increase in body weight leads to a 1.15% decrease in 25(OH)D. Consistently with these findings, a systemic review and meta-analysis of randomized and non-randomized controlled trials showed that weight loss can improve plasma 25(OH)D concentrations [121], and another meta-analysis reported that a weight loss of approximately 10 kg without VD supplementation could increase plasma 25(OH)D concentration by up to 6 nmol/L [122].

6. Relationship between VD and Obesity in Human Interventional Studies

Several randomized clinical trials (RCT) have been implemented and recently metaanalyzed in an effort to establish the causal link between low plasma VD levels and obesity (Table 2). Results contrasted between each RCT depending on experimental design (population recruited, 25(OH)D at baseline, duration of VD supplementation, VD dose, etc.). Two meta-analyses failed to show a beneficial effect of VD supplementation on measures of obesity (BMI, fat mass, percentage of fat mass or lean body mass) [123,124] whereas a third

meta-analysis pointed to improved BMI and waist circumference following VD supplementation [125]. This lack of clear-cut results is unfortunately common in RCT testing the therapeutic effect of VD, and Dr. BJ Boucher recently highlighted several highly pertinent ways to address this issue by improving RCT design and analysis to obtain definite answers on whether effects of VD translate into real health benefits [126].

Table 2. Relationship between VD and obesity in humans.

	In Humans	References
25(OH)D plasma levels	Reduced in obesity, inversely correlated to markers of obesity and adiposity	[94–99]
Free 25(OH)D plasma levels	Reduced in obesity	[85]
1,25$(OH)_2$D plasma levels	Reduced in obesity	[100,101]
Impact of obesity on VD supplementation	Obesity reduced the efficacity of VD supplementation	[106]
Low 25(OH)D predictor for obesity onset (Prospective studies)	Yes	[107–112]
Effect of polymorphisms in genes coding for proteins involved in VD metabolism (Genetic studies)	Relationship between obesity and SNP in VDR, VDBP, and Cyp27b1 in small cohorts No link between polymorphisms and obesity in larger cohorts	[113–117] [118,119]
Causal effect of low 25(OH)D on obesity (Mendelian randomization)	No	[120]
Causal effect of obesity on low 25(OH)D (Mendelian randomization)	Yes	[120]
Weight lost increase 25(OH)D plasma levels	Yes	[121]
Impact of VD supplementation on obesity (RCT)	Lack of clear-cut results (2 meta-analysis showing no effect, 1 meta-analysis showing improvement of obesity parameters following VD supplementation	[123–125]

VD: vitamin D; 25(OH)D: 25-hydrixyvitamin D; 1,25(OH)2D: 1,25-dihydroxyvitamin D; SNP: single nucleotide polymorphisms.

7. Maternal Vitamin D Deficiency and Links to Obesity and Adipose Tissue Biology

Investigations are starting to unravel the role of VD during pregnancy [127–130]. Adequate VD intake during pregnancy is essential for both maternal and fetal health. Indeed, epidemiological studies describe a large range of adverse maternal, fetal and neonatal outcomes associated with VD deficiency [131], such as increased risk for preeclampsia [132], gestational diabetes mellitus [133], higher risk of small-for-gestational-age at term and reduced-term birth weight, and lower head circumference [134–137].

7.1. Lines of Epidemiological and Clinical Evidence for a Link between VD and Obesity in Offspring

Recent studies on large mother–child cohorts have suggested associations between maternal plasma 25(OH)D content and various parameters characterizing obesity in children. In a cohort of 977 pregnant women, low 25(OH)D concentration at 34 weeks of pregnancy was associated with low percent fat mass at birth and high percent f fat mass in children aged 4 and 6 [138]. Similar results were observed in a cohort of 922 mother–child pairs where increased maternal 25(OH)D plasma levels at 15 weeks of pregnancy was associated with decreased percent body fat in offspring [139]. In another cohort including 4903 mothers and their children, severe maternal 25(OH)D deficiency during pregnancy was associated higher percent fat mass and lower percent lean body mass in children at 6 years [140]. In a cohort of 568 mother–child pairs, boys but not girls born to mothers with 25(OH)D deficiency had higher percent fat mass and lower percent fat-free mass at 5 years of age [141]. In a smaller cohort (292 mother–neonate pairs, low maternal 25(OH)D levels were associated with greater superficial and deep abdominal subcutaneous adipose tissue volume in neonates [142]. Other studies in humans have found that maternal VD status is associated with BMI, weight and waist circumference in offspring. Indeed, 25(OH)D concentrations during pregnancy were inversely correlated with BMI and waist circumference in children aged 4 and 6 years [143] but also with high risk for fetal and neonatal overweight [144].

Convergent evidence from all these epidemiological studies therefore tends to confirm the role of maternal VD status in developmental programming of obesity in the offspring. However, long-term studies (follow-up to adulthood) remain scarce; the only study with long-term follow-up (20 years) found no association between maternal VD status and cardiometabolic risk factors [145], and the early mechanisms involved in this programming remain to be elucidated.

RCTs to evaluate the effect of VD supplementation during pregnancy have been conducted and recently meta-analyzed (3725 participants from 22 trials) [146]. Supplementation with VD during pregnancy probably reduced the risks for preeclampsia, gestational diabetes, and babies with low birth weight at term. Another recent meta-analysis (3960 participants from 11 RCTs) found that VD supplementation was also associated with lower BMI and BMI z-score in

children aged 3–6 years [147]. However, these data remain weak and the certainty level is only moderate, and so further large RCTs are needed to confirm these results.

7.2. Lines of Preclinical Evidence for a Link between VD and Obesity in Offspring

A study conducted in rats showed that maternal VD deficiency induced before and during gestation appears to promote adipocyte and preadipocyte differentiation and proliferation in VD-deficient male offspring [148]. This phenomenon seems to be associated with epigenetic changes (differential methylation of promoters and CpG islets) leading to an obese phenotype (increased body mass and adiposity) in the offspring from VD-deficient females [148]. In mice, maternal VD deficiency is also associated with an obese phenotype in male offspring characterized by higher body mass, adiposity and glucose intolerance but did not translate over to a second generation [149]. Another study also reported glucose intolerance and slower growth in mice born from VD-deficient dam [136]. In a transgenerational study, maternal VD deficiency induced by a VD-deficient diet (from 5 weeks before mating until weaning) lead to disturbances in DNA methylation in somatic liver and germ cells (sperm) over two successive generations. These epigenetic changes were associated with differences in body weight and lean mass-to-fat mass ratio over the two generations [150].

However, this association between maternal VD deficiency and obese phenotype in the offspring is not systematically observed. A study of male Sprague Dawley rats from dams deficient in VD during gestation found no difference in body mass [151], but the offspring showed insulin resistance (high HOMA-IR and lowered glucose tolerance) associated with persistent inflammation (with high plasma and liver levels of IL-1β, IL-6, IL-8 and TNFα). Interestingly, the persistently increased inflammation was explained by the continuously increased IκBα expression related to methylation modifications [151]. Likewise, male mice from VD-deficient dams with intrauterine growth retardation and accelerated growth early in life did not have a higher mass in adulthood [152]. Nevertheless, these animals were predisposed to develop adipocyte hypertrophy in response to a high-fat diet.

We recently reported that juvenile males born to VDD dams had lower body weight and higher energy expenditure compared with controls, whereas females showed no change in body weight [153], which, for the first time, highlights a strong sex-specific metabolic response. Furthermore, we showed that challenging offspring with a HF diet strongly increased adiposity index and insulin resistance in males born to VDD mice, which correlated with insulin resistance, whereas the HF diet-challenged females born to VDD mice had a similar adiposity index and insulin sensitivity to control-diet females. These phenotypes (adiposity and insulin sensitivity) were associated with different transcriptomic profiles in white adipose tissue, prompting us to posit that the specific phenotypic response in females was linked to 17β-estradiol concentrations increased by maternal VD deficiency.

Taken together, these observations support a detrimental role of VD deficiency in terms of mediation of obesity and adiposity that appears to be sex-dependent and also appears to be further exacerbated by HF diet in rodents. However, the molecular mechanisms mediating this phenotype remain elusive.

8. Conclusions and Perspectives

The past decade of research has generated very interesting data in transgenic mice and rodents subjected to vitamin D supplementation or restriction, but still without convergent findings. It is vital to pinpoint the origin of these discrepancies, but also to keep in mind that VD deficiency during adulthood is totally different from global embryonic invalidation of the VDR (due to VDR ligand-independent activities and non-genomic effects of VD, among other factors).

Curative approaches based on VD supplementation have recently been implemented in mice but failed to improve obesity or adiposity. Similar results have been found in RCTs on supplementation to patients with obesity, which have mostly failed to confirm a beneficial role of VD supplementation on weight management. Several points for improvement are regularly proposed and should obviously be applied in future RCT designs, in line with Heaney's guidelines [154]. Trial designs would ideally always include people with measured baseline 25(OH)D and possibly 25(OH)D deficiency in order to highlight benefits of VD supplementation benefit, but these would be ethical issues to address. It would also require supplementing subjects with substantial doses of VD in order to observe a large change in VD status (not just VD intake). Co-nutrient status should also be optimized to limit risks of cofounding factors in the biological response. Even if the curative role of VD supplementation remains to be established, its preventive role is supported by prospective studies that converge to define low plasma 25(OH)D levels as a predictor of body weight gain. Rodent supplementation studies also partly support the preventive role of VD supplementation in obesity and adiposity. Some studies have nevertheless failed to demonstrate the preventive effect, which makes it vital to understand where such divergence comes from. The inconsistent findings could be linked, at least in part, to an inconsistent ability of VD supplementation to increase plasma 25(OH)D in mice models. Note that this preventive effect of VD is also supported by observational studies describing the impact of vitamin D deficiency in pregnant women on the metabolic programming of their offspring, which are supportive of a preventive metabolic effect of vitamin D sufficiency. Rodent models recently implemented will enable deeper exploration of the metabolic phenotype of offspring from VD-deficient female mice, and raise prospects for unravelling the molecular and epigenetic mechanisms involved in metabolic programming.

To conclude, several lines of evidence are supportive of a preventive effect of VD adequacy on obesity/adiposity, but a potential therapeutic role of VD supplementation for obesity and adiposity remains uncertain. In clinical practice, it is clearly necessary to keep 25(OH)D status within the normal range to avoid potentially associated risks in terms of obesity and adiposity. Well-designed clinical studies and fundamental research are urgently needed to confirm these assumptions.

VD exists in two forms: vitamin D3, which is produced in the skin and found in food, and vitamin D2, which is produced by plants and mushrooms. In the skin, 7dehydrocholestrol is converted to cholecalciferol following sun exposure. Once synthetized, VD is transported into the circulation bound to VDBP or albumin, whereas dietary VD is absorbed in the median part of the small intestine in a process that requires apical membrane receptors (SR-B1, NPC1-L1, CD36), and is then transported into the circulation incorporated in chylomicrons. For VD to be biologically activated, it needs to undergo two hydroxylations. The first one takes place in the liver, and is catalyzed by enzymes that display 25-hyrdroxylation (CYP27A1, CYP2R1, CYP3A4, CYP2J2) activity. This reaction results in the formation of 25(OH)D which is then transported into the circulation in its free form, and bound to VDBP or to albumin. The second hydroxylation is performed in the kidney and is catalyzed by 1α-hydroxylase CYP27B1, leading to the formation of 1,25(OH)2D. VD metabolism can be regulated: 25-hydroxylation is inhibited by 1,25(OH)2D concentration and PTH, and 1α-hydroxylation is stimulated by PTH but inhibited by FGF23, calcemia, and 1,25(OH)2D via a negative feedback mechanism. Furthermore, VD metabolism is also self-regulated via an inactivation pathway that involves a CYP24A1mediated 24-hydroxylation, leading to the conversion of 25(OH)D and $1,25(OH)_2D$ into 24,25(OH)D and $1,24,25(OH)_3D$ which is catabolized into inactive calcitroic acid.

1,25(OH)2D ultimately has many effects, including: (1) genomic effect that requires VDR and RXR and results in regulation of gene expression; (2) non-genomic effects via its association to the receptor 1,25-MARRS and subsequent activation of signaling pathways such as phospholipase C and phospholipase A2, phosphoinositide 3-kinase, protein kinase A, and mitogen-activated protein kinases; (3) epigenetic effects, including miRNA regulation, modulation of DNA methylation, histone acetylation/deacetylation and histone methylation/demethylation.

Author Contributions: Writing—original draft preparation, I.B. and N.H.; writing—review and editing, F.S., L.M. and J.-F.L. All authors have read and agreed to the published version of the manuscript.

Funding: This work has been granted by INRAE, INSERM and AMU.

Conflicts of Interest: The authors declare no conflict of interest.

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