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THÈSE

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Régulation du métabolisme adipocytaire de la vitamine D : Effet de l'obésité et d'une supplémentation en vitamine D

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Durant mon enfance, je me rappelle passer des heures à chercher, dans la célèbre BD « où est Charlie », ce petit personnage dans une foule immense … C'est drôle, parce qu'après toutes ses années, je fais un peu la même chose, je cherche où se cache la vitamine D (VD) chez les personnes obèses.

La VD peut être apportée par l'alimentation en consommant des poissons gras comme par exemple le saumon mais on peut aussi la fabriquer lorsque les rayons du soleil sont en contact de notre peau. La VD sert à fixer le calcium sur les os mais ce n'est pas cette fonction-là qui m'intéresse puisque j'étudie la relation entre la VD et l'obésité. L'obésité est une maladie qui n'est pas à prendre à la « légère » puisqu'elle touche quand même 15% de la population en France et 35% aux Etats-Unis. Il a été montré que toutes les personnes obèses sont en insuffisance de VD. Si on supplémente ces personnes avec de la VD, on n'observe aucune augmentation de ce taux, mais alors « où est la VD » chez les obèses ? Plusieurs hypothèses ont été émises afin d'expliquer cette insuffisance et nous en proposons une originale basée sur le métabolisme de cette VD au niveau du gras appelé aussi tissu adipeux (TA). Le métabolisme, c'est le nom donné à toutes les étapes qui permettent d'aboutir à la forme active de la VD. Je m'explique, quand la VD arrive dans notre corps, elle n'est pas active sous cette forme-là, elle va devoir subir plusieurs transformations afin de pour pouvoir assurer ses fonctions.

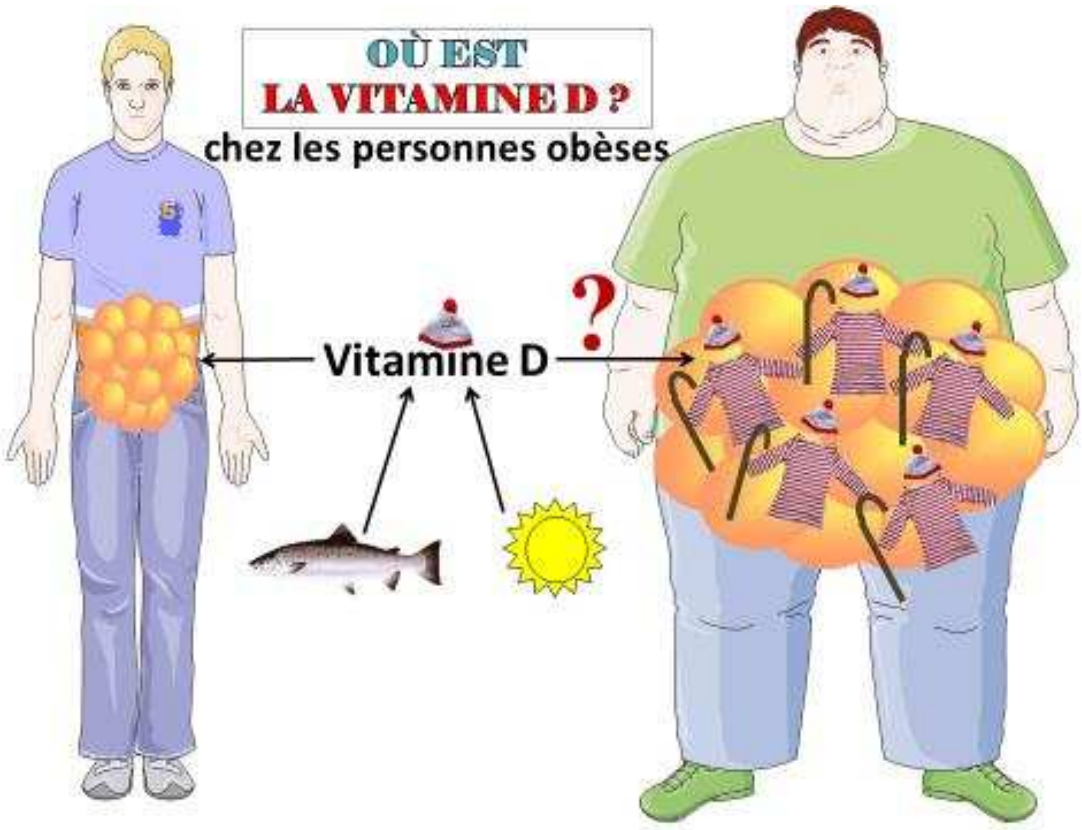
Mais le métabolisme de la VD n'a encore jamais été décrit au niveau du TA des personnes obèses, est-il différent des personnes non-obèses ?

Pour répondre à cette question, je nourris des souris avec une alimentation enrichie en graisse pendant plusieurs jours et déjà au bout de 4 jours de régime, alors que mes souris ont le même poids que celles nourries avec une alimentation contrôle, j'observe des changements dans leur métabolisme ! C'est un peu comme Charlie qu'on retrouve souvent avec sa canne, la VD semble être associée avec une modification de son métabolisme au niveau du TA au cours de l'obésité.

La 2ème partie de mes travaux consiste à quantifier cette VD dans le TA à la fois chez mes souris nourries avec une alimentation riche en graisse mais aussi, et surtout j'ai envie de dire, chez

l'Homme afin de comparer ces taux de VD entre patients obèses et non-obèses. Cette VD n'est pas habillée d'un bonnet blanc et rouge comme le fameux personnage mais j'ai à ma disposition plusieurs machines qui me permettent de la repérer parmi des milliards d'autres molécules.

La VD n'est certainement pas la recette miracle pour maigrir mais connaître au mieux son métabolisme donnera des pistes afin de prévenir de l'obésité. En tout cas, j'ai toujours réussi à trouver Charlie étant plus jeune, alors je sais que j'arriverai à retrouver la VD elle aussi !



LISTE DES ABREVIATIONS

AMN : Amnioless

ANC : Apports nutritionnels conseillés

ANSES : Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail

CCL2 : Chemokine ligand 2

CD36 : Cluster of differentiation 36

C-EBP : CCAAT-enhancer binding proteins

CNV : Copy-number variations

CYP : Cytochrome p450

Dab2 : Disabled 2

DESIR : Data Epidemiological Study on the Insulin Resistance syndrome

EFSA : Agence européenne des aliments

GNS : German nutrition society

GWAS : Genome-wide association study

HAT : Histones acétylases

HDAC : Histones désacétylases

IL : Interleukine

IMC : Indice de masse corporelle

INCA : Etude Individuelle Nationale des Consommations Alimentaires

IOM : Institute of Medecine

JNK : Jun N-terminale kinase

LC-MS : Chromatographie en phase liquide couplée à la spectrométrie de masse

LC-MS/MS : Chromatographie en phase liquide couplée à la spectrométrie de masse en tandem

LDL : Low-density lipoprotein

LPS : Lipopolysaccharide

MAPK : Mmitogen-activated protein kinases

MCP1 : Monocyte chemoattractant protein 1

microARN : miR

MONICA : Multinational MONItoring of trends and determinants in Cardiovascular disease

NF-κB : Nuclear factor-kappa B

NPC1L1 : Niemann-Pick C1-Like 1

NPY4R : Neuropeptide Y receptor

OCDE : Organisation de coopération et de développement économiques

OMS : Organisation mondiale de la santé

Pdia3 : Protein disulfide isomerase family A member 3

PPARγ : Peroxisome proliferator-activated receptor γ

PTH : Parathormone

RAP : Receptor Associated Protein

RXR : Retinoid X Receptor

SNP : single-nucleotide polymorphism

TA épi : Tissu adipeux épidymal

TAv : Tissu adipeux viscéral

TAsc : Tissu adipeux sous cutané

TOF-SIMS : Spectrométrie de masse à ionisation secondaire

TNFα : Tumor necrosis factor α

UVB : Rayonnements ultraviolets B

VDBP : Vitamin D binding protein

VDR : Vitamin D Receptor

VDRE : Vitamin D Element Response

25(OH)D : 25-hydroxyvitamine D

$1,25(OH)_2D$: 1,25-dihydroxyvitamine D

RESUME

La vitamine D est une hormone aux multiples potentialités. Elle est notamment impliquée dans de nombreux processus physiologiques, dont le contrôle de certains paramètres de la biologie du tissu adipeux, son site principal de stockage. Ainsi elle y exerce des effets anti-inflammatoires et métaboliques forts, mais de nombreux aspects n'ont jamais été étudiés concernant la régulation du métabolisme adipocytaire de la vitamine D. De plus, ce métabolisme semble être régulé par la vitamine D elle-même et par l'obésité, cependant les mécanismes mis en jeu ne sont pas clairement établis. Le but de cette thèse est d'avancer dans la connaissance de l'interrelation entre la vitamine D, le tissu adipeux et l'obésité.

Il a récemment été montré au laboratoire que la vitamine D présentait un effet anti-inflammatoire en modulant l'expression de cytokines et de chimiokines au niveau du tissu adipeux et de l'adipocyte. Puisque les miRs constituent un nouveau mécanisme de régulation de l'inflammation au niveau adipocytaire, nous avons étudié l'impact de la vitamine D sur l'expression des miRs dans les adipocytes soumis à un stress inflammatoire. Nous avons donc mis en évidence que la vitamine D limitait l'expression de 3 miRs (miR-146a, miR-150 et miR-155) en inhibant la voie de signalisation NF-κB, in vitro et in vivo, au niveau du tissu adipeux et de l'adipocyte, lors d'un stress inflammatoire.

De plus, nous nous sommes intéressés à l'impact de la vitamine D dans la régulation de son propre métabolisme et nous avons montré que la vitamine D modulait l'expression génique de certaines enzymes au niveau du tissu adipeux et de l'adipocyte, alors que ces régulations n'étaient jusqu'alors connues qu'au niveau hépatique et rénal. De plus, l'implication de la cubiline dans l'absorption de la 25(OH)D au niveau des adipocytes a été mis en évidence.

Ensuite, nous avons étudié l'effet d'un régime riche en graisse, mimant une alimentation déséquilibrée et obésogène, pendant 4 jours, 7 et 11 semaines, sur la régulation du métabolisme de la vitamine D, notamment au niveau du tissu adipeux. Au bout de 4 jours de régime, nous avons pu mettre en évidence une modulation de la concentration plasmatique de certains métabolites de la vitamine D ainsi que des ARNm de certains acteurs impliqués dans le métabolisme de la vitamine D, suggérant un stockage de la vitamine D sous forme de 25(OH)D dans le tissu adipeux.

Au bout de 7 et 11 semaines de régime, le tissu adipeux des souris obèses présentait une modification des gènes codant les acteurs du métabolisme de la vitamine D allant dans le sens d'un stockage de celle-ci et de ses métabolites, comme nous avons pu le confirmer par quantification réalisée par LC-MS/MS. L'ensemble de ces résultats pourrait expliquer la diminution des concentrations plasmatiques en cholécalciférol et 25(OH)D libre observée chez les souris obèses.

Enfin, l'effet d'une prise de poids induite par un régime riche en graisse puis un retour à un régime contrôle a été étudié chez les souris. Les niveaux d'expressions géniques au niveau du tissu adipeux des différents acteurs mis en jeu dans le métabolisme de la vitamine D ainsi que les dosages plasmatiques et adipocytaires de la vitamine D et de ses métabolites montrent un retour à l'état basal chez les souris ayant subi une prise puis une perte de poids, démontrant ainsi la réversibilité des modifications induites par un régime obésogène sur le métabolisme de la vitamine D.

ABSTRACT

Vitamin D is a hormone with multiple many potentialities. It is notably involved in many physiological processes, including the control of parameters of adipose tissue biology, its main storage site. Thus, it exerts strong anti-inflammatory and metabolic effects, but many other aspects have never been studied concerning the regulation of the adipocyte metabolism of vitamin D. Indeed, this metabolism seems to be regulated by vitamin D itself and by obesity, however the mechanisms involved are not clearly established. The aim of this thesis is to advance in the knowledge of the interrelation between vitamin D, adipose tissue and obesity.

It has recently been shown in the laboratory that vitamin D presented an anti-inflammatory effect by modulation of cytokines and chemokines expression in adipose tissue and adipocyte. Since miRs are a new mechanism for regulation of inflammation in adipocyte, we have studied the impact of vitamin D on the expression of miRs in adipocytes undergoing inflammatory stress. We have therefore demonstrated that vitamin D limits the expression of 3 miRs (miR-146a, miR-150 and miR-155) by inhibition of NF-κB signaling pathway, in vitro and in vivo, in adipose tissue and adipocyte, during inflammatory stress.

We also investigated the impact of vitamin D on the regulation of its own adipocyte metabolism and showed that vitamin D modulated gene expression of enzymes involved in its own metabolism in adipose tissue and adipocyte, during a treatment of vitamin D, in vivo and in vitro. These data were known only in the liver and kidney. Moreover, the involvement of cubiline in 25(OH)D uptake in adipocytes has been demonstrated.

Then, we studied the effect of high fat diet, mimicked by an unbalanced and obesogenic diet, for 4 days, 7 and 11 weeks, on the regulation of vitamin D metabolism, in adipose tissue mainly. After 4 days of diet, we were able to demonstrate a modulation of plasma concentration of vitamin D metabolites as well as mRNAs of some actors involved in vitamin D metabolism, suggested a possible storage of vitamin D under 25(OH)D form in adipose tissue.. After 7 and 11 weeks of diet, adipose tissue of obese mice showed a modification of the genes coding for vitamin D metabolism. These data suggested a possible storage of vitamin D and its metabolites which we have been able to confirm by quantification carried out by LC-MS/MS. All of these results could

explain the decrease in cholecalciferol and free 25(OH)D plasma concentrations observed in obese mice.

Finally, the effect of weight gain induced by a high-fat diet followed by a return to a control diet was studied in mice. The levels of gene expression in the adipose tissue of the actors involved in vitamin D metabolism as well as the plasma and adipocyte dosages of vitamin D and its metabolites show a return to the basal state in mice having undergone weight loss. These results demonstrate the reversibility of changes induced by an obesogenic diet on vitamin D metabolism.

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INTRODUCTION

1. LA VITAMINE D

1.1 GENERALITES/HISTORIQUE/ORIGINES

La découverte de la vitamine D est liée à une maladie : le rachitisme, touchant les enfants vivant dans des régions pauvres et faiblement ensoleillées. Deux chercheurs, Mac Collum et Mellanbourg, en 1920, font la relation entre l'efficacité de l'huile de foie de morue dans la prévention et le traitement du rachitisme, et la présence dans cette huile d'une substance qu'ils nomment vitamine D.

La vitamine D appartient à la famille des stérols au même titre que le cholestérol, et plus particulièrement à la sous-classe des sécostéroïdes. La vitamine D est présente sous 4 principales formes dans l'organisme **(Figure 1)** : l'ergocalciférol, le cholécalciférol (forme native et majoritaire), la 25-hydroxyvitamine D ou calcidiol (25(OH)D) (forme circulante) et le calcitriol ou la 1,25-dihydroxyvitamine D ($1,25(OH)_2D$) (forme active). Le terme « vitamine », c'est-à-dire un produit « vital » que l'organisme ne peut pas produire, est très largement inapproprié pour la vitamine D. En effet, elle a deux origines : exogène mais aussi endogène, contrairement aux autres vitamines [1].

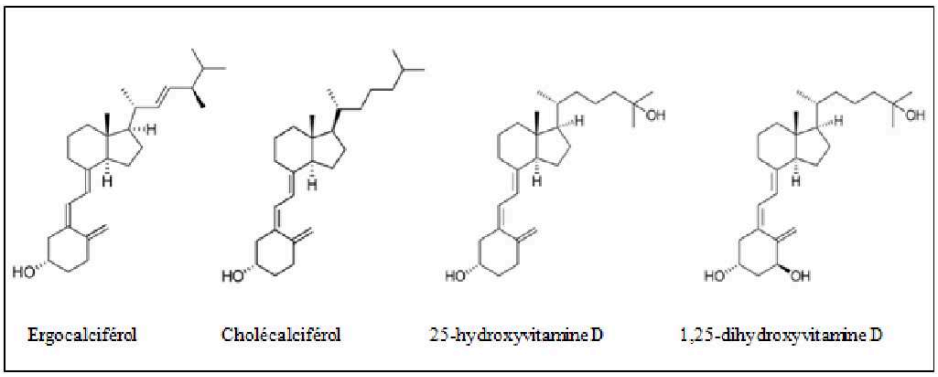

Figure 1 : Structure des différentes formes de la vitamine D [2].

La synthèse endogène de vitamine D se déroule au niveau de l'épiderme qui est capable de la produire en grande quantité, après une exposition aux rayonnements ultraviolets B (UVB) fournis par l'ensoleillement. Elle est réalisée à partir du 7-déhydrocholestérol, un dérivé du cholestérol, présent dans les membranes des cellules du derme et de l'épiderme [3]. L'énergie fournie par les rayons ultraviolets B permet sa transformation en pré-vitamine D3, elle-même rapidement convertie sous l'effet de la chaleur en vitamine D3, libérée dans la circulation. Cette synthèse de vitamine D est donc étroitement liée à l'exposition solaire. On considère classiquement que 80 % et 90% de la vitamine D de l'organisme provient de l'exposition solaire [4]. Elle est cependant plus faible l'hiver (du fait de l'incidence des rayons solaires et de la plus faible quantité de rayons UVB) par rapport à l'été. La synthèse est maximale entre 10 h et 15 h. Environ 5 à 15 minutes d'exposition, sans crème solaire, de 20 % de la surface corporelle par jour de mai à septembre permettent une synthèse suffisante.

La seconde origine de la vitamine D est exogène. Elle est apportée par l'alimentation, et 80 % de celle-ci est absorbée relativement lentement (environ 40 % sur 12 heures). La vitamine D est présente soit sous la forme de vitamine D2 (ergocalciférol), produite essentiellement par les végétaux, soit sous la forme de vitamine D3 (cholécalciférol) d'origine animale. Elles sont liposolubles et relativement stables, notamment à la chaleur.

Les aliments contenant de la vitamine D3 sont peu nombreux. On la trouve essentiellement dans les huiles de foie de poissons, dans certains poissons gras (saumons, sardines, harengs, maquereaux), dans le jaune d'œuf ou encore dans le foie **(Tableau 1)**. La vitamine D3 est également présente en petite quantité naturellement dans le lait et le jaune d'œuf, et en plus grande quantité quand ces aliments sont enrichis (dans la limite de 1,25 µg/100 g). Un paramètre largement sous-estimé dans le calcul des apports alimentaires en vitamine D est la contribution de la 25(OH)D, naturellement présente dans les aliments. En effet, cette dernière n'est jamais prise en compte dans le calcul des apports exogènes de vitamine D. Pourtant ce métabolite est présent en quantité variable mais non négligeable dans un grand nombre d'aliments de consommation courante [5, 6]. De plus, il semble que l'absorption de cette 25(OH)D soit plus efficace que celle de la vitamine D. Toutefois la contribution réelle de cette molécule dans le maintien des taux plasmatiques notamment chez l'Homme n'est pas complètement établie [5] et devra faire l'objet d'études approfondies, afin d'en tenir éventuellement compte dans les calculs d'apports vitaminiques.

Tableau 1 : Principales sources alimentaires de vitamine D3 (1 µg = 40 UI) (D'après la table Centre d'informations sur la qualité des aliments, 2012)

Aliments	Vitamine D3 (en µg/ 100g)	Vitamine D3 (en UI/ 100g)
Huile de foie de morue	250	10 000
Saumon, Hareng, Anchois	12-20	480-800
Sardine, Maquereau	8-12	320-480
Thon	4-7	160-280
Foie de veau	2-3	80-120
Jaune d'œuf	2-3	80-120
Laitages enrichis	1,25	50
Beurre	0,6-1,5	24-60

1.2 APPORTS RECOMMANDES

1.2.1 EN FRANCE

Les Apports Nutritionnels Conseillés (ANC) en 2001 pour la population adulte française étaient de 5 µg/jour (soit 200 UI/jour) **(Tableau 2)**. Ils ont été définis en considérant que la production endogène cutanée couvre 50 à 70% des besoins quotidiens en cette vitamine. A l'heure actuelle, l'ANSES (Agence Nationale de Sécurité Sanitaire de l'alimentation, de l'Environnement et du travail) a fixé les valeurs suivantes :

Tableau 2 : Apports nutritionnels conseillés en vitamine Den fonction de l'âge

(1µg = 40 UI) (D'arpès [7])

Tranche d'âge	Apports conseillés (µg/jour)	Apports conseillés (UI/jour)
Enfants (1 à 3 ans)	10	400
Eκants (4 à 12 ans)	5	200
Adolescents (13 à 19 ans)	5	200
Adultes	5	200
Personnes âgées	10 à 15	400 à 600
Femmes enceintes et allaitantes	10	400

En France, l'étude INCA (Etude Individuelle Nationale des Consommations Alimentaires) 2 [8] a permis de mettre en évidence, d'une part que l'apport alimentaire en vitamine D n'est que de 2,6 µg/jour (104 UI/jour) chez l'adulte et 1,9 µg/jour (76 UI/jour) chez l'enfant, ce qui est loin de couvrir les ANC. D'autre part cette étude a également montré que 38% de l'apport en vitamine D chez l'adulte provient de la consommation de poisson, 10% de la consommation d'œufs et 18% de la consommation de fromages (31 %, 9% et 7% respectivement chez l'enfant).

La vitamine D est connue depuis plus d'un siècle pour son action antirachitique et c'est sur cette base qu'ont été définis les apports recommandés. Cependant, devant les résultats des études montrant un lien entre vitamine D et santé extrasquelettique et les observations indiquant des apports généralement insuffisants, les réflexions s'intensifient sur la nécessité de revoir les recommandations à la hausse.

1.2.2 DANS LES AUTRES PAYS

L'agence européenne des aliments (EFSA) est une agence européenne indépendante fondée en 2002 par l'Union européenne « afin de constituer une source impartiale de conseils scientifiques

et de communication sur les risques associés à l'alimentation ». Elle publie régulièrement depuis 2010 sur les apports conseillés en nutriments. L'EFSA recommande un apport journalier de 15 µg de vitamine D (600 UI) pour toute les populations (et 10 µg pour les nourrissons), le triple des apports recommandés actuellement par l'ANSES **(Tableau 3)** [9]. L'agence a établi ces valeurs seuils d'apport en vitamine D afin d'améliorer la santé musculaire et osseuse mais n'a pas tenu compte d'autres bénéfices supposés de la vitamine D comme les maladies cardiovasculaires et cancers notamment. Selon l'EFSA, cet apport permet à presque tous les européens d'atteindre un taux sanguin minimum de 20 ng/mL (équivalent à 50 nmol/L).

D'autres pays en Europe ont eux aussi revu leurs recommandations, c'est notamment le cas de l'Allemagne où la German Nutrition Society (GNS) conseille un apport de 20 µg/jour (800 UI/jour) pour la majorité des groupes de populations **(Tableau 3)** [10]. De même en Outre-Atlantique, l'Institute Of Medicine (IOM) a fixé les recommandations entre 15 et 20 µg/jour (600 et 800 UI/jour) selon les groupes de populations [11].

Tableau 3 : Les recommandations en vitamine D des pays occidentaux (1µg = 40 UI)

Apports journaliers recommandés (en microgramme/jour)	**18-50 ans**	**+70 ans**
IOM (USA) et Health Canada (2011)	15	20
Australie (2006)	5	15
ANSES (France, 2001) **ANSES (France, 1992)**	5 12	10
EFSA (Europe, 2016)	15	20
GNS (Allemagne, 2012)	20	20-25

Institute Of Medicine (IOM), Agence Nationale de Sécurité Sanitaire de l'alimentation, de l'Environnement et du travail (ANSES), Agence européenne des aliments (EFSA) et German Nutrition Society (GNS)

1.3 EVALUATION DU STATUT EN VITAMINE D

Le niveau sanguin de 25(OH)D totale est l'indicateur de référence du statut en vitamine D. Lorsque le taux sérique de 25(OH)D est supérieur à 30 ng/mL (75 nmol/L), les réserves sont dites « suffisantes » et le statut vitaminique D peut être qualifié d' « optimal ». À l'inverse, le terme de statut vitaminique D « suboptimal » est souvent utilisé lorsqu'il est inférieur à 30 ng/mL. Cette valeur de 30 ng/mL est en effet considérée comme le seuil en deçà duquel apparaît une hyperparathyroïdie secondaire à l'hypovitaminose D, impliquant un remodelage osseux accéléré et une diminution de la densité minérale osseuse en particulier au niveau de l'os cortical. Ce taux est défini comme optimal par « The Endocrine Society » pour les personnes âgées afin de limiter le risque de chutes [12]. On distingue l'insuffisance, définie par un taux de 25(OH)D compris entre 10 et 30 ng/mL, de la carence, définie par un taux inférieur à 10 ng/mL (25 nmol/L) **(Tableau 4)**.

Tableau 4 : Valeurs déterminant les différents statuts en vitamine D

Statut en vitamine D	Niveau plasmatique de 25(OH)D (ng/mL)	Niveau plasmatique de 25(OH)D (nmol/L)
Carence en vitamine D	<10	<25
Insuffisance en vitamine D	10-30	25-75
Valeurs cibles optimales et plage d'apport idéale en vitamine D	30-90	75-225
Surdose	>100	>250
Intoxication	>150-200	>375-500

Parallèlement à la mesure du taux plasmatique de 25(OH)D totale, il existe d'autres marqueurs reflétant l'insuffisance ou la carence en vitamine D et notamment le taux de parathormone (PTH) [13], ou encore l'apparition de conséquences cliniques [12, 14, 15]. Chez le nourrisson, une concentration sérique élevée en phosphatase alcaline peut aussi indiquer une carence en vitamine D puisque la phosphatase alcaline est augmentée chez les patients atteints de rachitisme **(Tableau 5)**.

Récemment, un nouveau marqueur du statut en vitamine D plasmatique a été évoqué, il s'agit de la mesure des taux sériques en 25(OH)D libre [16]. En effet, la 25(OH)D est soit liée à sa Vitamin D Binding Protin (VDBP) (88%), soit à l'albumine (12%), soit une faible partie est sous forme libre (<1%) [17]. Il a été montré que les taux plasmatiques en 25(OH)D libre étaient fortement corrélés avec les taux plasmatiques en 25(OH)D totale chez l'Homme et que cette corrélation était la même suivant les différentes saisons de l'année [18]. Cependant, des études appropriées seront nécessaires afin de définir l'intérêt clinique du dosage de la 25(OH)D libre plutôt que de la 25(OH)D totale chez des sujets normaux ainsi que dans diverses pathologies [19]. Il semble, par ailleurs, exister un consensus selon lequel la plupart des cellules, en dehors des cellules tubulaires rénales, sont exposées à la forme libre de la 25(OH)D plutôt qu'à sa forme totale qui reste à « piéger » dans le milieu plasmatique [16]. Par conséquent, la 25(OH)D libre pourrait être responsable de la production locale de $1,25(OH)_2D$. Les données existantes entre la 25(OH)D libre et la PTH ne sont pas très claires et montrent dans certains cas une relation inverse entre les deux paramètres [20] et dans d'autres cas, aucune corrélation [21]. Plusieurs méthodes sont répertoriées afin de déterminer la concentration sérique de 25(OH)D libre : 1) par calcul en fonction de la mesure des concentrations sériques de la 25(OH)D totale, de la protéine de liaison à la vitamine D (VDBP) ou de l'albumine, 2) par la mesure de l'affinité entre 25(OH)D et ses protéines de liaison dans situations physiologiques 3) par mesure directe de la dialyse à l'équilibre, de l'ultrafiltration 4) ou par immunodosage. Cependant, ces différentes méthodes doivent encore être standardisées afin d'optimiser les résultats.

Tableau 5 : Indicateurs pour l'évaluation du statut en vitamine D à l'échelle de la population

Indicateur	Echantillon	Groupe de population	Seuil de définition de la carence	Remarques
25-hydroxyvitamine D totale	Sérum	Applicable à tous les groupes de population	20 à 25 nmol/l	Le taux sérique de 25(OH)D totale, en association avec le taux d'hormone parathyroïdienne, est un bon indicateur du statut en vitamine D
25-hydroxyvitamine D libre	Sérum	Applicable à tous les groupes de population	Actuellement pas de seuil généralement reconnu	Le taux sériques de 25(OH)D libre devrait être corréler avec les taux de sériques de VDBP pour de meilleurs résultats
Hormone parathyroïdienne	Sérum	Applicable à tous les groupes de population	Actuellement pas de seuil généralement reconnu	Le taux sérique d'hormone parathyroïdienne est en relation inverse avec celui de 25(OH)D et pourrait être un bon indicateur du statut en vitamine D
Phosphatase alcaline	Sérum	Applicable à tous les groupes de population	Actuellement pas de seuil généralement reconnu	Augmenté en cas d'ostéomalacie ou de rachitisme

1.4 CARENCE ET INSUFFISANCE

Malgré la double origine (endogène et exogène) de la vitamine D, la population française dans sa globalité est fortement en déficience ou insuffisance (**Tableau 6**). En effet, à la valeur seuil plasmatique de 25(OH)D de 20 ng/ml, on se rend compte qu'environ 40% de la population est en

insuffisance, et si l'on se fixe au seuil de 30 ng/ml, près de 80% de la population sont alors en situation d'insuffisance [22]. Ceci s'explique d'une part par le faible nombre d'aliments riches en vitamine D et leur relative faible consommation. D'autre part, si la néosynthèse cutanée a très longtemps été considérée comme pouvant couvrir 50 à 70 % des besoins en vitamine D, un certain nombre de facteurs dont certains fortement liés à nos modes de vie actuels, font que cette néosynthèse tend à diminuer, le chiffre de 10 à 25 % des besoins en vitamine D couverts par la synthèse endogène a récemment été avancé [23].

Tableau 6 : Statut plasmatique en vitamine D de la population française (D'après [22])

25(OH)D	< 10 ng/ mL (< 25 nmol/ L)	< 20 ng/ mL (< 50 nmol/ L)	< 30 ng/ mL (< 75 nmol/ L)
Hommes	3,6 %	35,8 %	78,7 %
Femmes	5,9 %	49,0 %	81,4 %
18 – 29 ans	7,5 %	45,9 %	79,2 %
30 – 54 ans	5,2 %	41,4 %	79,1 %
55 – 74 ans	1,9 %	41,7 %	82,4 %

On sait depuis longtemps que la synthèse endogène de vitamine D est influencée par la saison, l'horaire d'exposition et la latitude [1]. La saison hivernale est associée à une quasi-absence de néosynthèse. Les UVB ne sont présents en France que quelques mois par an (entre avril et octobre à Paris par exemple) et ce n'est qu'à ces périodes que la synthèse cutanée de vitamine D3 est possible. D'autres paramètres anthropomorphiques tels que l'âge, la pigmentation de la peau, l'obésité ou le surpoids tendent à réduire la synthèse [10]. En effet, la concentration de 7-déhydrocholestérol dans les couches profondes de l'épiderme diminue avec l'âge; une personne âgée de 70 ans produit 4 fois moins de vitamine D qu'un sujet âgé de 20 ans. La mélanine (pigment de la peau) constitue un écran solaire naturel et l'augmentation de cette pigmentation mélanique peut réduire la synthèse de vitamine D. Ainsi, la prévalence de l'insuffisance en vitamine D est plus importante chez les sujets de peau noire. Certains facteurs liés au mode de vie moderne

favorisent également l'insuffisance, c'est notamment le cas de la sédentarité conduisant à une moindre exposition au soleil, ainsi que l'augmentation de l'utilisation de crèmes solaires, liée à l'application des consignes de photoprotection en prévention des cancers cutanés [1]. En effet, la synthèse de vitamine D peut être réduite de plus de 90% par les crèmes solaires présentant un index de protection supérieur ou égal à 15, ce qui conduit à une prévalence de l'insuffisance en vitamine D paradoxalement plus élevée dans les pays où l'ensoleillement est important du fait d'une forte protection solaire. Le fait de vivre dans une région ensoleillée n'est donc pas obligatoirement synonyme de production optimale de vitamine D. La pollution atmosphérique en bloquant une partie du rayonnement UVB participe aussi à la réduction de la synthèse de vitamine D. Enfin, des aspects socioculturels tels que le port de vêtements couvrants limitent également la synthèse endogène.

Les signes cliniques sont liés à l'ampleur de la déficience ou de la carence. Ils peuvent s'observer au niveau osseux en provoquant l'ostéomalacie chez l'adulte ou le rachitisme chez l'enfant. Cela est particulièrement fréquent lorsque ce déficit est associé à une malabsorption de la vitamine D [24]. Cette carence a également des effets au niveau musculaire (baisse du tonus musculaire, douleurs, dégradation de la démarche) et peut s'accompagner de crises de tétanie, de convulsions (en relation avec une hypocalcémie) et de fractures. Chez les personnes âgées, la carence en vitamine D constitue un terrain favorable à la perte osseuse et donc à l'ostéoporose entraînant ainsi un risque plus important de chutes. Un déficit en vitamine D peut également aggraver l'effet hypocalcémiant de certains médicaments. Dans certains cas, la carence en vitamine D est associée avec un risque plus important de maladies cardio-vasculaires [25] ainsi qu'un risque plus important de cancers du sein, du colon et de la prostate. Une insuffisance en vitamine D entraîne une diminution de l'absorption intestinale du calcium et une tendance hypocalcémique, elle-même compensée par une élévation de la PTH [26]. Cette hyperparathyroïdie secondaire va stimuler le remodelage osseux, ce qui sur le long terme diminuera la densité minérale osseuse en particulier au niveau de l'os cortical, et stimulera la 1-alpha hydroxylase, augmentant la concentration sérique en $1,25(OH)_2D$.

1.5 SUPPLEMENTATION EN VITAMINE D

Avant d'envisager le recours à la supplémentation pharmacologique, plusieurs recommandations diététiques et comportementales peuvent être envisagées. L'exposition aux UVB est une façon simple d'augmenter la synthèse de la 25(OH)D qui n'expose pas à un risque d'intoxication, l'excès de vitamine D3 et de prévitamine D3 étant transformé en métabolites inactifs [27]. Une exposition au soleil, bras et jambes, 5 à 30 minutes, 2 fois par semaine, entre 10 et 15 heures au printemps, été et automne, accroît significativement le taux de 25(OH)D. Une exposition au soleil minimale sur le corps entier apporte en un jour, 20 000 UI de vitamine D. L'utilisation de lampes UV a donc été proposée [28]. Toutefois, cette recommandation présente tout de même des limites, notamment liées au risque accru de mélanome.

Il est difficile de compenser une carence en vitamine D grâce à l'alimentation puisque peu d'aliments en contiennent naturellement de façon significative. De plus ces quelques aliments sont des aliments qui ont une forte teneur en lipides, il n'est donc pas recommandé d'en ingérer en grande quantité.

La supplémentation pharmacologique est nécessaire dans certains cas. Le seuil de 75 nmol/l est actuellement proposé comme une référence en termes de bénéfice osseux, mais des taux supérieurs pourraient parfois être nécessaires pour l'obtention de certains effets extra-osseux. La forme naturelle de la vitamine D est de loin là plus largement utilisée pour corriger une insuffisance, mais ses modalités de prescription restent débattues. Des interrogations persistent en effet quant à la forme à administrer. Il existe en effet, des effets différenciels entre vitamine D2 et D3. Des résultats récents montrent que vitamine D2 et D3 sont également efficaces pour augmenter les niveaux plasmatiques en 25(OH)D après une forte dose (100 000 IU). À long terme, vitamine D3 semble mieux appropriée pour maintenir les taux plasmatiques en 25(OH)D au-dessus du seuil optimal, ce qui pourrait être plus pertinent pour les effets classiques et non classiques connus de la vitamine D [29]. De plus, de nombreuses études menées au cours des dix dernières années ont montré que la vitamine D3 était mieux absorbée et utilisée que D2 [30].

Le rythme de prescription est également une question redondante (quotidienne ou plus espacée, voie orale ou injectable. . .), ainsi que la posologie. Une posologie minimale de 800 UI/j de vitamine D3 apparaît nécessaire « en entretien » pour protéger l'os ; des doses plus fortes (100 000

à 200 000 UI tous les deux mois, pendant six mois par exemple) peuvent en revanche être requises pour corriger un déficit franc [31]. Dans ces cas, un contrôle du taux de 25(OH)D à quatre ou six mois permet de s'assurer de la correction de l'insuffisance et d'adapter la supplémentation ultérieure. Mais au-delà de ces considérations pratiques, le message actuel est de prendre en compte la fréquence du déficit en vitamine D, tant dans la population générale que plus spécifiquement chez les patient(e)s à risque d'ostéoporose, d'en vérifier l'intensité et de la corriger. Un apport quotidien minimal de 800 UI/j apparaît aujourd'hui comme un pré-requis utile et sûr en termes de santé publique [31]. Binkley et al. ont rapporté que le taux de 25(OH)D était plus élevé chez les sujets recevant une dose mensuelle (50 000 UI, vitamine D2 ou D3) qu'une dose quotidienne (1600 UI, vitamine D2 ou D3) [32]. Les suppléments de vitamine D administrés par voie intramusculaire sont utiles pour les patients souffrant de troubles d'absorption et de faible compliance et dans les domaines où les suppléments par voie orale ne sont pas possibles. Cependant, il existe des inquiétudes quant à la sécurité [33] et à l'efficacité de la vitamine D administrée par voie intramusculaire. Zabihiyeganeh et al. ont montré que la réponse à la supplémentation en 25 (OH) D était meilleure sous forme orale que par voie intramusculaire (+ 90,0 ± 11,2 nmol/L contre + 58,8 ± 8,9 nmol/L respectivement, p = 0,03) [34] mais, après 6 mois, il n'y avait plus de différence de concentration en 25(OH)D entre les deux groupes. Les auteurs ont suggéré qu'une période plus longue serait nécessaire pour observer des changements significatifs dans les concentrations sériques de 25(OH)D lorsque des suppléments de vitamine D sont administrés par voie intramusculaire.

Cependant, la vitamine D est potentiellement toxique. C'est pourquoi les doses recommandées pour la supplémentation (800 UI/j) sont relativement modestes [35-37]. Un excès de vitamine D a pour conséquence une augmentation de l'absorption intestinale du calcium. La tendance hypercalcémique qui en résulte freine la sécrétion de PTH ce qui augmente la calciurie avec des risques rénaux potentiels (lithiase, néphrocalcinose). Cette situation est cependant un fait très rare. La revue de Vieth [38] suggère qu'une intoxication à la vitamine D n'apparaît jamais pour des concentrations de 25(OH)D inférieures à 250 nmol/L (100 ng/mL) et que des doses de 10 000 UI/j sont sans danger. Des études complémentaires, en particulier sur des périodes longues, sont nécessaires pour que ces doses puissent être considérées comme acceptables.

1.6 METABOLISME DE LA VITAMINE D ET DE SES METABOLITES

1.6.1 ABSORPTION ET TRANSPORT DE LA VITAMINE D

La vitamine D d'origine alimentaire est incorporée dans les micelles mixtes et absorbée dans la partie proximale de l'intestin grêle. Ce processus a longtemps été considéré comme exclusivement passif, jusqu'à la mise en évidence de l'implication de transporteurs du cholestérol dans cette absorption. Ainsi, Cluster of Differentiation 36 (CD36), Niemann-Pick C1-Like 1 (NPC1L1) et Scavenger Receptor class B type 1 (SR-B1) participent également à l'absorption de la vitamine D [39]. Après son absorption, le transport plasmatique de la vitamine D alimentaire semble être majoritairement dépendant de son incorporation dans les chylomicrons, au sein desquels la vitamine D est véhiculée jusqu'au foie. Les vitamines D2 et D3 ont un métabolisme sensiblement identique et dépendant des mêmes complexes enzymatiques chez l'Homme. La vitamine D néosynthétisée semble être très majoritairement liée à la VDBP [40].

La VDBP est une α2-globuline synthétisée par le foie [41]. Elle appartient à la famille génique de l'albumine, de l'α-fétoprotéine et de l'afamine. Cette protéine lie à la fois la vitamine D mais également ses métabolites (25(OH)D et 1,25$(OH)_2$D) et constitue leur principal transporteur plasmatique. Ces différents métabolites sont très majoritairement liés à la VDBP (environ 88 %) dans la circulation sanguine. La VDBP est présente en très large excès molaire par rapport à ses ligands, ce qui pourrait permettre de limiter l'accessibilité des métabolites aux cellules utilisatrices. En effet, lorsque les métabolites de la vitamine D sont liés à la VDBP, ils semblent être moins accessibles que les formes libres circulantes, ce qui permettrait ainsi de prolonger leur demi-vie plasmatique et de stabiliser leurs concentrations plasmatiques [42]. Ainsi, il a été montré que des souris invalidées pour le gène codant la VDBP présentent une très rapide élimination rénale des métabolites de la vitamine D à l'origine de leurs très faibles taux plasmatiques. Enfin, les complexes VDBP-métabolites de vitamine D seraient internalisés dans les cellules utilisatrices par endocytose [41].

1.6.2 25-HYDROXYLATION

Après son transport dans la circulation sanguine, liée aux chylomicrons ou à la VDBP, la vitamine D est captée au niveau hépatique et hydroxylée sur le carbone 25 pour former la 25-hydroxyvitamine D (25(OH)D) **(Figure 2)** dont la demi-vie est relativement longue (3 à 4 semaines) et la concentration plasmatique moyenne comprise entre 20 et 50 ng/ml (50 à 125 nmol/L). Plusieurs enzymes sont capables de catalyser cette réaction chez l'Homme et la souris comme la CYP2R1, CYP27A1, CYP3A4 (ou CYP3A11 chez la souris) et CYP2J2 (ou CYP2J6 chez la souris) [43]. Elles appartiennent à la famille des cytochromes P450 (CYP) et ont une localisation mitochondriale (CYP27A1) ou microsomale (CYP2R1, CYP2J2 et CYP3A4) **(Figure 2)**. Les cytochromes P450 sont impliqués dans de nombreuses réactions dont les mécanismes de détoxification des xénobiotiques (composés étrangers à l'organisme) en greffant un groupement hydroxyle sur les xénobiotiques afin de faciliter leur élimination urinaire. De récents travaux révèlent que CYP2R1 semble être l'enzyme clé de la réaction de 25- hydroxylation de la VD [44]. Il est classiquement admis que cette étape hépatique de 25-hydroxylation est peu régulée [45] qu'une étude ancienne ait montré que la $1,25(OH)_2D$ et la PTH puissent l'inhiber [46].

La 25(OH)D circulante dans le sang, ainsi que la $1,25(OH)_2D$, sont majoritairement liées à la VDBP (environ 88% de la 25(OH)D totale présente dans le plasma) [17]. Dans la circulation sanguine, ces deux métabolites sont également liés à l'albumine (12% de la 25(OH)D totale), ou sous forme libre qui correspond à environ 1% de leur quantité totale [17]. Depuis quelques années, il est suggéré que seule la 25(OH)D libre correspond à la forme biologiquement active (hypothèse connue sous le terme de « free hormone hypothesis ») [47]. Ainsi, certains auteurs considèrent que seule la 25(OH)D libre devrait être quantifiée au niveau plasmatique puisqu'elle seule serait corrélée aux effets biologiques de la vitamine D [16].

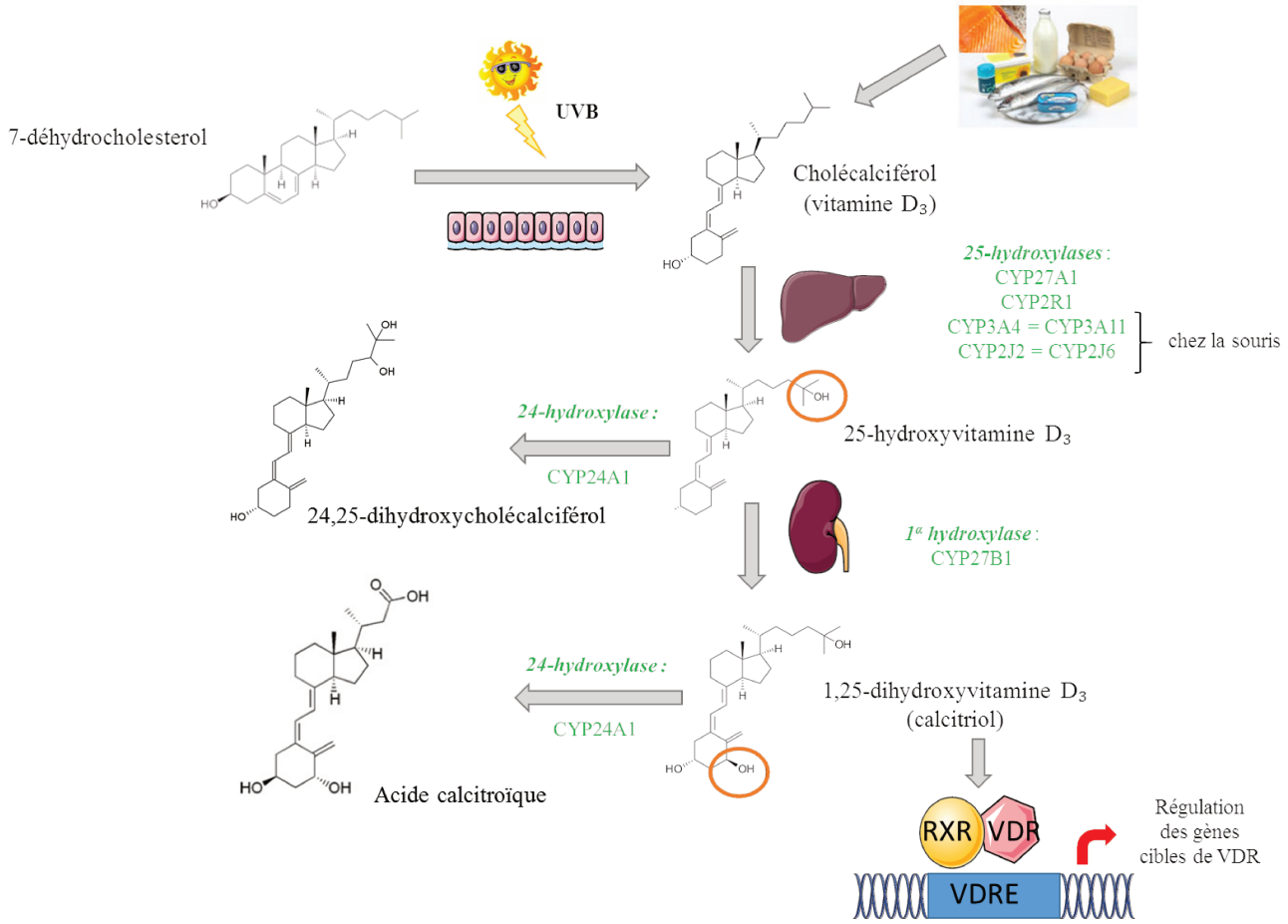

Figure 2 : **Métabolisme général de la vitamine D.** La vitamine D provient de l'alimentation mais surtout de l'exposition aux ultraviolets B qui entraînent au niveau de l'épiderme la conversion du 7-déhydrocholestérol en vitamine D3 native. La vitamine D native subit une 25- puis une 1α-hydroxylation pour devenir biologiquement active, au niveau du foie et du rein respectivement. Le métabolisme de la vitamine D est autorégulé via une voie d'inactivation impliquant la CYP24A1. Le calcitriol exerce ses effets en se liant à un récepteur spécifique appelé Vitamin D Receptor (VDR). Le complexe VDR-1,25$(OH)_2$D est transloqué dans le noyau de la cellule où il s'associe au récepteur de l'acide rétinoïque, le Retinoid X Receptor (RXR). L'hétérodimère RXR-VDR en présence de ligand se lie à l'ADN en des sites appelés éléments de réponse à la vitamine D (VDRE), dans les régions promotrices des gènes dont l'expression est ainsi activée ou réprimée.

1.6.3 ENDOCYTOSE DE LA VITAMINE D DANS LES CELLULES

Le complexe 25(OH)D/VDBP est endocyté par un complexe faisant intervenir les protéines mégaline, cubiline, Amnioless (AMN) et Disabled 2 (Dab2). Ce processus bien que décrit initialement au niveau des cellules du tubule proximal du rein et dans les cellules musculaires [48] pourrait également être actif dans d'autres types cellulaires.

1.6.3.1 MEGALINE / DAB2

La mégaline est une glycoprotéine à haut poids moléculaire (600 kDa, 4 655 acides aminés), membre de la famille des récepteurs des lipoprotéines à faible densité (low-density lipoprotein, LDL), qui s'exprime principalement dans les cellules revêtues de clathrine [49]. La mégaline joue un rôle essentiel dans la réabsorption et le métabolisme des substances filtrantes glomérulaires, y compris l'albumine et les protéines de faible poids moléculaire. La liaison au ligand peut se faire grâce à la présence de plusieurs éléments appelés « complement-type repeats » et l'endocytose est facilité par la présence de motifs NPXY à la partie C-terminale de la mégaline **(Figure 3)** [50]. Les complexes mégaline-ligand sont internalisés par des puits revêtus de clathrine médiés par de multiples protéines intracellulaires facilitatrices d'endocytose telles que les molécules Dab2 et des molécules motrices pour former des vésicules endosomales [51]. L'acidification du lumen intravésiculaire dissocie les ligands de la mégaline qui sont transportés vers des lysosomes pour soit être dégradés, stockés ou transférés vers le cytosol pour un traitement ultérieur. De plus, il a été montré que les souris mégaline$^{-/-}$ développent une déficience en vitamine D, de l'hypocalcémie et une ostéomalacie [52]. La mégaline peut être inhibée par le Receptor Associated Protein (RAP), d'environ 40 kDa et membre de la famille des récepteurs des LDL [53]. La mégaline interagit avec la cubiline, un autre récepteur mise en jeu dans l'endocytose de la vitamine D [54].

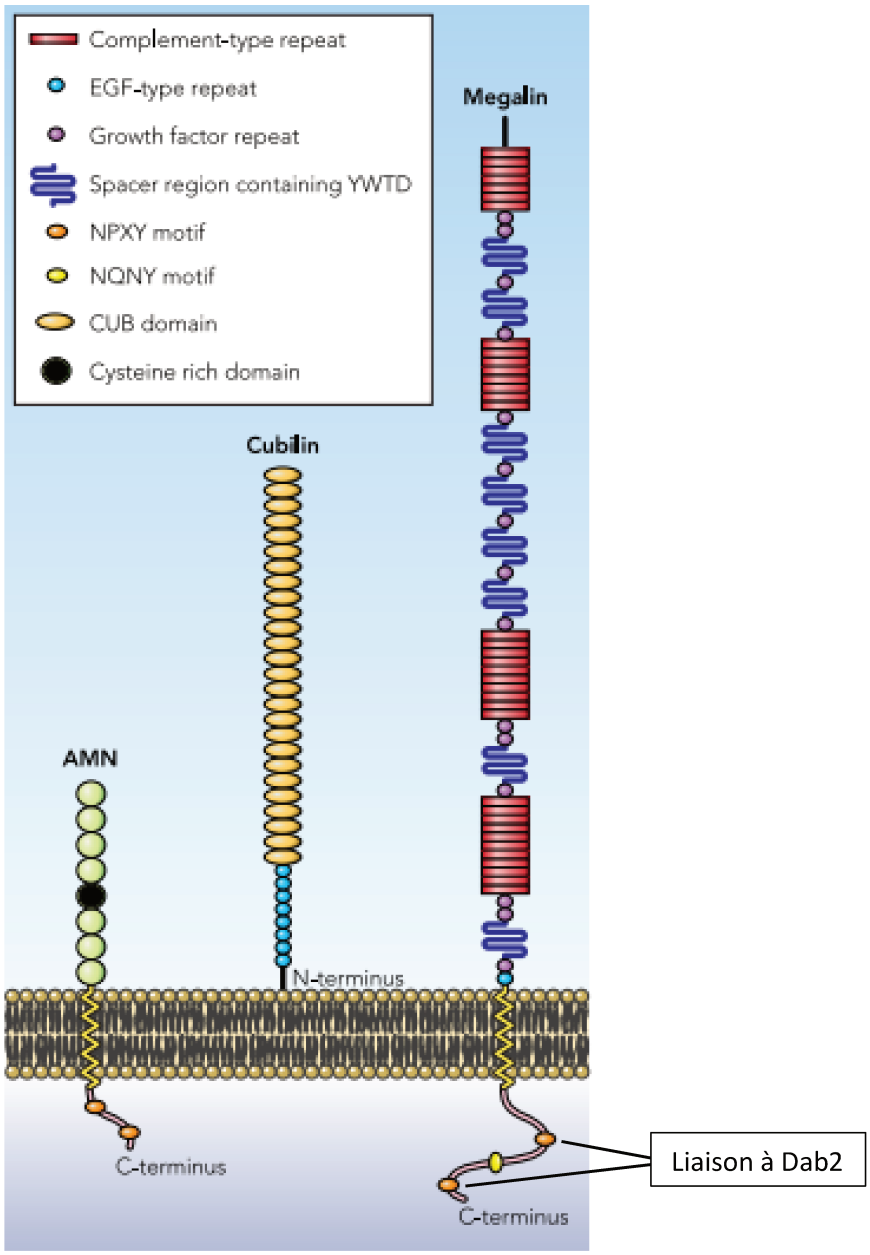

Figure 3: Représentation schématique de mégaline, cubiline et AMN. Les trois protéines sont exprimées dans le tubule proximal, où elles coopèrent pour éliminer les ultrafiltrats des protéines, vitamines, lipides et hormones. La mégaline et la cubiline peuvent se lier à de nombreux ligands avec leurs domaines de liaison (complement-type repeats et domaines CUB, respectivement). La capacité à effectuer l'endocytose semble s'appuyer sur des motifs à la partie C-terminale de la mégaline, qui contiennent les motifs d'endocytose (NPXY). La réabsorption des ligands liés par la cubiline dépend donc du complexe formation avec mégaline et/ou AMN. AMN agit aussi dans la translocation de la cubiline du réticulum endoplasmique à la membrane plasmique [50].

1.6.3.2 CUBILINE /AMN

La cubiline a été originelement identifiée comme le récepteur intestinal intrinsèque de la vitamine B_{12} en 1988 [55]. La cubiline est une glycoprotéine périphérique de 460 kDa dépourvue de segments transmembranaires et intracellulaires, mais qui est ancrée aux membranes apicales des cellules proximales du rein [56]. La cubiline est également impliquée dans l'absorption de plusieurs protéines présents dans les filtrats glomérulaires, y compris l'albumine et la transferrine [56], et ces interactions sont calcium dépendantes. La liaison au ligand peut se faire grâce à la présence de domaines CUB mais la cubiline est une protéine périphérique sans motif cytoplasmique **(Figure 3)** [50]. Le fonctionnement normal de la cubline est dépendant d'une protéine transmembranaire appelé AMN) de 50 kDa, formant le complexe CUBAM [57] et qui présente des motifs d'endocytose (NPXY) à sa partie C-terminale **(Figure 3)**. Dans le tubule proximal du rein, AMN est co-localisé avec la cubiline et est essentiel pour le transport de la cubiline vers la membrane apicale, comme en témoigne la rétention intracellulaire de la cubiline dans des tubules proximaux retrouvée chez l'Homme lors du syndrome d'Imerslund-Gräsbeck caractérisé par la mutation des gènes codant la cubiline et AMN [58]. Le rôle physiologique de ces récepteurs dans le tubule proximal du rein a été étudié pour la première fois sur des chiens déficients en cubiline (malabsorption de la vitamine B12, un des ligands de cubiline) [59] ainsi que par l'utilisation d'animaux knock-out. La première tentative de production de souris cubiline$^{-/-}$ dans toutes les cellules a montré que la suppression homozygote était létale entre le 7,5^{ème} et le 13,5^{ème} jour embryonnaire, démontrant le rôle important de la cubiline pendant le développement murin [60].

1.6.3.3 MECANISME D'ACTION

Au niveau du tubule proximal du rein, il a été mis en évidence que le complexe 25(OH)D/VDBP se fixe au récepteur membranaire mégaline par le biais de la cubiline. La cubiline y joue le rôle de facilitateur d'endocytose par séquestration du complexe à la surface de la cellule avant son association avec la mégaline et l'internalisation du complexe cubiline/25(OH)D/VDBP. Seulement quelques complexes 25(OH)D/VDBP se lient directement à la mégaline. Le complexe cubiline/25(OH)D/VDBP est dirigé vers les lysosomes où la VDBP est dégradée et la 25(OH)D est libérée dans le cytosol de la cellule. Cette 25(OH)D est soit sécrétée soit hydroxylée dans la

mitochondrie en 1,25(OH)2D avant son relargage dans la circulation sanguine où elle sera prise en charge par la VDBP **(Figure 4)** [61].

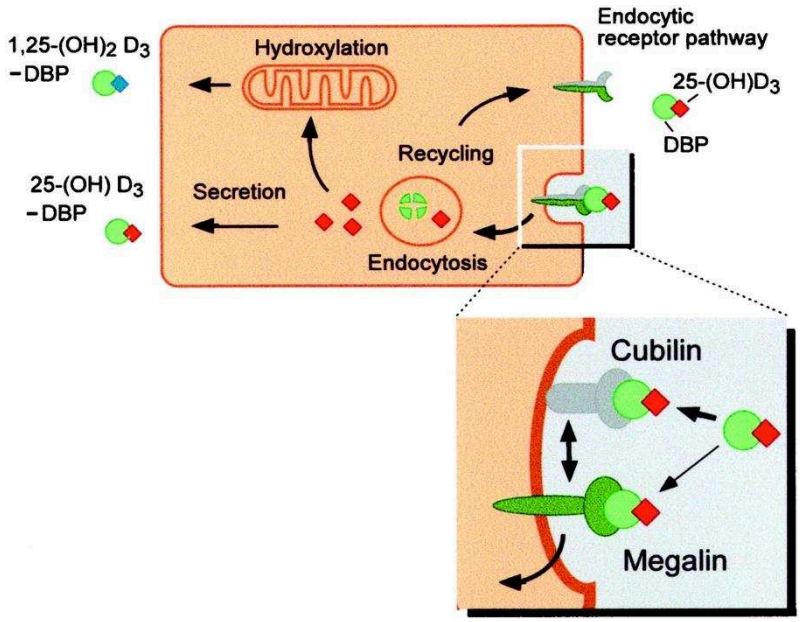

Figure 4 : **Rôle de la mégaline et de la cubiline dans l'endocytose du complexe 25(OH)D lié à la vitamine D binding protein (VDBP)** (D'après [61]).

Le transport intracellulaire de la 25(OH)D pourrait faire intervenir des transporteurs intracellulaires, les Intracellular Vitamin D Binding Protein identifiés chez des primates [62] mais dont l'existence chez l'Homme ou le rongeur n'a à ce jour pas été confirmée.

1.6.4 1α-HYDROXYLATION

Au niveau du tubule proximal du rein, la 25(OH)D va être hydroxylée sur le carbone 1 pour former la 1,25$(OH)_2$D ou calcitriol, considérée comme la principale forme active de la vitamine D [63]. Cette hydroxylation en position 1 est assurée par le cytochrome p450 27B1 (CYP27B1) **(Figure 2)** [43]. L'activité du CYP27B1 est très étroitement régulée par différents paramètres du métabolisme phosphocalcique. Elle est principalement stimulée par la PTH et une calcémie basse, tandis qu'elle est inhibée par le Fibroblast Growth Factor 23 et la concentration circulante de

$1,25(OH)_2D$, selon un mécanisme classique de rétrocontrôle négatif [12]. La demi-vie de la $1,25(OH)_2D$ est très courte (environ 4 heures) et sa concentration est mille fois inférieure à celle de la 25(OH)D.

La $1,25(OH)_2D$ peut exercer des effets endocrines lorsqu'elle est produite par le rein puis transportée via la circulation jusqu'à ses tissus cibles. Cette $1,25(OH)_2D$ peut également avoir des effets autocrines, paracrines et intracrines. En effet, de nombreux tissus et types cellulaires expriment la CYP27B1. C'est notamment le cas des lymphocytes, des macrophages, des adipocytes ou encore des kératinocytes. Dans ce cas, la 25(OH)D internalisée dans ces types cellulaires peut y être hydroxylée en $1,25(OH)_2D$ qui y agit localement [64]. Contrairement à la synthèse rénale, la synthèse extrarénale de $1,25(OH)_2D$ ne semble pas être régulée par la PTH ou la calcémie [45].

1.6.5 24-HYDROXYLATION

Le métabolisme de la vitamine D est autorégulé via une voie d'inactivation [43]. En effet, la $1,25(OH)_2D$ induit l'expression de la 24-hydroxylase (CYP24A1) qui convertit la 25(OH)D et la $1,25(OH)_2D$ en métabolites inactifs ($24,25(OH)_2$ vitamine D et $1,24,25(OH)_3$ vitamine D) transformés ensuite en acide calcitroïque inactif **(Figure 2)** [12]. D'autres enzymes de la famille des cytochromes P450 comme le CYP3A4 peuvent également dégrader le calcitriol dans le foie et l'intestin [65].

1.7 MECANISMES D'ACTION DE LA VITAMINE D

Le métabolite actif de la vitamine D, le $1,25(OH)_2D$ présente à la fois des effets génomiques, non-génomiques et épigénétiques.

1.7.1 EFFETS GENOMIQUES DE LA VITAMINE D

Les effets génomiques de la vitamine D sont bien connus et font intervenir le récepteur de la vitamine D (VDR), appartenant à la superfamille des récepteurs nucléaires des hormones

stéroïdes/thyroïdiennes [66]. Ce VDR est exprimé dans la plupart des types cellulaires et est donc exprimé dans tous les tissus [64], ce qui signifie que toutes les cellules ou presque sont des cibles potentielles du $1,25(OH)_2D$ [67]. La distribution ubiquitaire du VDR permet d'expliquer le grand nombre de gènes dont la régulation est sous la dépendance directe ou indirecte de la $1,25(OH)_2D$. Ceci se traduit par des effets de la vitamine D sur la régulation de gènes impliqués dans des voies métaboliques aussi variées que le métabolisme du calcium, la prolifération, la différenciation cellulaire, l'inflammation, l'apoptose ou encore l'angiogenèse pour ne citer que quelques exemples.

Dans la cellule, la $1,25(OH)_2D$ se lie au VDR [64]. Le complexe VDR-$1,25(OH)_2D$ est transloqué au noyau de la cellule où il s'associe au récepteur de l'acide rétinoïque, le Retinoid X Receptor (RXR) [68]. L'hétérodimère RXR-VDR en présence de ligand se lie à l'ADN en des sites appelés VDRE, dans les régions promotrices des gènes dont l'expression est ainsi activée ou réprimée **(Figure 2 et 5)** [66]. Cet effet inducteur ou répresseur est un phénomène complexe qui implique le recrutement de coactivateurs ou de corepresseurs lors de la fixation du ligand au VDR [69]. De même, le niveau de méthylation et d'acétylation de la chromatine sont des éléments qui vont orienter la régulation génique dans le sens de l'induction ou de la répression. Enfin, le VDR a également la capacité de réguler l'expression génique indépendamment de la présence de ligand, en s'hétérodimérisant avec RXR sur des régions promotrices. On voit donc que le mode de régulation génique par le VDR et la vitamine D est largement multifactoriel et fait intervenir de nombreux cofacteurs de transcription.

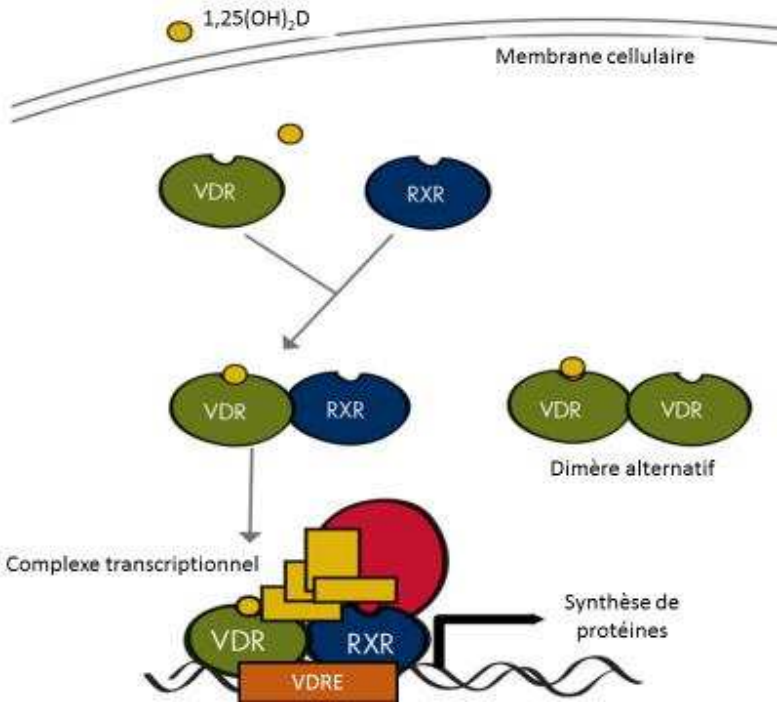

Figure 5 : Voie de signalisation de la vitamine D. La $1,25(OH)_2D$ se lie à son récepteur VDR qui va s'hétérodimériser avec RXR au niveau des éléments de réponse à la vitamine D (VDRE) situé sur les promoteurs des gènes cibles de VDR (Source [45]).

1.7.2 EFFETS NON GENOMIQUES DE LA VITAMINE D

La vitamine D et ses métabolites sont également responsables d'effets non-génomiques. Les effets du $1,25(OH)_2D$ dépendent d'un récepteur membranaire, la Protein disulfide isomerase family A member 3 (Pdia3), également connue sous les noms ERp57, GRP58 et 1,25D3MARRS [70]. Le rôle de ce récepteur a été bien décrit dans l'entérocyte, où il participe au captage rapide du calcium [71]. Ce phénomène a également été décrit dans d'autres types cellulaires tels que les ostéoblastes, les hépatocytes ou les cellules β du pancréas, cependant le caractère ubiquitaire de ce type de régulation n'est pas encore établi. Le récepteur Pdia3, après fixation et activation par la $1,25(OH)_2D$, active de nombreuses voies de transduction du signal parmi lesquelles, les phospholipases C et A2, les Mitogen-Activated Protein Kinases (MAPK), la protéine kinase C ainsi que les canaux calciques qui vont être à l'origine des réponses très rapides (de quelques secondes à quelques minutes) médiées par ce récepteur en réponse au calcitriol. Il est important de

souligner que des travaux ont montré l'implication du VDR dans cette voie de signalisation rapide [72], ce qui confirme le rôle central de VDR dans la médiation des effets de la vitamine D.

1.7.3 EFFETS EPIGENETIQUES

Il a été mis en évidence, via la régulation génique médiée par le VDR, que la vitamine D pouvait avoir des effets épigénétiques. En effet, en régulant l'expression d'histones méthylases et de DNA méthyltransférases, la vitamine D exerce des effets sur le niveau de méthylation des histones et des ilots CpG [73]. De même, la vitamine D agit sur la régulation mais également le recrutement d'histones acétylases (HAT) et d'histones désacétylases (HDAC), permettant ainsi de jouer sur le niveau d'acétylation des histones **(Figure 6)** [74]. Il a récemment été montré que la concentration plasmatique en 25(OH)D chez l'Homme était corrélée positivement avec le degré de méthylation du gène codant pour VDR, ainsi que pour les gènes codant pour les enzymes impliquées dans le métabolisme de la vitamine D (CYP2R1, CYP27B1 et CYP24A1) [75].

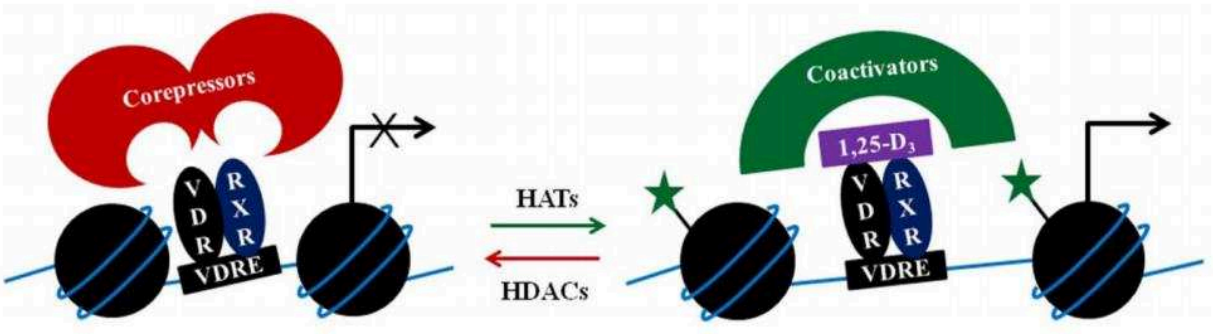

Figure 6 : **Illustration simplifiée d'un modèle de co-régulation à deux étapes** (D'après [76]). Le complexe VDR/RXR en l'absence du 1,25$(OH)_2$D peut se lier aux corépresseurs qui pourraient conduire ou maintenir la répression des gènes, en partie par l'attraction des histones désacétylases (HDAC). Lorsque le ligand se lie au complexe VDR/RXR, les corépresseurs sont remplacés par des coactivateurs, comme par exemple les histones acétyltransférases (HAT). L'acétylation des histones (indiquée par les étoiles vertes) permet la relaxation de la chromatine et la transcription du gène.

Enfin, la vitamine D via le VDR est également capable de réguler l'expression de microARN (miRs), soit directement soit indirectement en modulant l'activité de facteurs de transcription [77].

L'effet de la vitamine D sur la régulation de l'expression des miRs in vitro, in vivo et chez l'homme sera détaillé prochaine (chapitre 3.3.2).

1.8 SITE DE STOCKAGE DE LA VITAMINE D

La vitamine D est principalement stockée au niveau du tissu adipeux sous forme native (cholécalciférol) ou sous forme de 25(OH)D **(Tableau 7)** [2, 78] ainsi que dans le muscle. L'équipe de Rosenstreich et al. est la première, en 1971, à montrer que la vitamine D était stockée dans le tissu adipeux [79]. En effet, la vitamine D3 radiomarquée ayant été administrée par voie orale à des rats sevrés et déficients en vitamine D, il a pu être mis en évidence qu'une quantité significative de radioactivité apparaît dans le tissu adipeux 24h après son administration. Après arrêt du traitement, le tissu adipeux libère la vitamine D très lentement comme en témoigne la diminution progressive de la radioactivité présente dans le tissu adipeux. Cette libération est proportionnelle à la concentration de vitamine D déjà présente dans le tissu adipeux.

Plus récemment, il a été montré que le tissu adipeux viscéral (TAv) contient 20% de plus de vitamine D que le tissu adipeux sous cutané (TAsc) [80]. De plus, 65% de la forme totale de vitamine D présente dans l'organisme est sous forme de vitamine D3 (73% et 16% au niveau du tissu adipeux et du muscle, respectivement). En ce qui concerne la 25(OH)D, 34% se trouve dans le tissu adipeux, 30% dans le sérum et 20% dans le muscle [78].

Tableau 7 : Principaux sites de stockage de la vitamine D (D'après [78])

	Vitamine D (UI)	25(OH)D (UI)	Total (UI)
Tissu adipeux	6960	1763	8723
Muscle	1527	1055	2581
Foie	168	214	382
Sérum	271	1559	1830
Autre	571	578	1149
Total	9426	5169	14665

A ce jour, quelques d'études ont rapporté la quantification de la vitamine D et de ces métabolites dans le tissu adipeux **(Tableau 8)**. La première a été réalisée par l'équipe de Blum et al. qui a

montré qu'il est possible de quantifier le cholécalciférol dans le tissu adipeux sous cutanée (TAsc) en utilisant la chromatographie en phase liquide couplée à la spectrométrie de masse (LC-MS) chez des sujets obèses (102,8 ± 42 nmol/kg). Les auteurs ont mis en évidence une corrélation positive entre le taux du cholécalciférol dans le sérum et dans le tissu adipeux [81]. L'équipe de Lipkie et al. a quant à elle utilisée la chromatographie en phase liquide couplée à la spectrométrie de masse en tandem (LC-MS/MS) pour quantifier le cholécalciférol et la 25(OH)D dans plusieurs tissus comme le foie, le muscle et le tissu adipeux épididymal (TA épi) chez des rats carencés en cholécalciférol. Leurs données suggèrent que ces trois organes accumulent des concentrations similaires de 25(OH)D, lesquels peuvent servir de réservoir temporaire et/ou de substrat pour la production du $1,25(OH)_2D$. En ce qui concerne le TA, la quantité de vitamine D2 et D3 retrouvée chez les rats est de 30,6 ± 1,3 ng/tissue et la quantité de 25(OH)D est de 2,0 ± 1,0 ng/tissue [82]. La même année, Piccolo et al. ont réussi à détecter le 25(OH)D dans le tissu adipeux de patients obèses et en surpoids par LC-MS/MS (5,8 ± 2,6 nmol/kg tissue) [83]. Plus récemment, Malmberg et al, ont mesuré le cholécalciférol, le 25(OH)D et la $1,25(OH)_2D$ dans le tissu adipeux de sujets de poids normal comparés à des sujets obèses par spectrométrie de masse à ionisation secondaire (TOF-SIMS). Le cholécalciférol et ses métabolites ont été localisés dans les gouttelettes lipidiques des adipocytes des différents sujets. Cette étude suggère la présence d'un niveau faible en cholécalciférol et la 25(OH)D dans le TAsc des femmes obèses comparé à des femmes de poids normal et d'un niveau plus élevé en cholécalciférol dans TAsc comparé au TAv chez les sujets obèses [84]. Toutefois, le nombre de sujets par groupe n'étant que de trois, cette étude comporte de nombreuses limites et ne permet pas de conclure à de réelles différences. De plus, les différences en teneur en cholécalciférol et ses métabolites sont relatives entre les deux groupes, et aucune valeur absolue n'a été rapportée. Enfin, Carrelli et al. ont quantifié le cholécalciférol dans le TAsc et TAv de patients obèses et non-obèses par LC-MS/MS [85]. Leurs résultats démontrent que les sujets obèses ont un stock de vitamine D plus important dans le tissu adipeux.

Tableau 8 : Quantification de la vitamine D et de ces métabolites dans le tissu adipeux.

Articles	Types d'échantillons	Forme de vitamine D dosée	Quantification	Méthode/machines
Blum, 2008	TA sc patients obèses	Cholécalciférol	102,8 ± 42 nmol/kg tissu = 39,5 ± 16 ng/g tissu	LC-MS
Lipkie, 2013	TA épi rats supplémentés en vitamine D	Ergocalciférol et cholécalciférol	30,6 ± 1,3 ng/g tissu	LC-MS/MS
		25(OH)D	2 ± 1 ng/g tissu	
Piccolo, 2013	TAsc patients obèses et en surpoids	25(OH)D	5,8 ± 2,6 nmol/kg tissu = 2,23 ± 0,9 ng/g tissu	LC-MS/MS
Malmberg, 2014	TAsc et TAv patients obèses et non-obèses	Cholécalciférol, 25(OH)D3 et $1,25(OH)_2D3$	Pas de valeurs absolues	TOF-SIMS
Carrelli, 2016	TAsc patients obèses	Ergocalciférol et cholécalciférol	34,2 ± 9,1 ng/g tissu	LC-MS/MS
	TAsc patients non obèses		25,7 ±12,3 ng/g tissu	
	TAv patients obèses		50,6 ± 13,1 ng/g tissu	
	TAv patients non obèses		29,7 ± 17,6 ng/g tissu	

Tissu Adipeux (TA) épididymal (épi), sous-cutanée (sc) et viscéral (v)

2. LE TISSU ADIPEUX

Le tissu adipeux est un organe à part entière qui participe activement au maintien de l'homéostasie générale, et dont l'expansion est intimement liée au développement de l'obésité. Il existe deux principaux types de tissus adipeux, le tissu adipeux blanc et le tissu adipeux brun. Le tissu adipeux brun intervient essentiellement dans le contrôle de la thermogenèse. Le tissu adipeux blanc est un organe vital de stockage de l'énergie qui peut être redistribuée à l'organisme en fonction des besoins au cours du temps [86]. Au cours de cette thèse, nous nous focaliserons sur le tissu adipeux blanc en étudiant sa composition et sa fonctionnalité au cours de l'obésité.

2.1 DESCRIPTION ET COMPOSITION DU TISSU ADIPEUX BLANC

Le tissu adipeux blanc représente la quasi-totalité du tissu adipeux chez l'Homme adulte. Ce tissu se développe chez le fœtus au deuxième trimestre de la grossesse au niveau des joues, du cou, des épaules et des reins [87]. L'augmentation de masse se fait par deux phénomènes : l'hyperplasie qui correspond à l'augmentation numéraire des adipocytes par différenciation, et l'hypertrophie qui correspond à l'augmentation de la taille des adipocytes, ce dernier phénomène semble prépondérant par rapport au premier [88]. En effet, des études ont montré que le nombre d'adipocytes et leur taille augmentent depuis la naissance jusqu'à l'âge de 9 ans. A l'âge adulte, le nombre et la taille des adipocytes restent relativement constants le reste de la vie.

Environ 80% du tissu adipeux est localisée en territoire sous-cutané et les 20% restants en intra abdominal (tissus profonds) [89]. Le tissu adipeux intra-abdominal est constitué de viscéral (80%) et de tissu adipeux rétropéritonéal et périrénal (20%) [90]. La localisation du tissu adipeux varie chez l'Homme en fonction du sexe. Il est plus présent chez la femme (environ deux fois plus) où il se retrouve principalement au niveau des parties inférieures du corps (hanches, bassin et cuisse) [91]. On appelle cette répartition du tissu adipeux, l'adiposité gynoïde **(Figure 7)**. A contrario, une adiposité localisée au niveau de l'abdomen et du torse, plus présente chez l'homme, est dite androïde.

Figure 7 : Répartition du tissu adipeux en fonction du sexe : adiposité gynoïde ou androïde [91].

Ce tissu adipeux blanc est composé de plusieurs types cellulaires : les cellules souches mésenchymateuses multipotentes (les cellules précurseurs qui donnent naissance aux nouveaux adipocytes), les adipocytes (les cellules du tissu adipeux) et divers autres types de cellules appelé fraction stroma vasculaire. Quantitativement, la plus importante population de cellules correspond aux adipocytes. Il s'agit de cellules de grande taille possédant une grosse gouttelette lipidique uniloculaire (contenant des triglycérides) et peu de cytoplasme. La fraction stroma-vasculaire est composée quant à elle de macrophages, leucocytes, préadipocytes, fibroblastes et de cellules endothéliales et neuronales **(Figure 8)** [92].

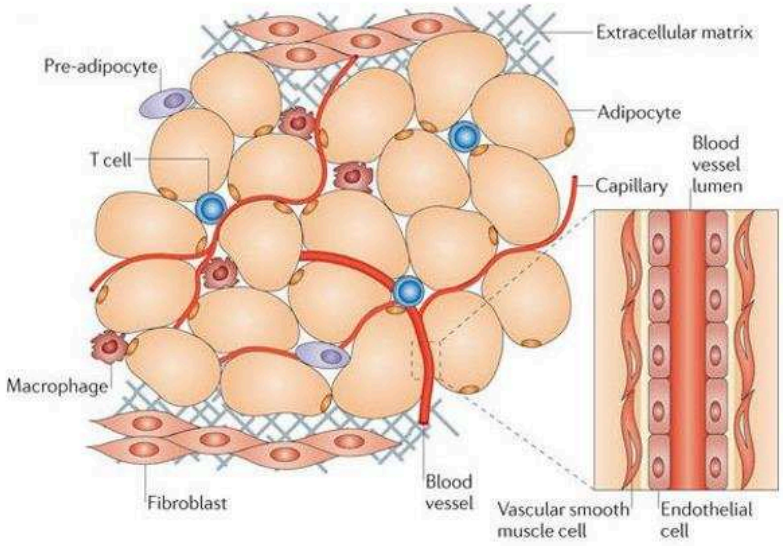

Figure 8 : Le tissu adipeux : composition cellulaire. Les adipocytes sont la principale composante cellulaire du tissu adipeux. La fraction stroma-vasculaire comprend les autres types cellulaires dont les cellules précurseurs (préadipocytes), les fibroblastes, les cellules vasculaires et immunitaires. Les cellules vasculaires comprennent les cellules endothéliales et les cellules musculaires lisses qui sont associées aux vaisseaux sanguins [92].

2.2 L'ADIPOCYTE

L'adipocyte, issu de la différenciation de préadipocytes est une cellule ronde, extensible, dont la majorité de l'espace intracellulaire est composé de plusieurs vacuoles lipidiques qui fusionnent en une seule refoulant ainsi le noyau et les autres organites vers la périphérie. Les adipocytes stockent 85% de la fraction lipidique sous forme de triglycérides [93].

Le processus de différenciation cellulaire selon lequel des pré-adipocytes deviennent des adipocytes est appelé adipogénèse. Les principaux facteurs de transcription impliqués dans ce processus sont les membres de la famille CREB (cAMP Response Element Binding protein), les CCAAT-Enhancer binging Proteins (C-EBPα), le Peroxisome Proliferator Activated Receptor γ (PPARγ) et le Sterol Regulatory Element Binding Protein-1 (SREBP-1) [94] **(Figure 9)**. L'expression séquentielle de ces facteurs de transcription conduit à l'expression de gènes permettant l'acquisition par l'adipocyte de fonctions caractéristiques comme la capacité à synthétiser et à hydrolyser les triglycérides mais aussi à sécréter des adipokines.

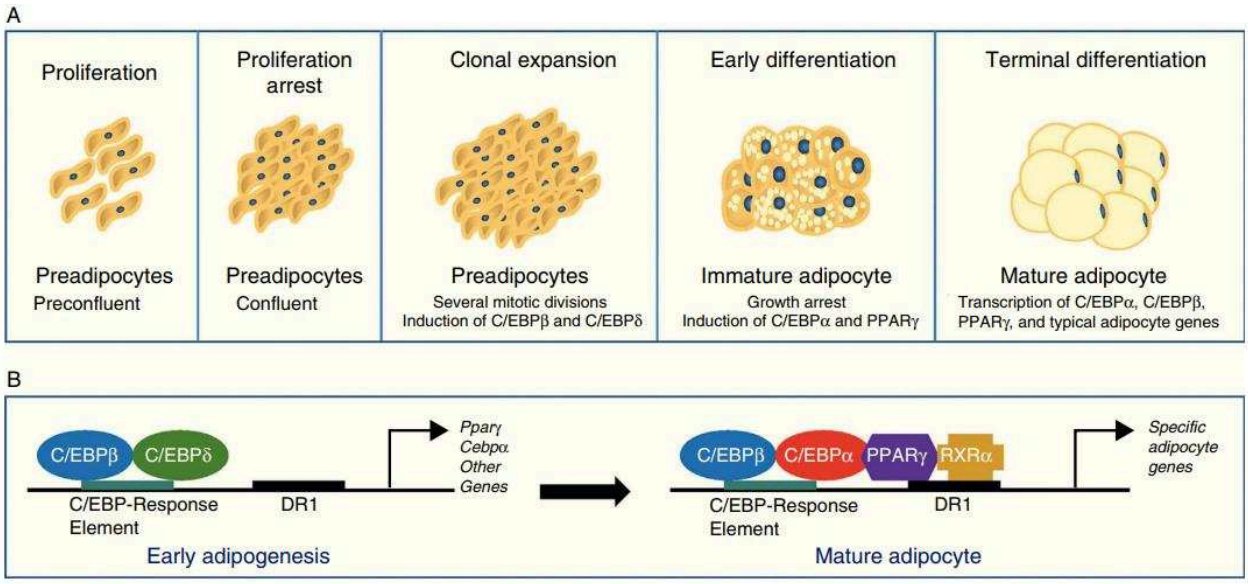

Figure 9 : Différenciation des préadipocytes en adipocytes. (A) Schéma du processus de différenciation, du préadipocyte à l'adipocyte mature. (B) Modèle séquentiel de contrôle transcriptionnel de l'adipogenèse.

Les adipocytes exercent des actions pléïotropes par l'intermédiaire de la production de nombreux facteurs agissant de façon autocrine, paracrine ou endocrine. Les produits sécrétoires du tissu adipeux sont appelés « adipocytokines » ou « adipokines ». Par extension, le mot « adipokine » est largement utilisé pour désigner les sécrétions du tissu adipeux quelle que soit l'origine cellulaire de ces adipokines. Parmi ces adipokines, on peut distinguer notamment des hormones, des cytokines, des chimiokines ou des facteurs de croissance. Il est important de préciser que l'adipocyte ne participe que pour partie à la totalité du sécrétome du tissu adipeux. En effet, si certaines adipokines sont spécifiquement produites par l'adipocyte lui-même, d'autres sont issues de la fraction stroma-vasculaire ou des deux [95, 96]. Ces facteurs, dont la liste ne cesse de croître, sont capables d'influencer les comportements, la régulation énergétique, l'oxydation des lipides, la fonction immune, les fonctions vasculaires, la situation hormonale, etc. La découverte de la première sécrétion adipocytaire remonte à 1964 avec la découverte de la lipoprotéine lipase (LPL) [97]. Puis en 1985, il a été montré que le tissu adipeux était la source principale d'adipsine [98]. Mais les découvertes les plus importantes concernant les adipokines ont eu lieu en 1994 avec la découverte de la leptine [99] et en 1995 avec la découverte de l'adiponectine ouvrant un nouveau champ de recherche dans le domaine de l'obésité **(Figure 10)**.

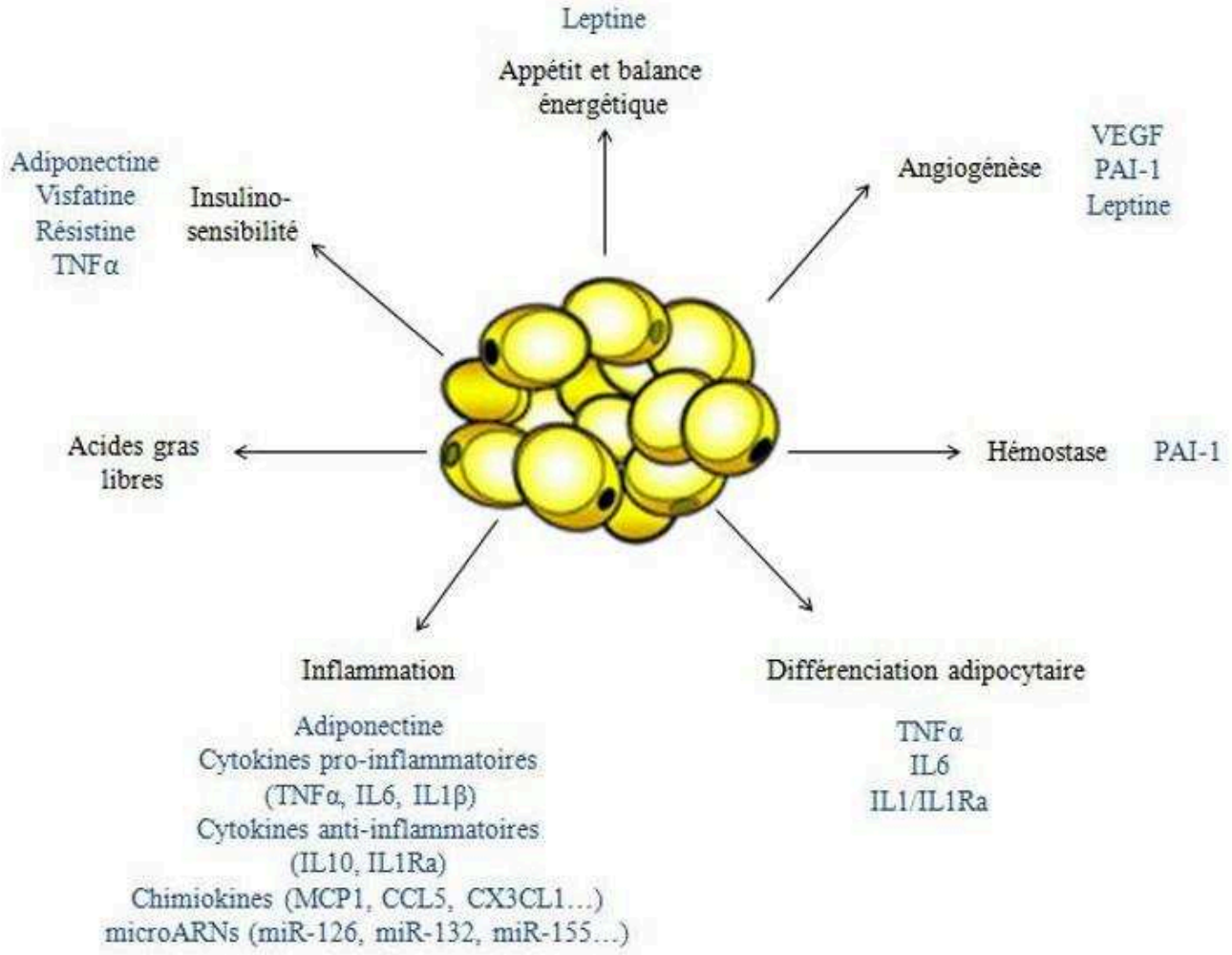

Figure 10 : Les sécrétions du tissu adipeux. Le tissu adipeux blanc produit une large variété de molécules ayant un rôle de régulateur dans le métabolisme et l'inflammation. En effet, elles contribuent au maintien de l'homéostasie de la dépense énergétique mais sont aussi impliquées dans la pathogenèse de complications associées à l'obésité.

2.3 TISSU ADIPEUX AU COURS DE L'OBESITE

Au cours de l'obésité, l'augmentation de la masse adipeuse peut résulter d'une augmentation de la taille des adipocytes (hypertrophie) ou de leur nombre (hyperplasie), soit des deux. L'hypertrophie précède généralement l'hyperplasie **(Figure 11)** [100]. L'hypertrophie résulte d'une accumulation de triglycérides. Au-delà d'une certaine taille, la cellule adipeuse ne grossit plus, l'augmentation des capacités de stockage nécessite une augmentation du nombre de cellules. C'est l'hyperplasie, dans ce cas, les précurseurs sont recrutés afin d'augmenter le nombre d'adipocytes.

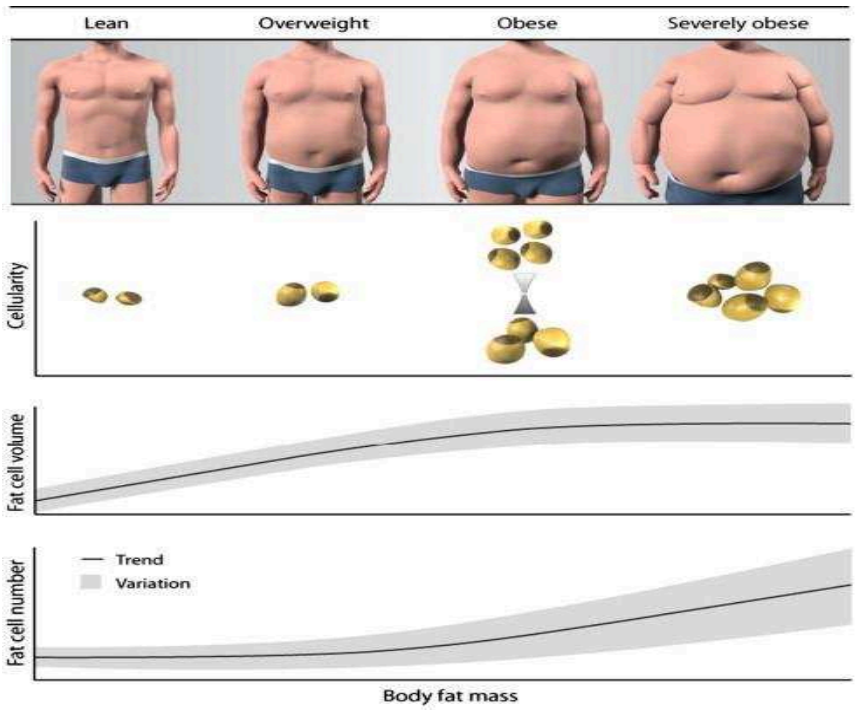

Figure 11 : Evolution macroscopique du tissu adipeux au cours de l'obésité [100].

Le tissu adipeux en général et l'adipocyte en particulier vont être confrontés à de nombreuses perturbations physiologiques lors de l'obésité parmi lesquelles un phénomène d'hypoxie due à l'expansion des adipocytes, le développement d'une inflammation à bas bruit (se traduisant par une dérégulation du sécrétome des adipocytes), un stress du réticulum endoplasmique, un stress oxydant, une accumulation d'acides gras libres ou encore d'une infiltration leucocytaire.

2.3.1 INFLAMMATION DU TISSU ADIPEUX AU COURS DE L'OBESITE

La première description de l'inflammation dans le tissu adipeux de souris obèse à constituer en la mise en évidence d'une augmentation de la quantité de cytokine pro-inflammatoire Tumor Necrosis Factor α (TNFα) [101], consécutivement à une infiltration accrue de macrophages de type M1 [95]. Il s'en est suivi de nombreuses études décrivant les différences phénotypiques observées entre TA obèses et non-obèses à la fois chez l'Homme et chez l'animal. Il est maintenant connu que non seulement le TNFα mais tout un réseau de cytokines inflammatoires sont induites au cours de l'obésité. En effet, le tissu adipeux produit de nombreuses cytokines pro-inflammatoires (transforming growth factor β et interferon γ), des interleukines (IL1, IL6, IL10 et IL8), des facteurs procoagulants (PAI-1, fibrinogène) et des chimiokines (monocyte chemotatic protein-1 ou MCP-1, et macrophage inflammatory protein 1 α) [102-104]. Le concept d'« adipokines » a été proposé pour qualifier les protéines sécrétées par le tissu adipeux, susceptibles de circuler dans le plasma et d'avoir une influence systémique [105]. Parmi celles-ci on distingue des substances produites spécifiquement par l'adipocyte (comme l'adiponectine et la leptine [106]) et d'autres produites en abondance par ce tissu mais qui ne lui sont pas spécifiques comme les protéines de l'inflammation, par exemple IL-6 dont la grand majorité provient de la fraction stroma-vasculaire [107] ou encore IL1β et IL10 qui sont sécrétées par les macrophages majoritairement [108, 109]. L'obésité est également associée à une diminution de la production d'adiponectine, qui est considérée habituellement comme un facteur ayant des propriétés anti-inflammatoires [105] et insulino-sensibilisatrices, ainsi qu'à une résistance à la leptine dû à une hyperleptinémie.

2.3.2 ROLE CLE DE L'ACTIVATION DES VOIES JNK/NFKB

De nombreux désordres physiopathologiques que l'on observe au cours de l'obésité semblent être à l'origine de régulations du niveau d'expression des adipokines qui vont participer à la survenue de l'insulino-résistance. Tous ces différents types de stress convergent vers les voies de signalisation impliquant les protéines Jun N-terminale kinase (JNK) et Ικκβ, dont les effecteurs finaux sont respectivement activator protein-1 et Nuclear Factor-Kappa B (NF-κB), et qui jouent un rôle fondamental dans la genèse de l'insulino-résistance par le biais de leur fonction de relais de signalisation [110].

En effet, il a été rapporté chez la souris obèse une augmentation de l'activité JNK1 dans le tissu adipeux. A l'inverse, les souris invalidées pour le gène codant JNK [111] sont protégées contre une insulino-résistance induite par un gavage en acides gras et voient leur adiposité diminuer. Cette induction de JNK1 au cours de l'obésité peut être reliée à l'augmentation du taux circulant des cytokines pro-inflammatoires (IL-6 ou TNFα) [112, 113] et ou des acides gras libres [114, 115], mais également à l'augmentation du stress oxydant dans le tissu adipeux [116]. Il en est de même pour Ikκβ qui, au cours de l'obésité, va voir son activité induite par ces différents stress et dont l'implication de l'insulino-résistance a été démontrée [117]. En effet, des souris hétérozygotes *Ikκβ*$^{+/-}$ résistent au développement d'une insulino-résistance, soit d'origine génétique (souris ob/ob), soit induite par un régime riche en graisses. De plus, la surexpression ou l'activation d'Ikκβ in vitro, inhibe en partie la voit de signalisation de l'insuline.

De nombreuses études menées sur des souris présentant une invalidation tissu spécifique de JNK1 ou Ikκβ ont permis de mettre en évidence la contribution spécifique du TA, du foie et de l'hypothalamus dans le contrôle de la sensibilité à l'insuline ainsi que dans le métabolisme général par le biais de ces kinases [110].

2.3.3 ROLE DES MICROARNS DANS L'INFLAMMATION

Les miRs sont des ARN de petite taille (19-25 nucléotides) transcrits à partir de régions non codantes du génome. Ils sont considérés comme des éléments majeurs du contrôle de l'expression génique au niveau post-transcriptionnel. Chaque miR peut réguler une centaine de gènes et un gène peut inversement être la cible de plusieurs miRs.

De nombreuses études ont montré que les miRs régulent l'expression de certaines cytokines mises en jeu dans l'inflammation au niveau du tissu adipeux ainsi qu'au niveau des adipocytes humains [118-120]. Par exemple, il a été montré récemment que la transcription d'un miR (1275), impliqué dans la maturation des préadipocytes humains, était régulée par des facteurs inflammatoires (IL-6 et TNFα) par l'implication de la voie NF-κB au niveau d'adipocytes humains [121]. A l'inverse, l'inflammation, induite par un traitement au TNFα ou au lipopolysaccharide, régule elle aussi l'expression de certains miRs [122]. D'autres études se sont intéressées aux miRs dérégulés dans le tissu adipeux au cours de l'obésité et à leurs associations avec les paramètres de l'obésité : 21

miRs sont différentiellement exprimés dans TA épi de souris minces et obèses [123] et 50 miRs ont été quantifiés dans le TAsc de sujets normo-pondéraux et obèses. Parmi ces miRs, 17 sont fortement corrélés avec l'Indice de Masse Corporelle (IMC) et les paramètres métaboliques (taux de glucose et de triglycérides) [124]. Ces résultats sont cohérents avec ceux obtenus par Kloting et al. qui ont montré des corrélations significatives entre l'expression de certains miRs et la morphologie du tissu adipeux et les paramètres métaboliques (taux de glucose, la quantité de masse grasse viscérale et taux circulants de marqueurs inflammatoires (IL6, leptine et adiponectine)) [125]. De plus, le miR155 a récemment été mis en évidence par notre équipe comme étant impliqué dans l'inflammation adipocytaire médié par la voie de signalisation NF-κB [126].

3. EFFETS DE LA VITAMINE D SUR LA BIOLOGIE DU TISSU ADIPEUX

3.1 METABOLISME DE LA VITAMINE D

Le tissu adipeux exprime de nombreuses enzymes nécessaires au métabolisme de la vitamine D **(Figure 12)**. La 25-hydroxylation semble être fonctionnelle dans le tissu adipeux. Zoico et al. [127] ont récemment montré que la sécrétion de 25(OH)D dans le milieu de culture des adipocytes augmente après leur incubation avec la vitamine D. Cette production de 25(OH)D pourrait être due à la présence de CYP27A1 qui est positivement régulée par le traitement à la vitamine D. De façon intéressante, il a été montré dans des biopsies de tissu adipeux humain, que 3 enzymes sur 4 impliquées dans la 25-hydroxylation (CYP27A1, CYP2R1 et CYP2J2) y sont exprimées, ce qui suggère que le tissu adipeux humain et les adipocytes sont capables de convertir la vitamine D en 25(OH)D [128].

La capacité des adipocytes à convertir la 25(OH)D en $1,25(OH)_2D$ a été initialement démontrée dans les cellules 3T3-L1 suite à l'identification de 1,25(OH)2D radiomarquée produite à partir de 25(OH)D radiomarquée [129]. La production de 1,25(OH)2D à partir de 25(OH)D a été ensuite confirmée par Ching et al. [130] et Nimitphong et al. [131]. La CYP27B1, l'enzyme responsable de la production de la $1,25(OH)_2D$, a également été détectée dans les adipocytes murins [129] et les biopsies de tissu adipeux humain [128]. A l'heure actuelle, on ne sait pas si la 1α hydroxylase, au niveau du tissu adipeux, est régulée.

La CYP24A1, enzyme impliquée dans la dégradation des métabolites de la vitamine D, a été détectée dans les adipocytes murins et humains [129, 131]. De plus, les niveaux d'ARNm codant pour la CYP24A1 sont fortement induits par la $1,25(OH)_2D$. L'expression de CYP24A1 a été également confirmée dans les biopsies de tissu adipeux humain [128].

L'expression du gène récepteur nucléaire VDR a été retrouvée dans des adipocytes 3T3-L1 [132] ainsi que des pré-adipocytes humains et des adipocytes différenciés en culture [133]. De plus, VDR est exprimé dans les tissus adipeux humain sous cutanée et viscéral [134] et adipocytes mammaires humains [130]. Le récepteur nucléaire VDR est lui aussi détecté dans les biopsies de tissu adipeux humain d'après Wamberg et a1. ainsi que dans les pré-adipocytes où l'expression de son gène est régulée positivement par le calcitriol lui-même [132].

Les mécanismes moléculaires impliqués dans l'absorption de la vitamine D et sa sécrétion par le tissu adipeux n'ont pas encore été clairement identifiés. Ils pourraient impliquer la voie mégaline/cubiline [48], et/ou les transporteurs du cholestérol décrits dans l'intestin [39] ou ceux décrits dans le tissu adipeux pour d'autres micronutriments lipophiles comme le CD36 [135].

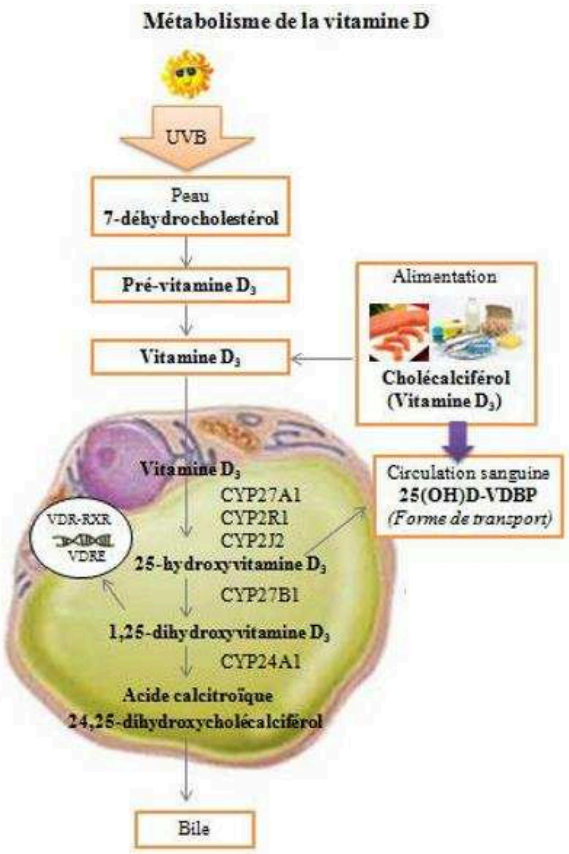

Figure 12 : Métabolisme de la vitamine D dans le tissu adipeux. La vitamine D est 25- et 1α-hydroxylée au niveau de l'adipocyte. Les métabolites obtenus sont inactivés par la CYP24A1. Le calcitriol exerce ses effets en se liant à un récepteur spécifique appelé Vitamin D Receptor (VDR). Le complexe VDR-1,25$(OH)_2$D est transloqué dans le noyau de la cellule où il s'associe au récepteur de l'acide rétinoïque, RXR.

3.2 ROLE DE LA VITAMINE D SUR L'INFLAMMATION

Les études réalisées ces 30 dernières années ont permis de mettre en évidence les effets de la vitamine D sur le système immunitaire [136, 137]. Plus spécifiquement, une bibliographie abondante a notamment été consacrée à la relation entre la vitamine D et l'inflammation adipocytaire associée à l'obésité. Les premières études menées au niveau des adipocytes humains et murins ont montré que la vitamine D entraînait une surexpression de cytokines pro-inflammatoires et une diminution de l'expression de cytokines anti-inflammatoires dans les deux types cellulaires [138-140]. Ces résultats très paradoxaux par rapport à l'effet anti-inflammatoire

de la vitamine D bien décrit dans de nombreux types cellulaires [141] ne sont toujours pas bien compris.

Par la suite, il a été montré que la 1,25(OH)2D diminue significativement la libération d'IL8, MCP1 et IL6 par les préadipocytes humains [142] et MCP1 par les adipocytes humains [143], ainsi que dans des adipocytes murins [144]. Wamberg et al. ont montré que l'incubation d'adipocytes humains avec la 1,25(OH)2D in vitro permet de réduire l'expression d'IL6, IL8 et MCP1 induite par l'IL1β [145]. Les autres métabolites de la vitamine D sont également capables de réduire l'inflammation au niveau des adipocytes. En effet, une étude a récemment montré que le cholécalciférol et la 1,25(OH)2D ont des propriétés anti-inflammatoires similaires au niveau d'adipocytes humains soumis à une inflammation par le lipopolysaccharide [127]. De plus, notre groupe a récemment montré que l'expression et la sécrétion de nombreuses chimiokines, induites par le TNFα, était fortement diminuée dans les cultures d'adipocytes humains et murins (modèle 3T3-L1) prétraités avec la $1,25(OH)_2D$. Cette $1,25(OH)_2D$ est aussi capable de limiter la migration macrophagique induite par le milieu conditionné dans les adipocytes murins **(Figure 13)** [146]. L'effet anti-inflammatoire de la vitamine D est médié par une inhibition de la voie de signalisation NF-κB et des MAPK **(Figure 13)** [144, 146-148]. Enfin, in vivo, l'effet anti-inflammatoire de la vitamine D a été rapporté dans une étude où il a été montré qu'un traitement avec la $1,25(OH)_2D$ réduit le contenu en protéine IL6 dans le TA épi de souris obèses [149]. Notre groupe a aussi montré qu'un modèle murin d'inflammation chronique soumis à une supplémentation en vitamine D entraîne une limitation de l'expression des chimiokines et de l'infiltration des leucocytes au sein du tissu adipeux [146]. De plus, deux récentes études montrent qu'une supplémentation en vitamine D lors d'un régime riche en graisse chez le rat réduit la concentration de TNFα et de MCP1 dans le tissu adipeux [150] et augmente la concentration plasmatique en IL-10 [151]. A contrario, un régime riche en graisse déficient en vitamine D augmente, au niveau du tissu adipeux de rat, l'expression d'IL6 et de TNFα [152, 153] et le recrutement des macrophages [153].

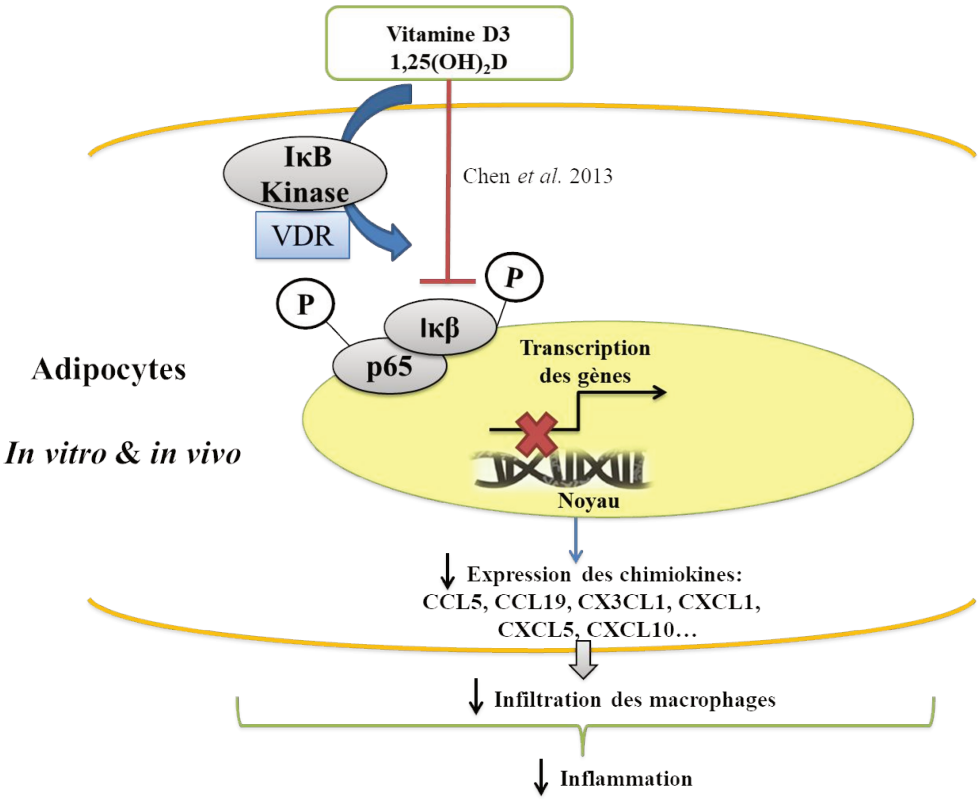

Figure 13 : Schéma de régulation de l'inflammation adipocytaire par la vitamine D.

Peu d'études cliniques décrivent le lien entre la vitamine D et l'inflammation associée à l'obésité [154] et les résultats sont globalement assez contrastés, à l'image des deux principales études réalisées dans ce domaine. En effet, Wamberg et al. ont étudié l'effet d'une supplémentation en vitamine D chez des sujets obèses ayant de faibles taux plasmatiques de 25(OH)D et n'ont obtenu aucune amélioration du statut inflammatoire [145], tandis que Zitterman et al. ont montré qu'une supplémentation permet de corriger le statut inflammatoire en diminuant le taux circulant de TNFα chez des personnes obèses [155]. Plus récemment, une étude a montré une association négative entre les niveaux plasmatiques en IL6 et TNFα et les concentrations plasmatiques en 25(OH)D chez des sujets normaux-pondéraux [156]. D'autres études seront nécessaires afin de confirmer l'effet d'une supplémentation en vitamine D sur le statut inflammatoire chez l'Homme.

En ce qui concerne la régulation de l'expression des miRs par la vitamine D in vitro et in vivo, aucune donnée n'était disponible lorsque nous avons entrepris nos travaux. Chez l'Homme, une

étude s'est intéressée à la relation entre une supplémentation en vitamine D et les profils plasmatiques des miRs sans pouvoir mettre en évidence de corrélation [157].

3.3 EFFETS METABOLIQUES ET ADIPOGENIQUE DE LA VITAMINE D

De nombreuses études se sont penchées sur le rôle de la $1,25(OH)_2D$ dans la prolifération et la différentiation des pré-adipocytes murins [158, 159]. Il a été montré que de faibles concentrations en $1,25(OH)_2D$ peuvent inhiber l'adipogenèse et réduire l'accumulation de triglycérides dans l'adipocyte [159]. Les mécanismes moléculaires font intervenir une diminution de l'expression de régulateurs clés de l'adipogenèse tels que C/EBPα et PPARγ [159, 160]. D'autres mécanismes sont également impliqués comme l'antagonisation de PPARγ et la stabilisation de la protéine VDR [159]. Des effets similaires ont été rapportés dans les adipocytes bruns [161]. Cet effet inhibiteur de la $1,25(OH)_2D$ sur l'adipogenèse reste cependant controversé. En effet, d'autres études ont montré que la $1,25(OH)_2D$ accroît la différenciation et l'accumulation lipidique dans l'adipocyte humain ainsi que dans des primocultures de préadipocytes murins [131]. Un effet inducteur de la $1,25(OH)_2D$ sur l'adipogenèse a également été rapporté dans des cellules souches humaines de tissu adipeux [162]. Les effets de la $1,25(OH)_2D$ sur l'adipogenèse dépendent donc largement du modèle, et des études complémentaires seront nécessaires afin d'évaluer ces effets in vivo.

Il est bien établi que le VDR et son ligand ($1,25(OH)_2D$) ont de nombreuses activités incluant la régulation de la biologie et du métabolisme de l'adipocyte. Une première étude a montré que la $1,25(OH)_2D$ induit une augmentation significative de l'activité de la lipoprotéine lipase et la quantité de ses ARNm dans des adipocytes 3T3-L1 [163]. En même temps, la fatty acid synthase qui catalyse la lipogenèse est régulée de façon négative par la $1,25(OH)_2D$ dans les adipocytes 3T3-L1 [159]. Des études in vivo ont été entreprises en ayant recours à des modèles murins transgéniques. Il a été montré que des souris VDR-/- ou CYP27B1-/- (qui ne synthétisent pas la $1,25(OH)_2D$) sont résistantes au régime obésogène [164]. Inversement, une surexpression du VDR humain dans le tissu adipeux chez la souris induit un phénotype obèse caractérisé par une augmentation du poids et de la masse grasse, due à une diminution de la dépense énergétique, une réduction de la β-oxydation des acides gras et une lipolyse [165]. De plus notre groupe a montré que la $1,25(OH)_2D$ améliore l'absorption du glucose par les adipocytes [144] qui pourrait être liée

à une induction de l'expression de la protéine glucose transporter 4 et sa translocation dans les adipocytes 3T3-L1 [166]. Tout ceci suggère donc fortement que VDR et la 1,25$(OH)_2$D ont un impact sur le métabolisme global en exerçant notamment leurs effets sur le tissu adipeux. De plus, notre groupe a montré que la supplémentation en vitamine D limite la prise de poids induite par l'alimentation obésogène, notamment en réduisant l'expansion des différents dépôts de tissus adipeux [167]. Ces effets sont visibles dès 5 semaines de régimes et plus marqués à 10 semaines. La supplémentation en vitamine D limite également l'augmentation du taux de glucose plasmatique ainsi que celui d'insuline. La limitation de prise de poids s'explique en partie par une augmentation de la dépense énergétique des animaux celle-ci n'étant pas due à une augmentation de l'activité physique mais plutôt à une oxydation des lipides accrue. L'expression, dans différents organes tels que le foie, le tissu adipeux brun ou encore le muscle, de gènes impliqués dans l'oxydation des lipides ainsi que dans le métabolisme mitochondrial est en effet plus importante chez les souris supplémentées en vitamine D. Ces résultats concernant l'activation de l'oxydation des lipides dans le TAv ont été récemment confirmés chez le poisson zèbre [168]. Des résultats similaires concernant la limitation de prise de poids en réponse à une supplémentation en vitamine D ont été rapportés chez des souris [169] et chez le rat jeune [150] et âgé [170] et de façon cohérente, une augmentation de la masse grasse a été observée chez des rats déficients en vitamine D [170, 171].

4. L'OBESITE

4.1 DEFINITION

L'obésité, en augmentation croissante, apparaît depuis quelques années comme un problème de société. Cette pathologie est causée par une augmentation de l'apport énergétique associée à diminution de la dépense énergétique ce qui induit une augmentation massive du tissu adipeux qui est néfaste pour la santé. La prévalence de l'obésité varie en fonction de nombreux paramètres tels que l'âge, le sexe, le statut socio-économique ou encore l'origine ethnique (étude ObEpi 2012, ROCHE). Selon l'Organisation Mondiale de la Santé (OMS), le surpoids et l'obésité sont définis comme « une accumulation anormale ou excessive de masse grasse constituant un risque pour la santé ». Chez l'adulte, l'IMC permet une estimation de l'importance de la masse grasse. Il

correspond à la masse de la personne (en kilogrammes) divisée par le carré de la taille (en mètres). L'IMC s'applique aux deux sexes et à toutes les tranches d'âge adultes. La valeur d'IMC obtenue permet de situer les personnes dans différentes catégories allant de la maigreur à l'obésité massive (**Figure 14**). Il doit toutefois être considéré comme une indication approximative car il ne reflète pas nécessairement correctement le pourcentage de masse graisseuse des individus et peut être biaisé par certaines situations physiologiques particulières (vieillissement, sportifs de haut niveau, grossesse…). L'IMC n'est pas utilisable dans le cas des enfants.

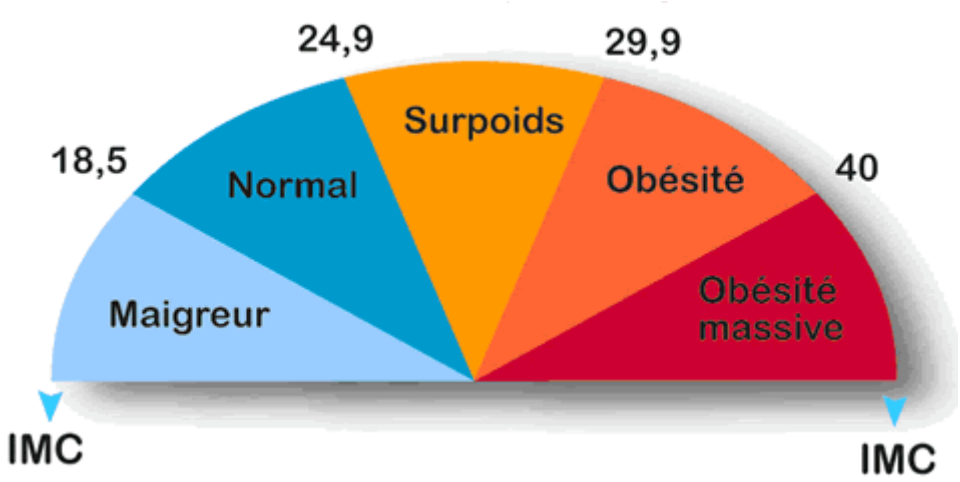

Figure 14 : Classification des individus en fonction de leur IMC.

Chez l'Homme, en complément de l'IMC, le tour de taille est un indicateur pertinent de l'obésité. Ainsi, un tour de taille supérieur à 102 cm chez l'homme et à 88 cm chez la femme (en dehors de la grossesse) est associé à un risque accru de diabète et de maladies cardiovasculaires. De même, le ratio taille/hanche est un paramètre complémentaire qui permet d'avoir une idée de la localisation abdominale ou sous-cutanée de la masse grasse. Le ratio taille/hanche doit pour sa part être inférieur à 1 chez l'homme et 0,8 chez la femme. En effet, indépendamment de l'IMC, l'excès de masse grasse au niveau abdominal, traduisant l'augmentation du tour de taille et du ratio taille/hanche, est particulièrement délétère pour la santé. Bien évidemment de nombreuses autres méthodologies existent pour quantifier de manière précise la masse grasse d'un individu, mais ces techniques font appel à des appareillages lourds et coûteux (DEXA, IRM, tomographie…), qui ne peuvent pas remplacer un indicateur facile à mettre en œuvre comme l'IMC dans le cadre d'études épidémiologiques incluant un très grand nombre d'individus, c'est pour cette raison que ce paramètre anthropomorphique reste une référence.

4.2 L'OBESITE EN QUELQUES CHIFFRES

4.2.1. DANS LE MONDE

En 2015, plus de 1,9 milliard d'adultes (personnes plus de 18 ans) à travers le monde étaient en surpoids et parmi eux 600 millions étaient obèses selon les données de l'OMS, ce qui correspond à l'échelle mondiale à 1 individu sur 5 en surpoids. Cela correspond à 39% des adultes âgés de plus de 18 ans pour les chiffres en lien avec le surpoids (38% des hommes et 40% des femmes) et 13% pour ceux en lien avec l'obésité (11% des hommes et 15% des femmes). Plus préoccupant encore, 42 millions d'enfants de moins de 5 ans étaient en surpoids en 2013. L'obésité et le surpoids constituent la 5ème cause de mortalité à l'échelle planétaire, ce qui correspond à 2,8 millions de morts chaque année. Autrefois considérés comme des problèmes spécifiques des pays à haut revenu, le surpoids et l'obésité sont désormais en augmentation dans les pays à revenu faible ou intermédiaire, en particulier en milieu urbain. En Afrique, le nombre d'enfants en surpoids ou obèses a pratiquement doublé, passant de 5,4 millions en 1990 à 10,6 millions en 2014. Près de la moitié des enfants de moins de 5 ans en surpoids ou obèses vivaient en Asie en 2014. À l'échelle mondiale, le surpoids et l'obésité sont liés à davantage de décès que l'insuffisance pondérale. Il y a plus de personnes obèses qu'en insuffisance pondérale, et ce dans toutes les régions à l'exception de certaines parties de l'Afrique subsaharienne et de l'Asie.

D'après l'Organisation de Coopération et de Développement Economiques (OCDE), un adulte sur cinq est atteint d'obésité dans les 35 pays membres en 2017. D'ici à 2030, l'OCDE estime que près de la moitié (47 %) des adultes américains pourraient être obèses. Le taux en France pourrait monter jusqu'à 21 %. Il est aujourd'hui à 15,3% **(Figure 15)**.

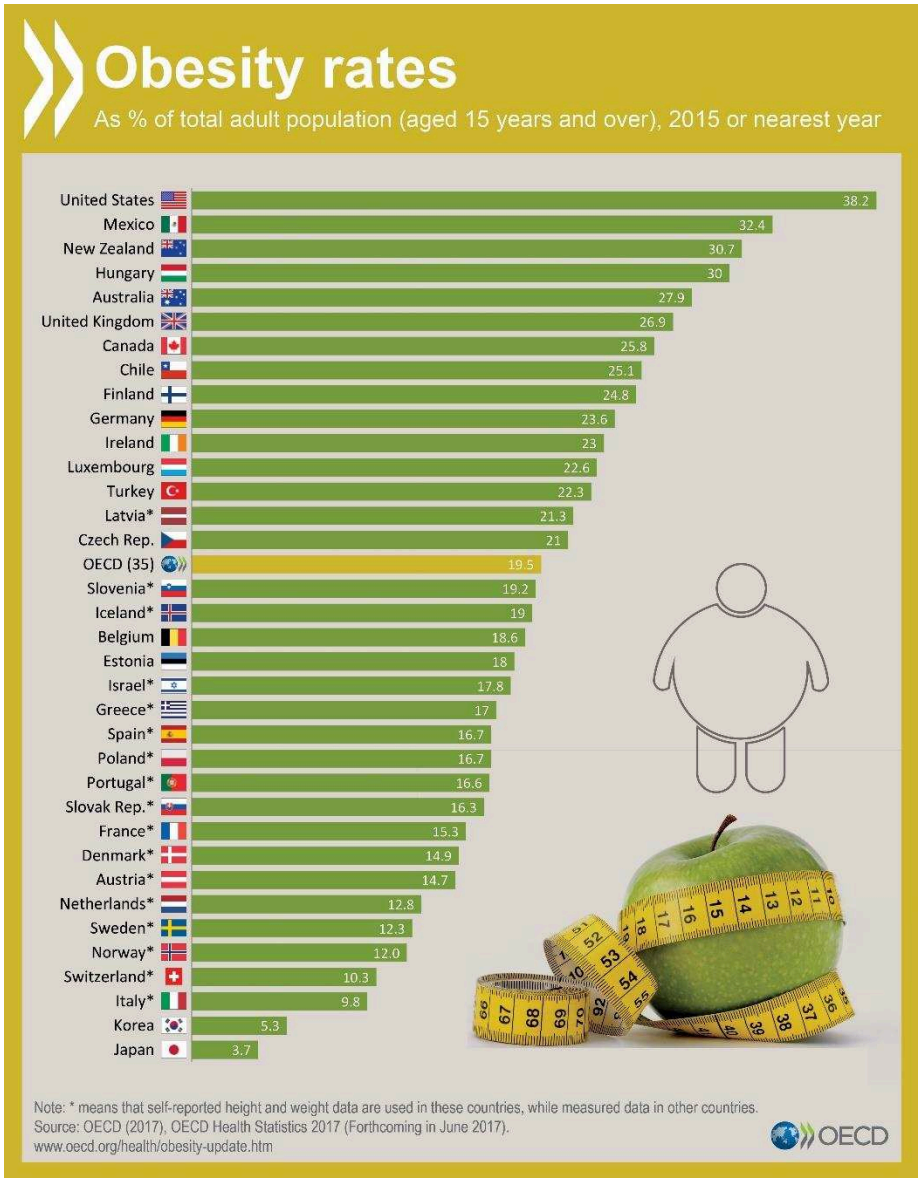

Figure 15 : Chiffre de l'obésité dans le monde par l'Organisation de coopération et de développement économiques (OCDE) en 2017.

4.2.2. EN FRANCE

En France, l'enquête épidémiologique ObEpi a permis d'obtenir depuis 1997 des chiffres très détaillés sur l'obésité et le surpoids. Les derniers résultats (ObEpi 2012) publiés en octobre 2012 ont montré que 15% des Français étaient obèses et 32,3% étaient en surpoids **(Figure 16)**.

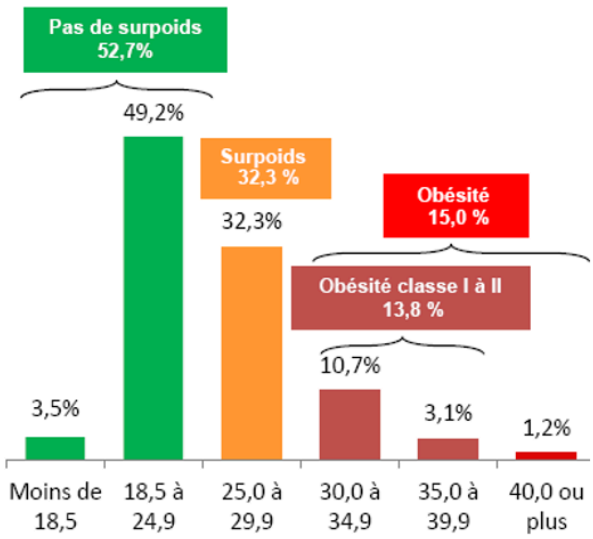

Figure 16 : Répartition de la population en fonction du niveau d'IMC (Issu d'ObEpi 2012).

Depuis 15 ans, la population française a vu son poids moyen augmenter de 3,6 kg. De même, le tour de taille moyen est passé de 85,2 cm en 1997 à 90,5 cm en 2012. Enfin, on observe une apparition de l'obésité de plus en plus précoce, de génération en génération. Il existe des différences de la prévalence de l'obésité en France en fonction des régions **(Figure 17)**. Le Nord est la région à la plus forte prévalence d'obésité en 2012 (21,8%). Suivent le Bassin Parisien (17,8%) et l'Est (17,1%). Les 5 autres régions ont des prévalences de l'obésité relativement semblables et toutes inférieures à la moyenne nationale.

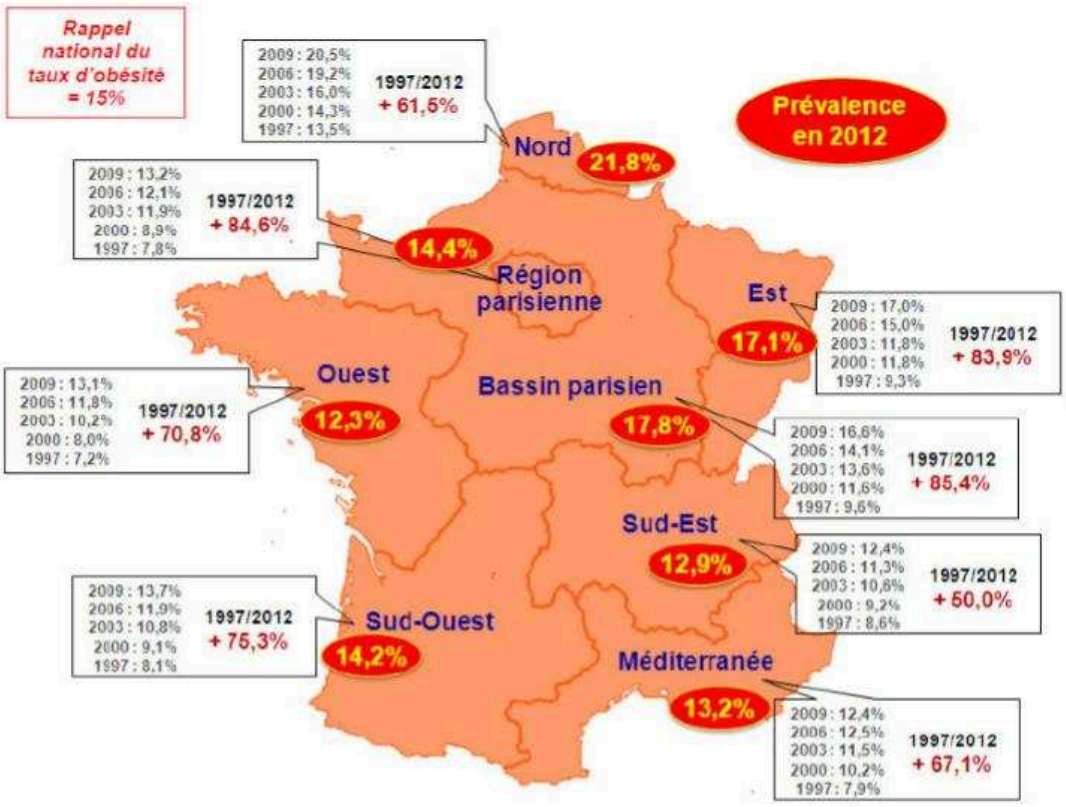

Figure 17 : Prévalence de l'obésité par région et son évolution depuis 1997 (Issu d'ObEpi 2012).

Les régions subissant les plus fortes évolutions de l'obésité entre 1997 et 2012 sont la Région Parisienne avec 84,6% d'augmentation, le Bassin Parisien avec + 85,4% et l'Est avec + 83,9%. Le Sud-Est, quant à lui, a la plus faible augmentation de prévalence ces 15 dernières années (+ 50%). L'augmentation de la prévalence de l'obésité se poursuit dans notre pays mais avec une tendance significative à la décélération. L'augmentation estimée entre 2009 et 2012 n'est que de 0,5 %, alors qu'elle avait toujours dépassé 1 % sur la période de 3 ans séparant deux études ObEpi jusqu'à présent. On observe en parallèle un ralentissement de la progression de l'indice de masse corporelle et du tour de taille moyens. Quoi qu'il en soit, malgré les campagnes d'informations et de sensibilisation du grand public diffusées ces dernières années, il reste préoccupant d'observer que la prévalence de l'obésité soit aussi élevée, compte tenu des nombreux risques et pathologies associés à l'obésité.

4.3 CAUSES DE L'OBESITE

L'obésité est une maladie complexe qui résulte d'une interaction entre une multitude de facteurs génétiques, biologiques et environnementaux. Les progrès combinés de la génétique quantitative, de la génomique et de la bioinformatique ont permis de mieux comprendre les bases génétiques et moléculaires de l'obésité.

4.3.1 FACTEURS ENVIRONNEMENTAUX

Notre environnement a considérablement évolué en quelques décennies, ainsi l'espèce humaine se trouve confrontée à de nombreux nouveaux facteurs de risques environnementaux, tels que la pollution environnementale, la prise de médicaments (antidépresseurs, neuroleptiques…), la pression socio-économique, les modifications alimentaires, une faible activité physique liée à la sédentarisation (internet et télévision notamment), une plus grande consommation d'aliments caloriques riches en graisses et en sucres mais pauvres en vitamines, en minéraux et autres micronutriments (l'augmentation de la taille des portions, la plus grande densité énergétique de l'alimentation, l'évolution des prix alimentaires ont favorisé l'excès de consommation calorique), le manque de sommeil, le stress, l'anxiété, etc., autant de facteurs de risques suspectés d'avoir un rôle causal dans l'épidémie d'obésité **(Figure 18)** [172]. Selon l'OMS, l'obésité et le surpoids ont pour cause essentielle un déséquilibre énergétique entre les calories consommées et dépensées. L'obésité constitue un facteur de risque important pour nombre de pathologies telles que les maladies cardiovasculaires (qui constituent la principale cause de mortalité dans le monde), l'insulino-résistance pouvant aboutir au diabète de type 2, l'arthrose, certains types de cancers (sein et colon notamment) [173]. L'obésité est également très souvent associée à l'hypertension artérielle, l'insulino-résistance et les dyslipidémies, cet ensemble de désordres physiologiques constituant le syndrome métabolique ou syndrome X [174].

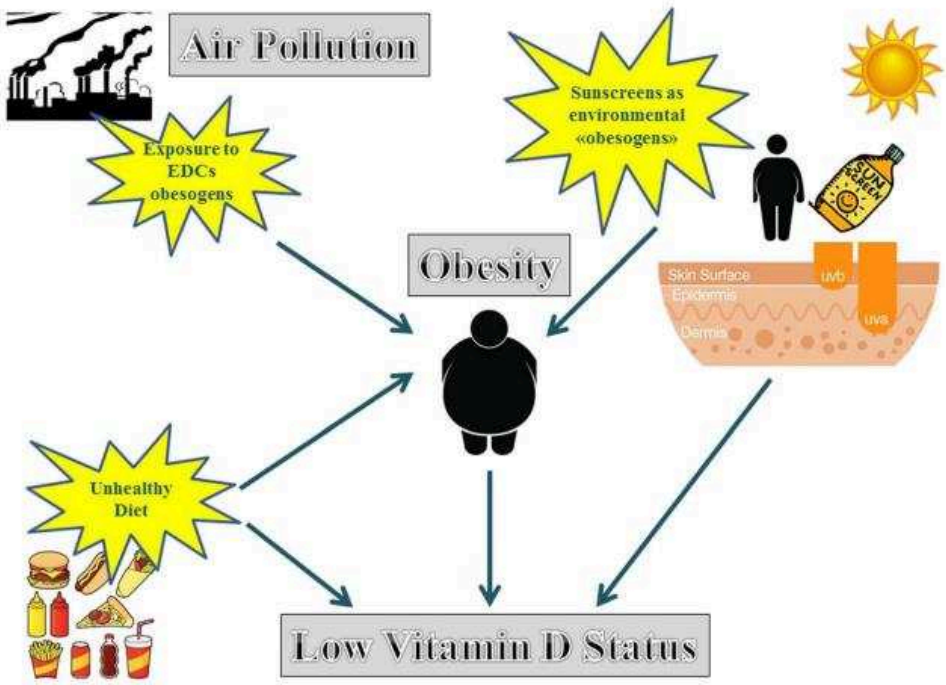

Figure 18 : Relation entre différents facteurs environnementaux dans la mise ne place de l'obésité [175]

4.3.2 FACTEURS BIOLOGIQUES

Certains facteurs biologiques prédisposent à l'obésité, c'est notamment le cas de l'âge et du sexe. Ainsi, la prévalence de l'obésité augmente régulièrement avec l'âge. De même, l'étude ObEpi 2012 a permis de mettre en évidence que l'obésité féminine tend à augmenter plus rapidement que l'obésité masculine en France. Certains désordres physiopathologiques peuvent également être considérés comme des facteurs causaux dans l'apparition d'une obésité dite secondaire, c'est notamment le cas de l'hypothyroïdisme, de l'hypercorticisme ou de l'hypogonadisme, qui aboutissent à une obésité dite endocrinienne [176]. Enfin, il est à présent bien admis que la modification du microbiote par l'alimentation constitue un facteur causal dans l'apparition de l'obésité [177]. En effet, la première démonstration de l'impact du microbiote sur la survenue de l'obésité a été apportée en 2004 [178]. Les auteurs ont montré qu'il est possible d'induire une augmentation de la masse grasse chez la souris axénique en lui transférant une flore bactérienne de souris conventionnelle. Par la suite, cette même équipe a montré que la transplantation d'un microbiote de souris hyperphage et génétiquement obèse (souris ob/ob) induit une augmentation

de masse grasse plus importante que la transplantation d'une flore de souris mince, suggérant l'existence d'un microbiote particulier associé à l'obésité [179]. Ces données ont par la suite été largement validées et complétées [180, 181]. En 2008, Cani et son équipe ont montré que 4 semaines de régime hyperlipidique chez la souris entraînaient une altération de la composition du microbiote et une multiplication par 2 à 3 de la concentration de lipopolysaccharide (LPS) circulant. Cette augmentation de la concentration de LPS a été appelée « endotoxémie métabolique » étant donné que les concentrations mesurées restent bien en-deçà de celles observées lors d'une endotoxémie associée à un choc septique. Lorsque l'endotoxémie métabolique était reproduite par une injection continue de LPS, les souris ont développé les mêmes anomalies métaboliques que celles induites par un régime hyperlipidique : prise de poids, diminution de la tolérance au glucose, augmentation du degré de stéatose, augmentation de l'expression de cytokines proinflammatoires dans le foie, le muscle et le tissu adipeux **(Figure 19)** [182].

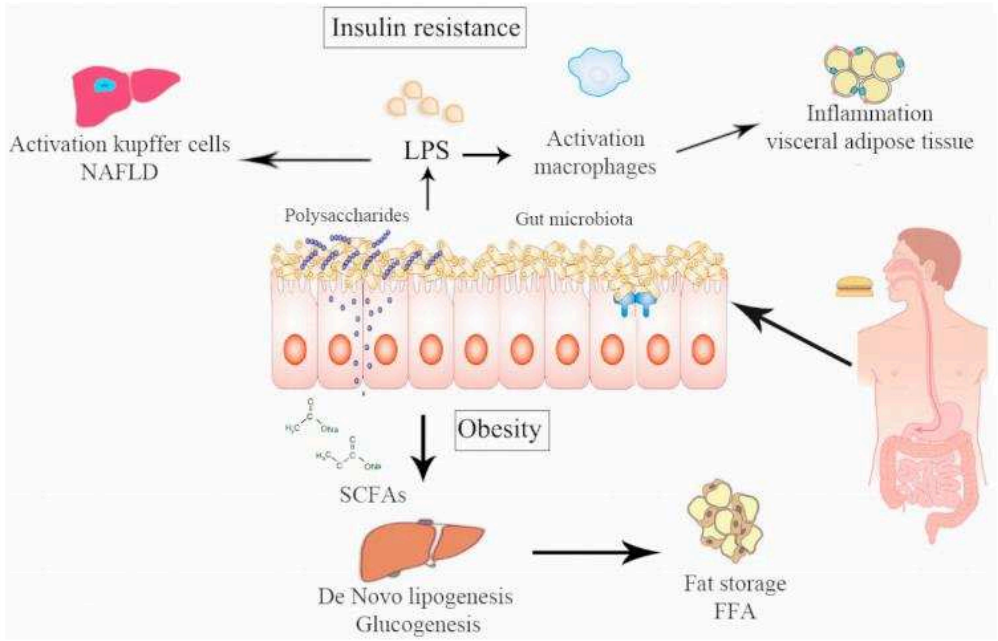

Figure 19 : Mécanismes d'altération du microbiote intestinal et développement de l'obésité. (1) La translocation bactérienne chronique due à l'augmentation de la perméabilité intestinale peut entraîner une inflammation systémique et par conséquent une infiltration de macrophages dans le tissu adipeux viscéral, l'activation des cellules hépatiques Kupffer et le développement de la résistance à l'insuline. (2) Les acides gras à chaîne courte altèrent la lipogenèse de novo et la glucogenèse [183].

Les études chez l'Homme ont elles aussi confirmé que les proportions des grandes familles bactériennes dans le microbiote intestinal diffèrent entre personnes obèses et personnes minces [179]. Des études récentes se sont intéressées à la relation entre la prise alimentaire, le microbiote intestinal et les phénotypes métabolique et inflammatoire. Pour ce faire, un régime hypocalorique et une période de stabilisation ont été réalisés chez des patients obèses ou en surpoids. Il a été montré qu'une flore intestinale appauvrie est associée à une inflammation plus marquée avec une augmentation de l'adiposité, le développement d'une insulino-résistance et une dyslipidémie par rapport à des sujets qui ont une flore plus riche en bactéries [184]. L'intervention alimentaire améliore l'état de la flore mais semble être moins efficace sur la variation de l'inflammation chez des individus ayant une flore appauvrie [185]. D'un point de vue mécanistique, la modification de la flore bactérienne semble agir sur son hôte via de nombreux effets métaboliques, tels que l'augmentation de la fermentation des polysaccharides aboutissant à la synthèse d'acides gras à courte chaîne, la modification du métabolisme des acides biliaires ou de la choline, l'augmentation de la perméabilité membranaire intestinale induisant une endotoxémie à l'origine d'une inflammation à bas bruit [177, 183].

4.3.3 FACTEURS GENETIQUES

La composante génétique dans la susceptibilité à l'obésité a été suspectée depuis la fin des années 1980 lorsque des études portant sur des jumeaux ont montré une forte héritabilité de l'adiposité [186]. D'autres études portant sur des familles ou des jumeaux ont estimé cette héritabilité de 40 à 70 % [187]. Dans les années 1990, certaines formes d'obésité monogénique ont été mises en évidence. Il s'agit la plupart du temps de mutations causant la perte de fonction de protéines-clés dans la régulation de la prise alimentaire comme la leptine, le récepteur à la leptine, la pro-opiomelanocortine (POMC), le récepteur 4 à la mélanocortine [188], la Proprotein Convertase Subtilisine/Kesine 1 (PCSK1) ou la Single minded homolog 1 (SIM1) [189]. Cependant de telles mutations restent très rares dans la population. Les études de liaison et de gènes candidats ont identifié un certain nombre de gènes et régions génomiques associés à l'obésité et ses complications [190, 191]. Les Genome-Wide Association Study (GWAS) ont permis d'avoir une approche sans hypothèse et avec une très bonne résolution. Cette méthode interroge plusieurs milliers de Single-Nucleotide Polymorphism (SNP) fréquents et répartis dans le génome humain

dans le but d'identifier ceux qui sont plus fréquents chez les obèses par rapport aux contrôles. C'est ainsi que depuis 2007, plusieurs vagues de GWAS et de méta-analyses de GWAS ont permis d'identifier une trentaine de loci/gènes de susceptibilité à l'obésité. Cependant, la combinaison de ces polymorphismes ne permet d'expliquer que 1,45 % de la variation interindividuelle de l'IMC, relativisant d'une part l'impact global de ces variations géniques sur la prévalence de l'obésité et suggérant d'autre part que de nombreux autres facteurs prédisposent à l'obésité [192]. D'autres événements génétiques tels que les copy number variations (CNV) résultant de réarrangements chromosomiques et se traduisant par des délétions, des duplications, des inversions et des translocations ont été associées à l'obésité. La réanalyse des puces à ADN utilisées dans les GWAS a permis d'identifier une large délétion de plus de 593kb au niveau du chromosome 16p11.2, augmentant de 43 fois le risque d'obésité, et présent chez 0,7 % des individus obèses morbides européens [193]. En 2014, une étude a mis en évidence des associations significatives entre des CNV multi-alléliques englobant le gène de l'amylase salivaire et l'obésité et l'IMC. Ces résultats ont donné lieu au premier lien entre le métabolisme des glucides et l'IMC [194]. Une étude très récente a permis de mettre en évidence une association significative entre le gène Neuropeptide Y Receptor (NPY4R) contenant le CNV 10q11.22 et la survenue de l'obésité [195].

4.4 COMPLICATIONS ASSOCIEES A L'OBESITE

L'essentiel de la surmortalité liée à l'obésité est dû aux complications qui lui sont associées. Celles-ci sont multiples, fréquentes et souvent sévères. Elles sont influencées par le degré d'obésité, la durée d'évolution de la maladie et par la répartition du tissu adipeux. En effet, dans les années 40, Jean Vague avait remarqué qu'il existait différents types d'obésité. Plus précisément, il avait noté que les personnes présentant une obésité dite androïde ayant une localisation abdomino-viscérale (ou centrale) du tissu adipeux avaient plus de risques de développer les pathologies connexes à l'obésité que les personnes présentant une obésité de type gynoïde présentant une localisation fémorale et sous cutanée du tissu adipeux [91]. Les complications associées à l'obésité les plus connus sont l'insulino-résistance qui peut aboutir au diabète de type 2 et le syndrome métabolique.

La sensibilité à l'insuline est généralement caractérisée par l'action de l'insuline sur le métabolisme du glucose. Elle intervient dans différents organes (les 3 tissus cibles étant le muscle, le foie, le tissu adipeux) et différents métabolismes (glucidique, lipidique, protéique) qui

interagissent entre eux. L'insuline joue un rôle anabolique majeur au niveau de l'organisme ainsi que dans la mise en réserve et l'utilisation des substrats énergétiques. Elle exerce également des fonctions pléïotropes sur le métabolisme protéique (augmentation de la synthèse et inhibition de la protéolyse), la croissance, le contrôle de l'apoptose et le développement **(Figure 20)** [196] dû à sa liaison avec son récepteur membranaire spécifique (IR) exprimé en priorité sur ses trois tissus cibles.

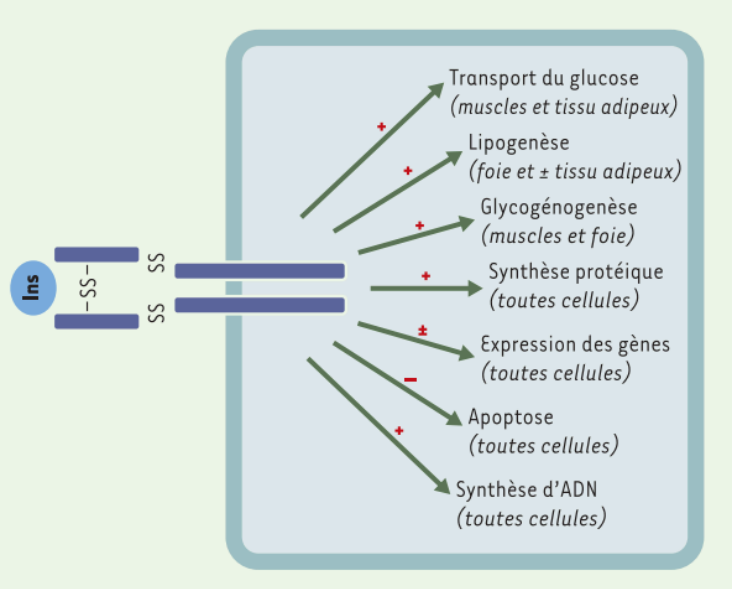

Figure 20 : Les effets pléïotropes de l'insuline. En se fixant sur son récepteur spécifique, l'insuline exerce ses effets dans de nombreux tissus. Ses trois principaux tissus cibles étant le foie, le tissu adipeux et le muscle [197].

L'effet de l'insuline sur le transport du glucose, représente sans doute l'un des effets les mieux étudiés de l'hormone. Il est à présent admis que l'obésité induit une perturbation forte de la signalisation intracellulaire à l'insuline à l'origine d'une baisse de sensibilité appelée insulino-résistance [198]. Ainsi, l'insulino-résistance est définie comme un défaut d'action de l'insuline sur ses tissus cibles (le muscle cardiaque, le muscle squelettique, le tissu adipeux et le foie). Cela va se traduire par une production « compensatoire » d'insuline par les cellules β du pancréas afin de maintenir une glycémie normale qui va persister jusqu'à épuisement. On bascule alors dans le diabète de type II. La perturbation de la signalisation à l'insuline joue un rôle majeur dans le développement du syndrome métabolique **(Figure 21)** [199].

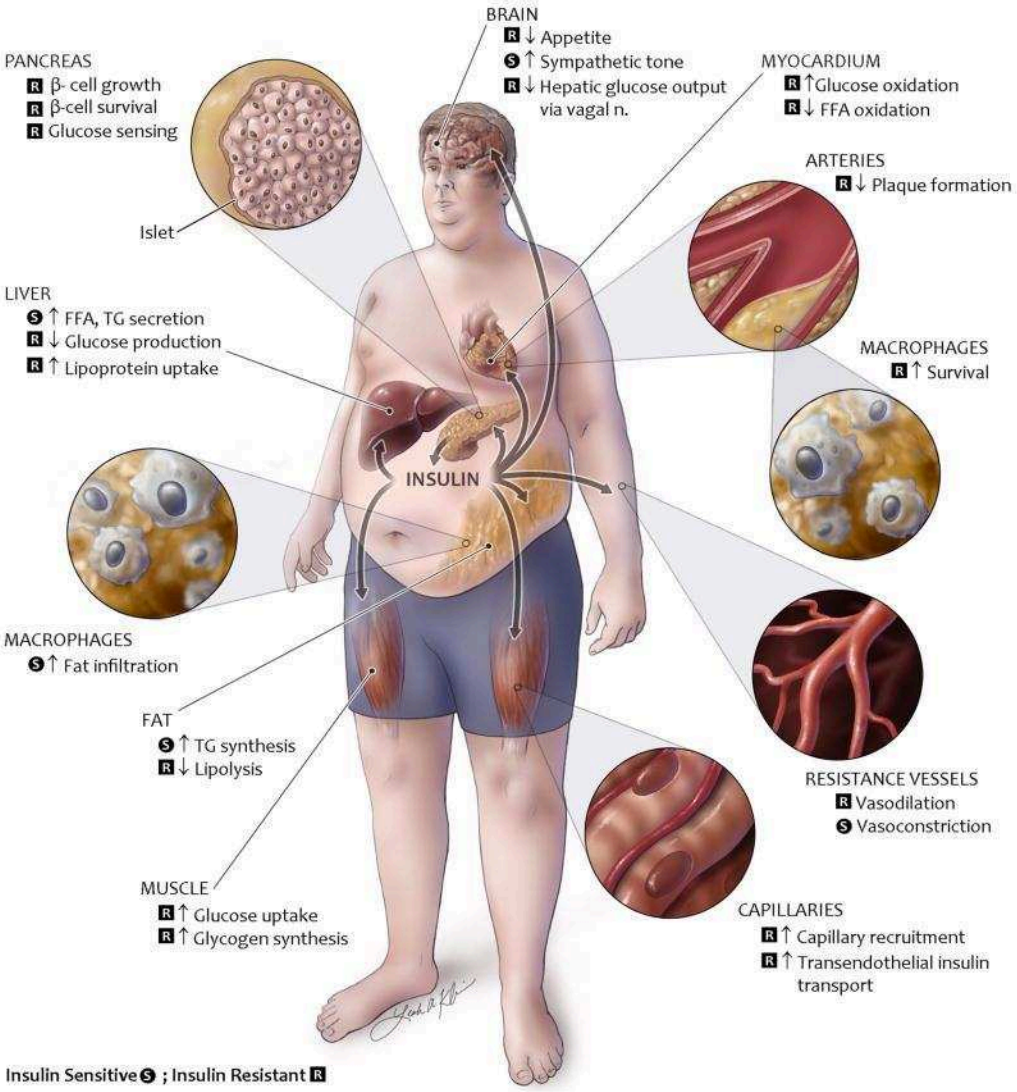

Figure 21 : Physiologie de la signalisation de l'insuline dans le syndrome métabolique [199].

Le syndrome métabolique est une entité clinique et biologique définie par l'association chez un même individu de plusieurs facteurs de risque, parmi lesquels une surcharge pondérale abdominale, une anomalie du métabolisme glucidique, une dyslipidémie et enfin une hypertension artérielle [200-202]. Toutes ces caractéristiques sont modulées par des facteurs environnementaux, mais également l'activité physique ou les habitudes alimentaires, et par des facteurs de susceptibilité génétique qui en favorisent l'expression [203]. En France, quelques données sont

disponibles, notamment au travers des études DESIR (Data Epidemiological Study on the Insulin Resistance syndrome) [204] et MONICA (Multinational MONItoring of trends and determinants in CArdiovascular disease). Celles-ci montrent que la prévalence du syndrome métabolique est élevée en France. Elle est estimée à 16 % (hommes) et 11 % (femmes) selon l'étude DESIR, et à 22,5 % (hommes) et 18,5 % (femmes) selon l'étude MONICA [205]. Comparativement à la prévalence des autres facteurs de risque cardiovasculaire, le syndrome métabolique occupe la troisième place, après l'hypertension artérielle et l'hypercholestérolémie et devant l'obésité et le diabète de type 2 [205]. Selon l'OMS, le syndrome métabolique est basé sur la présence d'une insulino-résistance, associée à au moins deux des anomalies suivantes : une dyslipidémie, une pression artérielle élevée, une microalbumminurie et une obésité centrale (voir les caractéristiques dans le Tableau11) [206]. La notion de syndrome métabolique a évolué au fil des années, et sa définition « officielle » a beaucoup varié [174]. La définition principalement utilisée aujourd'hui est celle proposée en 2001 par le NCEP ATP III (National Cholesterol Education Program Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults Treatment Panel III). Selon cette définition, le syndrome métabolique est caractérisé par la présence d'au moins trois des critères suivant : l'insulino résistance, l'hypertension, la dyslipidémie ou l'obésité viscérale et central.

A travers cette définition, on se rend compte du rôle central qu'occupe l'obésité abdominale et, par voie de conséquence, l'expansion du tissu adipeux abdominal dans la genèse de ce syndrome. En effet, afin de bien prendre conscience de l'importance de ce tissu, il est important de préciser que chez une personne mince, le tissu adipeux représente entre 8 et 18% du poids total chez l'homme et entre 14 et 28% chez la femme. Chez les personnes obèses, ce pourcentage peut être multiplié par 4 et représenter jusqu'à 60 à 70% du poids total dans les cas extrêmes.

5. RELATION ENTRE OBESITE ET VITAMINE D

5.1 ETUDES D'OBSERVATIONS

D'un point de vue épidémiologique, de très nombreuses études mettent en évidence une relation entre obésité et insuffisance plasmatique en 25(OH)D. Plus précisément, les taux circulants de 25(OH)D total d'individus obèses sont généralement plus bas que ceux d'individus normo-pondéraux [207]. Il a aussi été montré que les taux plasmatiques de 25(OH)D libre étaient eux aussi plus faibles chez les personnes obèses que chez les normo-pondéraux [208]. Une méta-analyse très récente regroupant 23 études a montré que les obèses ont 35% de chance de plus de développer un taux bas de 25(OH)D que les sujets normaux et 25% pour les sujets en surpoids [209]. Une étude transversale sur 250 patients en surpoids ou obèses a montré que le taux plasmatique en 25(OH)D est inversement corrélé à la plupart des paramètres de l'obésité tels que l'IMC, la masse grasse et le tour de taille [210]. De même, l'adiposité sous-cutanée mais surtout viscérale [211], ainsi que la masse grasse, mesurée par impédancemétrie, sont inversement corrélées avec le statut en 25(OH)D [212, 213]. La concentration plasmatique en PTH est, quant à elle, positivement corrélée avec la masse grasse [214] et à l'IMC [208]. De plus, il a été montré qu'une augmentation de l'apport alimentaire en vitamine D, entraînant des taux plasmatiques en 25(OH)D plus élevés, est associée à une adiposité viscérale plus faible [215]. Enfin, une étude utilisant l'analyse par randomisation mendélienne montre que l'obésité favorise la diminution de la vitamine D plasmatique. Ainsi, une augmentation d'1 kg de poids corporel entraîne la réduction de 1,15% la teneur plasmatique en 25(OH)D [216].

Si la relation entre la 25(OH)D totale ou libre et l'obésité a été mise en évidence depuis de nombreuses années, la relation entre l'obésité et la $1,25(OH)_2D$ est beaucoup moins claire. Des études chez l'Homme rapportent une relation inverse entre la $1,25(OH)_2D$ et l'IMC et la masse grasse [208, 217, 218]. Cependant, des études plus anciennes montrent une association positive entre la $1,25(OH)_2D$ et l'IMC [219]. Chez la souris, la concentration plasmatique en $1,25(OH)_2D$ est augmentée lorsqu'elles sont nourries avec un régime riche en graisse pendant 18 semaines [220].

L'ensemble de ces résultats contradictoires pourrait être à l'origine de biais méthodologiques dans le dosage du calcitriol ou de variabilité inter-individuelle de $1,25(OH)_2D$.

Depuis quelques années, le lien entre vitamine D et obésité dans les études des SNPs a été mis en évidence. En effet, il existe une association entre les polymorphismes de VDR et l'IMC [221], les marqueurs d'adiposité [222, 223] et l'obésité [224], mais aussi entre l'IMC et les SNP de VDBP [225] et de CYP27B1 [226]. Deux très grandes études suggèrent que les polymorphismes des gènes impliqué dans le métabolisme de la vitamine D pourraient avoir un lien avec les paramètres associés à l'obésité chez des femmes chinoises [227] et dans la population en général [228] mais aucun lien direct n'a été mis en évidence. Ces associations ne sont donc pas toujours retrouvées en fonction des populations étudiées. En effet, un polymorphisme de VDR (BsmI) a été significativement associé à la carence en vitamine D et à la résistance à l'insuline, mais pas à l'obésité dans une population d'adolescents vivant dans des pays tropicaux [229]. De plus, deux randomisations mendéliennes ont montré que les SNPs associés à un faible niveau de 25(OH)D circulant (DHCR7, CYP2R1, VDBP et CYP24A1) n'avait pas de rôle causal dans le risque de développer des maladies cardio-vasculaires [230] ni sur l'IMC [216].

5.2 ETUDES PROSPECTIVES

Des études prospectives récentes ont rapporté que de faibles taux plasmatiques de 25(OH)D sont associés à une forte prévalence de l'obésité chez les enfants [231], les adultes [232, 233] et les femmes âgées [234]. Les faibles apports en vitamine D ont été considérés comme un facteur prédictif de l'obésité [235]. De plus, la consommation de vitamine D serait un marqueur prédictif indépendant de l'IMC dans une population Norvégienne [236]. L'impact de la vitamine D sur la régulation du métabolisme énergétique n'est pas très clair chez l'Homme. Une étude a montré que le taux de 25(OH)D est positivement corrélé à la thermogenèse [237] ce qui pourrait expliquer pourquoi les faibles concentrations de 25(OH)D modifient la balance énergétique. Cependant, une autre étude n'a pas mis en évidence un effet de la supplémentation en vitamine D sur la dépense énergétique et le métabolisme lipidique mais il est important de noter que ce régime a été réalisé pendant une semaine uniquement [238].

5.3 ETUDES INTERVENTIONNELLES

Concernant les études interventionnelles pour étudier la causalité entre le statut plasmatique en vitamine D et l'obésité, quelques travaux ont étudié la co-supplémentation en vitamine D et calcium sur la perte de poids. Les résultats obtenus ne montrent pas de changement en ce qui concerne le poids corporel des individus obèses mais montrent plutôt une réduction de l'adiposité viscérale [239, 240]. Il est difficile de conclure à un potentiel effet de la vitamine D seule car elle est souvent associée au calcium. Néanmoins, une étude a permis de montrer qu'une supplémentation en vitamine D réduit la masse grasse chez des femmes en surpoids et obèses, sans modification du poids total [241]. Aucune autre étude clinique n'a démontré un effet amaigrissant (perte de poids) suite à une supplémentation en vitamine D chez des personnes obèses [155, 242, 243] et ces données ont été validées par une récente méta-analyse [244]. Cependant, un essai randomisé combinant un programme de perte de poids avec un placebo ou une supplémentation en vitamine D a mis en évidence que la supplémentation en vitamine D est associée à une réduction significativement plus élevée de l'IMC et du tour de taille chez les sujets avec un niveau élevé en 25(OH)D (80 nM/L) [245], suggérant ainsi les effets bénéfiques d'une supplémentation à haut niveau de 25(OH)D.

De même, il a récemment été rapporté dans une méta-analyse sur les 20 dernières années a montré qu'une perte de poids d'environ 10 kg, sans supplémentation en vitamine D, pourrait amener à une augmentation des taux plasmatique en 25(OH)D de 6 nmol/L [246]. De plus, une perte de poids comprise entre 5 et 10% du poids d'origine est nécessaire pour observer une augmentation du statut en vitamine D [247].

5.4 PRINCIPALES HYPOTHESES POUR EXPLIQUER LE LIEN

Afin d'expliquer la faible teneur en 25(OH)D observée chez les personnes obèses, plusieurs mécanismes ont été proposés. Tout d'abord, des différences de modes de vie entre personnes obèses et non-obèses pourraient être avancées. Si l'obésité est associée à une augmentation de la surface corporelle [248], on pourrait logiquement s'attendre à une augmentation de la synthèse cutanée de la vitamine D, or l'exposition cutanée des sujets obèses au soleil est limitée à cause de la diminution de leur activité physique à l'extérieur, à l'utilisation d'habits cachant leurs formes

ainsi qu'à la stigmatisation sociale de l'obésité [249]. La conversion du 7-déshydrocholestérol en vitamine D3 ne semble pas altérée les sujets obèses et les non-obèses [250]. Par contre, l'exercice en extérieur peut protéger les personnes en surpoids de l'hypovitaminose D [251]. Il a également été suggéré que l'accumulation de masse grasse pendant l'obésité pourrait découler d'une adaptation excessive de « la réponse hivernale », et que la diminution de la synthèse cutanée de la vitamine D, en raison de la réduction de l'exposition au soleil, contribuerait à l'augmentation de la masse graisseuse pendant les périodes plus froides de l'année [252]. L'alimentation pourrait être aussi mise en jeu dans ce phénomène. En effet, l'apport en vitamine D a été mesuré comme étant plus faible chez les hommes obèses, mais pas chez les femmes, comparé aux personnes non-obèses [235, 236]. L'absorption intestinale de la vitamine D est elle aussi suspectée d'être modifiée au cours de l'obésité. Étant donné que la vitamine D est liposoluble et que l'absorption de calcium est augmentée par les régimes riches en graisses [253], il est probable que l'obésité affecte l'homéostasie vitamine D-calcium par une absorption altérée de l'intestin. Cependant, à ce jour, aucune preuve n'a été apportée pour confirmer cette hypothèse [254]. Le métabolisme de la vitamine D pourrait être modifié pendant l'obésité. En effet, la concentration plasmatique en $1,25(OH)_2D$ et en PTH étant plus élevée au cours de l'obésité, elle pourrait réguler négativement la synthèse hépatique de la 25(OH)D [46].

D'autres hypothèses ont été émises afin d'expliquer la diminution en 25(OH)D chez les personnes obèses. En 2000, Wortsmann et a1. ont suggéré que l'insuffisance en 25(OH)D observée au cours de l'obésité résulterait d'une diminution de la biodisponibilité de la vitamine D à partir de ses sources exogène ou endogène en raison de sa séquestration dans le tissu adipeux. Du fait de l'obésité, l'expansion du tissu adipeux constituerait ainsi un réservoir plus important de stockage de vitamine D et/ou 25(OH)D aboutissant à une diminution des taux plasmatiques de 25(OH)D. Ce phénomène de séquestration mettrait en jeu un phénomène passif résultant uniquement de l'accroissement de la quantité de tissu adipeux [250]. Quelques années après, Drincic et a1. ont mis en relation les taux sériques de 25(OH)D avec la quantité de masse grasse ainsi que le volume corporel total des sujets étudiés. Les auteurs ont pu mettre en évidence que l'association entre la réduction du taux plasmatique et le volume total des individus est plus forte que l'association avec la quantité de masse grasse, suggérant ainsi que le volume total des individus est un meilleur marqueur prédictif que la quantité de masse grasse pour expliquer la diminution de la teneur plasmatique, remettant ainsi en cause l'hypothèse de la séquestration dans le tissu adipeux pour

donner lieu à l'hypothèse de la dilution volumétrique [255]. Plus récemment, Wamberg et al. montrent une modification du métabolisme de la vitamine D dans le tissu adipeux de patients obèses. Les auteurs ont en effet comparé l'expression adipocytaire des ARNm codant différentes enzymes (Cyp2r1, Cyp2j2, Cyp27a1, Cyp3a11, Cyp27b1 et Cyp24a1) et Vdr chez des patientes obèses et une population normo-pondérale. Il en ressort que l'obésité est caractérisée par une diminution de l'expression de la 25-hydroxylase Cyp2j2 dans le tissu adipeux sous-cutané, alors que l'enzyme Cyp24a1 n'est pas différentiellement exprimée chez les sujets obèses et minces [128]. En conclusion, le tissu adipeux qui présente la capacité de métaboliser localement la vitamine D peut voir le profil d'expression des gènes impliqués dans le métabolisme de la vitamine modifié en fonction de l'adiposité. Ceci a été confirmé expérimentalement aussi très récemment par Park et al. qui montrent que l'obésité, induite par une alimentation riche en graisse pendant 18 semaines chez la souris, modifie l'expression des ARNm codants pour les enzymes impliquées dans le métabolisme de la vitamine D au niveau du foie (augmentation de Cyp2r1, Cyp27a1 et Cyp2j3), du rein (augmentation de Cyp27b1 et diminution de Cyp24a1) et du TAv (augmentation de Cyp27a1, Cyp2j3 et Vdr) [220]. Ces deux dernières études très intéressantes ne permettent malheureusement pas de dire si ces modifications de profils d'expression ont un rôle causal dans la mise en place de la diminution plasmatique en 25(OH)D ou s'ils sont juste la résultante de phénomènes adaptatifs consécutifs à l'obésité.

OBJECTIF DE LA THESE

Nous venons de voir au cours de cette introduction qu'il existe une très forte interaction et interrelation entre la vitamine D, le tissu adipeux et l'obésité. En effet, la biologie du tissu adipeux, principal site de stockage de la vitamine D, est directement sous contrôle de cette vitamine et est également directement impactée par l'obésité. De plus, le métabolisme de la vitamine D semble être régulé dans le contexte de l'obésité notamment au niveau adipocytaire. Fort de ces données, l'objectif de cette thèse a été d'avancer dans la connaissance de cette interrelation vitamine D – tissu adipeux – obésité.

En préambule, des recherches bibliographiques approfondies, afin de connaître au mieux le contexte scientifique en lien avec nos objectifs, nous ont conduits à l'écriture d'une revue scientifique **(Article 1)**.

Les travaux réalisés au sein du laboratoire ont récemment montré que la vitamine D présente un effet anti-inflammatoire au niveau du tissu adipeux et de l'adipocyte en modulant l'expression de cytokines et de chimiokines. De plus, il a été mis en évidence que certains miRs sont impliqués dans l'amplification du signal inflammatoire via l'induction de l'expression de certaines cytokines et chimiokines. Dans ce contexte, il était pertinent d'étudier **l'impact de la vitamine D sur l'expression des miRs dans les adipocytes soumis à un stress inflammatoire** ainsi que les mécanismes moléculaires mis en jeu dans ces régulations **(Article 2)**.

Un verrou important pour nos études résidait dans la quantification de la vitamine D et ses métabolites au niveau plasmatique et tissulaire. Aussi un des objectifs de ce travail a été de mettre au point un dosage de ces molécules par la méthode de LC/MS-MS dans le plasma et le tissu adipeux. Ce travail réalisé sur la méthode de quantification est présenté dans l'**Article 3**.

La régulation du métabolisme adipocytaire de la vitamine D était jusqu'à présent largement méconnu. Afin d'apporter de nouvelles connaissances dans ce domaine, nous nous sommes intéressés à **l'effet d'une supplémentation en vitamine D sur la régulation transcriptionnelle du métabolisme adipocytaire de la vitamine D** (**Article 4**). Ce travail nous a permis d'identifier **le mécanisme d'absorption de la 25(OH)D au niveau de l'adipocyte** et de montrer le rôle de la cubiline dans ce phénomène et en étudier le mécanisme moléculaire (**Article 4**).

Nous avons également étudié **l'effet d'un régime riche en graisse, mimant une alimentation déséquilibrée et obésogène, sur la régulation de l'expression des ARNm des différents acteurs**

impliqués dans le métabolisme de la VD, notamment au niveau du tissu adipeux, soit durant une période courte (4 jours) afin d'étudier des évènements précoces (**Article 5**), soit durant une période plus longue (7 et 11 semaines) lorsque l'obésité est établie (**Article 6**). Enfin, l'effet d'une perte de poids, causée par le changement d'alimentation, sur la régulation de l'expression des gènes codant les protéines du métabolisme de la vitamine D, a été étudié et est décrit dans l'**Article 7**.

MATERIELS ET METHODES

1. CULTURE CELLULAIRE (Articles 2 et 4)

Des cellules préadipocytaires 3T3-L1 (d'origine murine) ont été cultivées dans un milieu de base DMEM (*Dulbecco's m*odified eagle Minimal Essential Medium) à 4,5 g/L de glucose supplémenté par 10% de sérum de veau fœtal, 1% d'antibiotiques (Pénicilline à 100 U/ml et Streptomycine à 100 µg/ml) et 1% de biotine (66 µM) / pantothénate (17 µM). La différenciation a été initiée 72 H après confluence par l'addition d'un cocktail de différenciation au milieu de base. Ce cocktail est composé d'insuline de pancréas bovin (1µg/ml), de dexaméthasone (0,25 µmol/L) et de 3-isobutyl-1-méthylxanthine (IBMX) (0,5 mM). Après 48 H d'incubation, le cocktail est retiré et les cultures sont maintenues en présence d'insuline (1µg/ml). Les adipocytes sont considérés comme matures 8 jours après l'induction de la différenciation.

Les adipocytes ont été traités pendant 24 H avec différentes doses d'1,25$(OH)_2$D (1, 10 et 100 nM). Les contrôles ont reçu la même quantité d'éthanol, utilisé comme solvant des micronutriments. Afin d'étudier l'effet des micronutriments sur l'expression des miRs/ARNm, les cellules ont été ensuite incubées ou non avec du TNFα à 15 ng/mL pendant 24 H. Afin d'identifier les mécanismes moléculaires mis en jeu dans la régulation de l'expression des miRs, les adipocytes ont été traités avec des inhibiteurs spécifiques des voies MAP Kinases (JNK (JNK Inhibitor II (10 µM)), p38 (SB202190 (20 µM)) et de la voie de signalisation NF-κB (BAY 117082 (10 µM)) (Calbiochem, Merck Millipore) pendant 1 H et stimulés ou non avec du TNFα pendant 24 H. Pour identifier les mécanismes moléculaires mis en jeu dans la régulation de l'absorption de la vitamine D, les adipocytes ont été traités avec un agoniste de VDR (EB1089 (10, or 100 nM)) pendant 24 H.

Des préadipocytes humains ont été obtenus commercialement (Promocell). Ils ont été isolés à partir du tissu adipeux de femmes caucasiennes non obèses (IMC: 25 kg/m^2). Après 15 jours de différenciation, ils ont été traités pendant 24 H avec la 1,25$(OH)_2$D (100 nM).

2. TRANSFECTIONS CELLULAIRES (Article 4)

Des adipocytes 3T3-L1 ont été transfectés avec soit des si-ARN ciblés contre VDR ou cubiline respectivement, soit des si-ARN non ciblés (Dharmacon, Lafayette, CO, USA) en utilisant INTERFERin (Polyplus-transfection) pendant 24H.

3. UPTAKE DE LA 25(OH)D DANS LES ADIPOCYTES (Article 4)

Les adipocytes 3T3-L1 sont incubés avec de la 25(OH)D radioactive ([3H]-25(OH)D) à la concentration de 11,25 nCi/mL et avec 50 nM de 25(OH)D non radioactive (Sigma-Aldrich, St Louis, MO, USA) dans du DMEM supplémenté avec 1 µg/mL d'insuline avec la présence soit de 1,9 µM de VDBP, soit de 0,125% d'albumine de sérum de veau (BSA) ou de l'éthanol (condition contrôle). Les adipocytes 3T3-L1 sont traités avec 10 ou 100 nM de $1,25(OH)_2D$ ou avec 100 ou 500 nM de RAP (receptor-associated protein) un inhibiteur du complexe mégaline/cubiline. Après 16H d'incubation, les cellules sont lysées et la radioactivité est mesurée grâce à du liquide scintillant. Les résultats sont exprimés par coups par minute (CPM) par puits.

4. EXTRACTION DES ARN TOTAUX (Articles 2, 4, 5, 6 et 7)

L'extraction est réalisée par l'ajout de 1 mL de TRI Reagent (Invitrogen) dans chaque puits de culture ou dans chaque prélèvement de tissu adipeux. Les échantillons sont mélangés à 200 µL de chloroforme et centrifugés (12000 g, 15 min, 4°C). La phase supérieure contenant les ARN est récupérée et mélangée à 500 µL d'isopropanol. Après une centrifugation (12000 g, 30 min, 4°C), le surnageant est éliminé et le culot d'ARN est lavé avec 1mL d'éthanol 80 %. Les échantillons sont ensuite centrifugés (10 min, 12000 g, 4°C) et les culots sont séchés avant d'être repris dans l'eau bidistillée. La quantité et la pureté des ARN extraits ont été mesurées par spectrophotométrie (nanodrop).

5. TRANSCRIPTION INVERSE

Transcription inverse pour miRs **(Article 2)** : La transcription inverse est réalisée dans un volume final de 20 µL. 1 µg d'ARN totaux est ajouté à un mélange contenant 4 µL de tampon miscript RT et 1 µL de miScript transcriptase reverse (Qiagen). La synthèse est réalisée à 37°C pendant 60 min suivie d'une inactivation pendant 5 min à 95°C. Les ADNc obtenus sont dilués dix fois.

Transcription inverse pour ARNm **(Articles 4, 5, 6 et 7)** : Le kit utilisé est le M-MLV RT (Moloney Murine Leukemia Virus Reverse Transcriptase, Invitrogen). La transcription inverse est réalisée dans un volume final de 20 µL. 1 µg d'ARN totaux est ajouté à un mélange contenant 4 µL de tampon 5X, 2 µL de dithiotréitol (0,1 M), 2 µL de dNTP (5 mM), 1 µL d'hexamères (0,3 µg/µL) et 1 µL de M-MLV RT (200 U/µL). La synthèse est réalisée à 37°C pendant 60 min puis les ADNc obtenus sont dilués cinq fois.

6. PCR QUANTITATIVE EN TEMPS REEL (QPCR)

qPCR spécifique des miRs **(Article 2)** : L'amplification est réalisée avec 1,25 µL d'ADNc dilués dans un volume final de 12,5 µL contenant 6,25 µL de SYBR Green Quantitect (Qiagen), 1,25 µL de miScript Universal Primer (Qiagen), 1,25 µL de miScript Primer Assay (Qiagen) et 2,5 µL d'eau bidistillée. Après 15 min à 95°C (activation de la polymérase), la réaction d'amplification s'effectue en 40 cycles comprenant 3 étapes : dénaturation de l'ADNc (15 sec à 94°C), hybridation des amorces (30 sec à 55°C) et une élongation (30 sec à 70°C). Les résultats sont exprimés par rapport au RNU6B ou SNORD68.

qPCR spécifique des ARNm **(Articles 4, 5, 6 et 7)** : L'amplification est réalisée avec 2,5 µL d'ADNc dilués dans un volume final de 12,5 µL contenant 6,25 µL de SYBR Green (Eurogentec), 0,375 µL de chacun des deux amorces sens et antisens à 10 pmol/ µL et 3 µL d'eau bidistillée. Après 2 min à 50°C puis 10 min à 95°C, la réaction d'amplification s'effectue en 40 cycles comprenant 2 étapes : dénaturation de l'ADNc (15 sec à 95°C), hybridation des amorces (1 min à 60°C). Les résultats sont exprimés par rapport à l'ARN ribosomal 18S.

7. MIRNA PCR ARRAYS (Article 2)

Les miRNA PCR arrays ont été réalisés sur des primocultures d'adipocytes humains prétraités pendant 24 H avec la forme active de la vitamine D (1, 25(OH)$_2$D, 100 nM). Les adipocytes ont été ensuite traités avec le TNFα à 15 ng/mL pendant 24 H. Les ARN totaux ont été extraits puis rétrotranscrits. La technique des miRNA PCR arrays permet de quantifier 84 miRs et 6 gènes de référence. Il s'agit d'une plaque précoatée avec les différents miRs d'intérêt **(Figure 22)**. L'amplification a été réalisée par PCR en temps réel.

hsa-miR-142-5p	hsa-miR-9	hsa-miR-150	hsa-miR-27b	hsa-miR-101	hsa-let-7d	hsa-miR-103a	hsa-miR-16	hsa-miR-26a	hsa-miR-32	hsa-miR-26b	hsa-let-7g
hsa-miR-30c	hsa-miR-96	hsa-miR-185	hsa-miR-142-3p	hsa-miR-24	hsa-miR-155	hsa-miR-146a	hsa-miR-425	hsa-miR-181b	hsa-miR-302b	hsa-miR-30b	hsa-miR-21
hsa-miR-30e	hsa-miR-200c	hsa-miR-15b	hsa-miR-223	hsa-miR-194	hsa-miR-210	hsa-miR-15a	hsa-miR-181a	hsa-miR-125b	hsa-miR-99a	hsa-miR-28-5p	hsa-miR-320a
hsa-miR-125a-5p	hsa-miR-29b	hsa-miR-29a	hsa-miR-141	hsa-miR-19a	hsa-miR-18a	hsa-miR-374a	hsa-miR-423-5p	hsa-let-7a	hsa-miR-124	hsa-miR-92a	hsa-miR-23a
hsa-miR-25	hsa-let-7e	hsa-miR-376c	hsa-miR-126	hsa-miR-144	hsa-miR-424	hsa-miR-30a	hsa-miR-23b	hsa-miR-151-5p	hsa-miR-195	hsa-miR-143	hsa-miR-30d
hsa-miR-191	hsa-let-7i	hsa-miR-302a	hsa-miR-222	hsa-let-7b	hsa-miR-19b	hsa-miR-17 hsa-miR-	hsa-miR-93	hsa-miR-186	hsa-miR-196b	hsa-miR-27a	hsa-miR-22
hsa-miR-130a	hsa-let-7c	hsa-miR-29c	hsa-miR-140-3p	hsa-miR-128	hsa-let-7f	hsa-miR-122	hsa-miR-20a	hsa-miR-106b	hsa-miR-7	hsa-miR-100	hsa-miR-302c
cel-miR-39	cel-miR-39	SNORD61	SNORD68	SNORD72	SNORD95	SNORD96A	RNU6-2	miRTC	miRTC	PPC	PPC

Figure 22 : Liste des miRs quantifiés par la technique des miRNA PCR arrays.

8. ACTIVATION DE LA VOIE DE SIGNALISATION NF-κB (Article 2)

Les niveaux de phosphorylation des sous unités p65 (Ser536) et IκB (Ser32/36) ont été quantifiés par le kit ELISA Instant One (eBioscience), selon le protocole du fournisseur.

9. ANALYSE IN-SILICO DU PROMOTEUR (Article 4)

Le logiciel MatInspector mis en œuvre dans le site Genomatix (www.genomatix.de) a été utilisé pour effectuer l'identification in-silico de l'élément de réponse au VDR dans les régions promotrices de la cubiline humaine et murine identifiées par le logiciel MatInspector.

10. PROTOCOLES IN VIVO

Des souris mâles C57BL/6J (Janvier) âgés de 6 semaines ont accès à la nourriture ad libitum (A04, Safe) et à l'eau. Les animaux ont une photopériode contrôlée de 12 H de lumière et 12 H d'obscurité et maintenus à une température constante de 22°C.

Modèle d'inflammation chronique **(Article 2)** : 3 groupes de souris ont été mis en place : des souris nourries soit avec un régime standard (10% d'énergie sous forme de lipides), un régime High Fat (HF) (45% d'énergie sous forme de lipides) ou un régime HF supplémenté en vitamine D (3000 UI/kg de poids corporel) pendant 10 semaines.

Souris supplémentées en vitamine D **(Article 4)** : Des souris ont été gavées avec du cholécalciférol (15000 UI/kg de poids corporel) pendant 4 jours.

Modèle d'obésité à court terme **(Article 5)** : 2 groupes de souris sont nécessaires pour de cette expérience : un groupe nourri avec un régime standard (A04, Safe) et un groupe nourri avec un régime HF (60% d'énergie sous forme de lipides) pendant 4 jours.

Modèle d'obésité à long terme **(Articles 6 et 7)** : 2 groupes de souris ont été identifiés pour cette expérience : un groupe nourri avec un régime standard (10% d'énergie sous forme de lipides) et un groupe nourris avec un régime HF (60% d'énergie sous forme de lipides) pendant 7 ou 11 semaines.

Modèle perte de poids **(Article 7)** : le groupe de souris est nourri avec un régime HF (60% d'énergie sous forme de lipides) pendant 7 semaines puis il change de régime (régime standard (10% d'énergie sous forme de lipides)) pendant 4 semaines.

Chez les souris, l'index d'adiposité est un excellent indicateur de la quantité de masse grasse présente dans le corps. Il se calcule par la somme des différents tissus adipeux retrouvés (épididymal, sous-cutané et viscéral).

11. QUANTIFICATION DES PROTEINES CHEZ LA SOURIS

Cubilin **(Article 4)** : un kit ELISA spécifique a été utilisé afin de quantifier la quantité de protéines cubiline dans le TA épi de souris (Mybiosource, San Diego, California, USA), en accord avec le protocole associé.

Forme libre de la 25(OH)D **(Articles 5, 6 et 7)** : un kit ELISA spécifique a été utilisé afin de quantifier la quantité de protéines de la 25(OH)D sous forme libre dans le sérum de souris (DIAsources, Louvain-La-Neuve, Belgique) en accord avec le protocole associé.

PTH **(Articles 6 et 7)** : un kit ELISA spécifique a été utilisé afin de quantifier la quantité de protéines de la PTH dans le sérum de souris (Euromedex, Strasbourg, France) en accord avec le protocole associé.

Calcium et phosphate **(Articles 6 et 7)** : un test colorimétrique a été utilisé afin de quantifier la quantité de protéines de calcium et de phosphate respectivement dans le sérum de souris (Clinisciences, Nanterre, France) en accord avec le protocole associé.

12. ANALYSES STATISTIQUES

Tous les calculs statistiques ont été effectués avec le logiciel StatView. La signification statistique a été déterminée par une analyse ANOVA, puis la significativité entre les différents groupes a été évaluée par le test post hoc de Tukey Kramer. Une valeur de $p<0,05$ est considérée comme statistiquement significative. La différence significative entre deux groupes a été évaluée par le test t de Student. Une valeur de $p<0,05$ est considérée comme statistiquement significative. Le coefficient de corrélation est R et le test p est calculé avec une probabilité « one-tailed ».

RESULTATS

ARTICLE 1 : Vitamin D modulates adipose tissue biology : possible consequences for obesity?

Publié dans Proceedings of the Nutrition Society, 2016.

Mes travaux de recherches bibliographiques m'ont permis de participer à l'élaboration d'une revue portant sur le rôle majeur de la vitamine D dans la modulation de la biologie de l'adipocyte.

Cette revue s'articule autour de cinq parties :

Une première partie décrit le rôle principal du tissu adipeux dans le stockage de la vitamine D.

Une deuxième partie décrit les différentes enzymes exprimées et impliquées dans le métabolisme de la vitamine D dans le tissu adipeux.

Une troisième partie expose les effets de la vitamine D sur la biologie de l'adipocyte et du tissu adipeux, notamment la régulation de l'adipogenèse, la régulation de l'expression des gènes en lien avec le métabolisme énergétique et la régulation de l'inflammation.

Une quatrième partie décrit le métabolisme de la vitamine D dans des modèles de souris transgéniques.

Une cinquième et dernière partie expose les effets de la vitamine D sur l'obésité et ses désordres associés chez l'Homme.

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Conference on 'Diet, gene regulation and metabolic disease'
Symposium 2: Micronutrients, phytochemicals, gene expression and metabolic disease

Vitamin D modulates adipose tissue biology: possible consequences for obesity?

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Cross-sectional studies depict an inverse relationship between vitamin D (VD) status reflected by plasma 25-hydroxy-vitamin D and obesity. Furthermore, recent studies *in vitro* and in animal models tend to demonstrate an impact of VD and VD receptor on adipose tissue and adipocyte biology, pointing to at least a part-causal role of VD insufficiency in obesity and associated physiopathological disorders such as adipose tissue inflammation and subsequent insulin resistance. However, clinical and genetic studies are far less convincing, with highly contrasted results ruling out solid conclusions for the moment. Nevertheless, prospective studies provide interesting data supporting the hypothesis of a preventive role of VD in onset of obesity. The aim of this review is to summarise the available data on relationships between VD, adipose tissue/adipocyte physiology, and obesity in order to reveal the next key points that need to be addressed before we can gain deeper insight into the controversial VD–obesity relationship.

Adipose tissue: Vitamin D: Obesity: Inflammation: Adipocytes: Nutrients: Nutrition

Vitamin D: a brief overview

Vitamin D (VD; calciferol) is a hormone mainly described for its role as a regulator of phosphate and calcium homeostasis[1]. It can be obtained through animal (VD_3, cholecalciferol) or plant (VD_2, ergocalciferol) food sources. Only a few foodstuffs contain significant amounts of VD, the main sources being fish liver oils, fatty fish (sardines, herring and mackerel) and egg yolk[2,3], but small quantities are also found in fortified milk, orange juice, bread and cereals. Alternatively, VD_3 is produced endogenously in the skin after UVB irradiation from the precursor 7-dehydrocholesterol to give pre-VD_3, which is further isomerised to VD_3 before being released into the circulation[4]. Classical estimates have assigned a majority (70–90 %) of VD supply to dermal synthesis, but a recent paper revised this figure down to just 10–25 % of VD supply[5] and posited that dietary intake of 25-hydroxy-vitamin D (25(OH)D) is a significant contributor to total VD input.

Adipose tissue is a major storage site for vitamin D

Despite limited data, it is widely accepted that adipose tissue is a reservoir for VD in human subjects and rats[6–10]. Interestingly, visceral fat was found to contain 20 % more VD than subcutaneous fat[11]. Heaney *et al.*[12] calculated that 65 % of total VD in the body is in the form of D_3, for which adipose tissue and skeletal muscle appear to be the main body stores (accounting for 73 and 16 %, respectively). Regarding 25(OH)D, 34 % of it is found in adipose tissue, 30 % in serum and 20 % in skeletal muscle. However, 25(OH)D was recently

Abbreviations: 25(OH)D, 25-hydroxy-vitamin D; $1,25(OH)_2D$, 1,25-dihydroxy-vitamin D; MCP, monocyte chemoattractant protein; CYP24A1, vitamin D 24-hydroxylase; DBP, vitamin D-binding protein; VD, vitamin D; VDR, vitamin D receptor.

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detected in human subcutaneous adipose tissue[13]. Fat tissue may thus contain about 60 % of total body VD, but the amount of VD present in fat tissue varies strongly between individuals and is not correlated to serum 25(OH)D levels[8], whereas 25(OH)D adipose tissue content seems to be correlated to 25(OH)D plasma levels[13]. Other factors such as VD status and amount of intake can also influence adipose tissue storage. Indeed, it was calculated that for low VD intakes and when serum 25(OH)D concentration is below 88 nM/l, almost all VD is converted to 25(OH)D in the liver with very little deposited in tissues[14], indicating that low 25(OH)D status is associated with limited tissue stores as well.

A recent pilot study using time-of-flight secondary ion mass spectrometry confirmed that both VD and 25(OH)D were present in human adipose tissue, and also reported for the first time that 1,25-dihydroxy-vitamin D ($1,25(OH)_2D$) was also detectable in fat tissue[15]. This pilot study also suggested that all these molecules were located in adipocyte lipid droplets and that VD and 25(OH)D concentrations in adipose tissue were lower in obese than lean subjects, although this last observation was only generated with a very limited number of samples[15].

Parallel between systemic vitamin D metabolism and adipose tissue metabolism

Uptake of vitamin D

VD from food is partially absorbed in the distal part of the small intestine, in emulsion with bile salts[16]. Its intestinal absorption occurs not only by passive diffusion but also via at least two cholesterol carriers[17,18]. VD and its metabolites are majorly transported in the plasma bound to vitamin D-binding protein (DBP), a globulin produced in the liver[19], but also to albumin, LDL and chylomicrons for dietary VD[20]. Plasma DBP is in large excess compared with VD and metabolites, thus leaving only a very limited amount of circulating unbound VD and metabolites[21]. Interestingly, only the unbound part of VD is considered biologically active and able to diffuse in any target cells[21], thus leading to the free-hormone hypothesis[22]. Indeed, despite having low plasma levels of the different forms of VD compared with wild-type animals, Dbp-null mice do not show any signs of disrupted calcium homeostasis, suggesting that free VD levels can cover the needs of physiological functions as long as diet is VD-sufficient[23]. Subsequent work showed that kidney content of $1,25(OH)_2D$ was not different from that of wild-type animals[24]. Taken together, these data suggest that tissues may uptake VD and metabolites from the free pool through a DBP-independent mechanism.

The molecular mechanisms involved in VD uptake/secretion by adipose tissue have not yet been investigated but may well involve the megalin/cubulin pathway (described later) as suggested by Abboud *et al.*[25] and/or cholesterol transporters as described in the intestine[17] or for other lipophilic micronutrients in adipose tissue[26].

25-hydroxylation

Whatever its origin (endogenous or exogenous), calciferol is taken to the liver via the circulation where the VD 25-hydroxylase enzyme catalyses the synthesis of 25(OH)D. 25(OH)D is the major circulating form of VD and its serum concentration is classically used as a marker of VD status. 25(OH)D has a relatively long half-life (15 d), and mean plasma 25(OH)D concentration varies between 20 and 50 ng/ml (50–125 nM/l)[27]. Several enzymes can accomplish this first hydroxylation of 25(OH)D, but CYP2R1 seems to be the key one[28,29]. Interestingly, $Cyp2r1^{-/-}$ mice only display a 50 % reduction in serum 25(OH)D compared with wild-type or heterozygous animals, suggesting that other enzymes help maintain circulating 25(OH)D levels and/or compensate for CYP2R1 dysfunction[30].

In human subjects, other P450 cytochromes such as CYP3A4[31], CYP2J2[32] and CYP27A1[33] display 25-hydroxylase activity towards VD molecules, but less efficiently (i.e. with a high K_M relative to physiological substrate concentration)[30]. CYP2J3[34], CYP2D25 and CYP2C11 also show VD 25-hydroxylase activity but are only expressed in pigs and male rats, respectively[35,36].

25-Hydroxylation seems to be functional in adipose tissue, as Zoico *et al.*[37] recently reported that 25(OH)D release in the cell culture medium increased after 24 h incubation of 3T3-L1 adipocytes with VD. This production of 25(OH)D could be due to the presence of Cyp27A1, which is up-regulated by VD treatment. Interestingly, human adipose tissue biopsies have confirmed the expression of CYP27A1, CYP2R1 and CYP2J2[38], suggesting that human adipose tissue and adipocytes are able to convert VD to 25(OH)D.

1α-hydroxylation

In renal proximal tubule cells, urinary loss of DBP–25(OH)D complexes is prevented by uptake via the membrane receptors megalin (also known as low-density lipoprotein receptor-related protein 2) and cubilin[39,40]. After internalisation into vesicles, DBP is degraded into lysosomes and 25(OH)D is handled by intracellular DBP. Intracellular DBP have been identified in VD-resistant new-world primates (four isoforms have been reported so far), are related to the human heat-shock protein 70 family, and are thought to mediate 25(OH)D interactions with intracellular proteins[41]. An additional binding protein termed cytosolic DBP has also been isolated from human intestinal cells[42]. 25(OH)D is then either secreted into circulation or directed towards mitochondrial 1α-hydroxylase CYP27B1 to be metabolised into $1,25(OH)_2D$, the active form of VD. CYP27B1 is the key enzyme of 1α-hydroxylation and its activity is regulated by parathyroid hormone, fibroblast growth factor 23, calcium and phosphorus and self-regulated by $1,25(OH)_2D$ via a negative-feedback mechanism[1]. $1,25(OH)_2D$ has a very short half-life (about 4 h) and is 1000 times less concentrated than 25(OH)D in the plasma.

The ability of adipocytes to convert 25(OH)D into $1,25(OH)_2D$ was initially demonstrated in 3T3-L1 cells

via the activation of a gene reporter system and through the identification of radiolabelled $1,25(OH)_2D$ derived from radiolabelled 25(OH)D[43]. The production of $1,25(OH)_2D$ from 25(OH)D was then confirmed by Ching *et al.*[44] and Nimitphong *et al.*[45]. CYP27B1 expression has also been detected in murine adipocytes[43] and in human adipose tissue biopsies[38].

24-hydroxylation

Finally, vitamin D 24-hydroxylase (CYP24A1) is in charge of inactivating $1,25(OH)_2D$. This inactivation is self-regulated, since $1,25(OH)_2D$ induces the expression of CYP24A1 that converts 25(OH)D and $1,25(OH)_2D$ into less-active metabolites (e.g. $24,25(OH)_2D$ and $1,24,25(OH)_3D$), which are further catabolised into inactive calcitroic acid[40].

In adipose tissue, CYP24A1 expression has been detected in murine and human adipocytes[43,45]. In addition, the mRNA levels of CYP24A1 are strongly induced by $1,25(OH)_2D$ incubation[43,45]. CYP24A1 expression has also been confirmed in human adipose tissue biopsies[38].

Vitamin D signalling

Even if few VD receptor (VDR)-independent effects of $1,25(OH)_2D$ have been documented[46], most biological activities of VD are mediated by the VDR, a member of the nuclear receptor superfamily that is the only nuclear receptor that binds $1,25(OH)_2D$ with high affinity[47,48]. VDR expression has been demonstrated in almost all human tissues[49], which means that all cells are potential targets of $1,25(OH)_2D$ action. The VDR–$1,25(OH)_2D$ complex is associated with the retinoid X receptor[50], and the retinoid X receptor–VDR–$1,25(OH)_2D$ complex binds to the DNA of sites called VD response elements in the promoter region of genes whose expression is either activated or repressed[47]. There are more than 1000 genes that are directly or indirectly regulated by $1,25(OH)_2D$ and involved in various physiological processes such as cell proliferation, differentiation, apoptosis and angiogenesis[51].

The presence of VDR in adipose tissue was first reported in the early 1990s[52] and has since been widely confirmed. Interestingly, it was recently found that VDR expression is increased in obese compared with lean subjects[38,53], but the physiological relevance of this up-regulation has not yet been elucidated.

Another VD-dependent signalling pathway has been described that involves ERp57 (also known as GRP58 or 1,25D3-MARRS), a protein disulfide isomerase involved in stress response[54] that mediates rapid cellular responses (i.e. within seconds or minutes) to $1,25(OH)_2D$ stimulation[55,56]. ERp57–$1,25(OH)_2D$ complexes are internalised, which opens the possibility that ERp57 might also participate in $1,25(OH)_2D$ intracellular trafficking, especially since ERp57 has been found to participate in nuclear complexes, including heat-shock protein 70, one of the human intracellular DBP. However, it is not yet known whether this signalling pathway is active in other tissues, particularly adipose tissue.

Taken together, these data demonstrate that on top of being a major storage site for VD, adipose tissue also expresses enzymes involved in VD metabolism and signalling, which points to the hypothesis that adipose tissue could be a target tissue that is also able to synthesise 25(OH)D and $1,25(OH)_2D$ that could be locally active via paracrine, autocrine or even intracrine processes[57]. The regulation of this local metabolism has never been studied but certainly warrants future investigation. Nevertheless, there is increasing evidence of the impact of VD and its active metabolites on adipose tissue, notably in terms of adipogenesis control, adipokine expression and a host of other metabolic regulations.

In vitro and *in vivo* effects of vitamin D on adipose tissue and adipocyte biology

Regulation of adipogenesis

Many studies have examined the role of $1,25(OH)_2D$ in the proliferation and differentiation of murine 3T3-L1 pre-adipocytes[52,58–61]. Low $1,25(OH)_2D$ concentrations were associated with an inhibition of adipogenesis and a reduction of TAG accumulation in 3T3-L1 cells, even if the opposite effects, i.e. induction of adipogenesis, have also been depicted in this cellular model[62]. The mechanism governing these effects implies $1,25(OH)_2D$-mediated down-regulation[59,60] of C/EBPα and PPARγ, the two master regulators of adipogenesis[63]. Other mechanisms such as antagonisation of PPARγ activity and stabilisation of the VDR protein are also components of this complex regulation[59]. Similar results, i.e. inhibited differentiation under $1,25(OH)_2D$, have also been reported in brown adipocytes[64].

However, these data have been recently challenged in human adipocytes where $1,25(OH)_2D$ enhanced adipocyte differentiation and lipid accumulation[45]. Interestingly, a similar activation of adipogenesis was found in primary mouse pre-adipocytes (albeit at a more advanced stage of differentiation compared with 3T3-L1 cells, suggesting that stage of differentiation is a key factor in the nature of the effect of $1,25(OH)_2D$ on adipogenesis). Similarly, $1,25(OH)_2D$ also increased adipogenesis in human adult stem cells derived from adipose tissue, as revealed by lipid accumulation and expression of adipogenic markers[65].

To summarise, the effects of $1,25(OH)_2D$ and VDR on adipogenesis are not fully consistent: VDR appears to act as a promoter of adipogenesis, but $1,25(OH)_2D$ has less clear effects. It is currently difficult to firmly conclude in favour of an anti- or pro-adipogenic effect, and further *in vivo* studies are required to clarify this point.

Regulation of gene expression linked to energy metabolism

VD and particularly $1,25(OH)_2D$ may also influence adipose tissue and systemic biology. Indeed, $1,25(OH)_2D$ directly up-regulated leptin expression and secretion,

independently of fat mass modifications[66]. 1,25(OH)$_2$D was found to promote glucose uptake by adipocytes[67], which could be related to the induction of GLUT4 protein expression and translocation observed in 3T3-L1 adipocytes[68]. We showed that VD supplementation in mice led to an increase of fatty oxidation (especially in brown adipose tissue) that could be responsible for a high-fat diet-induced limitation of body weight gain in VD-supplemented mice[69]. Note that similar weight gain limitations in response to VD supplementation have been reported in mice[70] while VD-deficient old rats showed fat mass gain[71]. Likewise, a global dietary vitamin restriction was associated with an increase of adiposity and a disruption of glucose homeostasis[72] in mice.

Taken together, these data suggest that VD regulates energy expenditure, notably via its impact on adipose tissue biology.

Regulation of inflammation

Initial studies performed on human and mouse adipocytes (3T3-L1) found that 1,25(OH)$_2$D up-regulates several inflammatory cytokines and down-regulates anti-inflammatory cytokines in both cell types[73,74]. These results have since been challenged by several groups[67,75–77] who consistently report anti-inflammatory effects of 1,25(OH)$_2$D whatever the model studied, which fits better with the well-described anti-inflammatory effect of VD in many other cell types[78]. Indeed, it was shown that 1,25(OH)$_2$D significantly decreased the release of IL-8, monocyte chemoattractant protein (MCP)-1 and IL-6 by human preadipocytes[76] and MCP-1 by human adipocytes[75]. These anti-inflammatory effects were associated with inhibition of the NF-κB signalling pathway[77]. We also demonstrated the anti-inflammatory properties of 1,25(OH)$_2$D in murine and human adipocytes[67]. In these various models, 1,25(OH)$_2$D was able to decrease the expression of inflammatory markers such as IL-6, IL-1β and MCP-1 in both basal and TNFα-stimulated conditions[67]. Similarly, 1,25(OH)$_2$D reduced the expression of IL-6, IL-8 and MCP-1 in human adipose tissue biopsies submitted to IL-1β stimulation *in vitro*[79]. The molecular mechanisms have been investigated, and VDR and NF-κB signalling pathways and p38 mitogen-activated protein kinases were shown to be involved in 3T3-L1 adipocytes[67]. Interestingly, Zoico *et al.* recently demonstrated that VD, similarly to 1,25(OH)$_2$D, was able to blunt the lipopolysaccharide-mediated pro-inflammatory effect in human adipocytes[37]. Using a microarray approach, we recently demonstrated that 1,25(OH)$_2$D was able to down-regulate a large set of chemokines[80] induced by inflammatory stimulus[81] in human and murine adipocytes. This effect was accompanied by a reduction of macrophage migration mediated by an adipocyte-conditioned medium[80].

These anti-inflammatory properties of 1,25(OH)$_2$D have also been established in several types of immune cells found in the adipose tissue, including lymphocytes and macrophages[78]. Furthermore, 1,25(OH)$_2$D reduced macrophage-induced inflammatory response in human adipocytes via inhibition of NF-κB and mitogen-activated protein kinase activation together with monocyte migration mediated by the adipocyte-conditioned medium[82]. These data suggest that VD not only blunts adipocyte response to inflammatory stimulus, but also interferes with macrophage/adipocyte cross-talk, a key element of the propagation of metabolic inflammation in obesity[83].

The anti-inflammatory effect of VD in adipose tissue has also been observed *in vivo*. It was reported that dietary treatment with 1,25(OH)$_2$D-reduced IL6 protein content in the epididymal adipose tissue of obese mice[84], whereas feeding with a VD-deficient high-fat diet was found to increase Il-6 expression in rat adipose tissue[85]. In addition, we recently demonstrated that VD supplementation of high-fat diet reduced the expression of proinflammatory cytokines and chemokines and inhibited macrophage infiltration in the adipose tissue of obese mice[80]. Similar effects of VD supplementation were found in an acute inflammation model (intraperitoneal injection of LPS) where no modification of body weight was measured[80], which strongly suggests that the decreased inflammatory status of adipose tissue observed in obese mice is not only a consequence of reduced fat mass[69] but is also driven by an anti-inflammatory effect of VD *per se*.

To summarise, it has been demonstrated *in vitro* and *in vivo* that VD has a limiting effect on adipose tissue inflammation, acting on both inflammatory status in pre-adipocytes and adipocytes and on leucocyte infiltration.

Energy metabolism in transgenic mice models impacting vitamin D metabolism

Several transgenic mice models generated over the last decade have been used to gain insight into the role of VD metabolism in body weight management and adipose tissue metabolism. Studies using transgenic mouse models have shown that Vdr$^{-/-}$ and Cyp27b1$^{-/-}$ mice (which are unable to synthesise 1,25(OH)$_2$D) are resistant to diet-induced obesity[86,87]. This phenotype was linked to the co-induction of fatty acid β-oxidation and uncoupling proteins in adipose tissue leading to increased energy expenditure in these mice. Conversely, overexpression of human VDR in mouse adipose tissue induced an obese phenotype characterised by increased weight and fat mass due to decreased energy expenditure, reduced fatty acid β-oxidation and lipolysis[88].

Taken together, these data strongly suggest that VDR or 1,25(OH)$_2$D has an impact on overall energy metabolism by acting on adipose tissue biology, but with a number of caveats. First of all, the use of global knockout models makes it difficult to attribute the observed phenotype to a specific tissue. In addition, these mice were fed a rescue diet containing large amounts of calcium and lactose, and calcium is strongly suspected to regulate energy homeostasis and well known to reduce intestinal lipid absorption[89]. Moreover, in wild-type mice, high-calcium rescue diet[86] has been shown to reduce 1,25(OH)$_2$D to extremely low levels similar to Cyp27b1$^{-/-}$ mice[90], making it unlikely that the observed effect on body weight is attributable to 1,25(OH)$_2$D.

In all these animal models ($Vdr^{-/-}$, $Cyp27b1^{-/-}$ and human VDR overexpression), phenotype appears tightly linked to uncoupling protein-1 modulation[86–88]. However, we now know that unliganded VDR can down-regulate uncoupling protein-1[91], which means the VDR knockout could trigger an increase in uncoupling protein-1 leading to obesity resistance independently of plasma or adipose tissue levels of $1,25(OH)_2D$ or other VD metabolites. In this case, the resistance to diet-induced obesity observed in $Vdr^{-/-}$ mice would be only VDR-dependent and not mediated by its ligand[87]. This would also be the case of human VDR overexpression where a down-regulation of uncoupling protein-1 is associated with weight gain[88]. In the case of $Cyp27b1^{-/-}$, the decrease in $1,25(OH)_2D$ plasma levels likely results in a decrease of VDR, since VDR is able to induce its own expression[92], leading to the observed phenotype[86]. The phenotype of these mice models could stem from other mechanisms too, e.g. modification of the bile acid pool, as recently evoked[93].

Further research is clearly needed to provide an explanation (if any) that could reconcile data from transgenic mice and nutritional approaches on the impact of VD on energy metabolism regulation. An important aspect to investigate would be the concentrations of VD and its metabolites in adipose tissue in the different mice models (notably between lean and obese animals) and the regulation of VD metabolism in adipocytes, which remains largely unknown.

Effects of vitamin D on obesity and associated disorders in human studies

Numerous cross-sectional studies have reported a correlation between VD deficiency and obesity. Indeed, serum 25(OH)D is consistently lower in obese than lean individuals[94]. A recent meta-analysis found that prevalence of VD deficiency was 35 % higher in obese and 25 % higher in overweight compared with lean subjects[95]. In addition, 25(OH)D plasma levels are inversely correlated to all the parameters of obesity, including BMI, fat mass and waist circumference[96,97], and increased dietary intake of VD, resulting in higher 25(OH)D plasma levels, is associated with a lower visceral adiposity[98].

The fact that adipose tissue is the main storage site for VD and/or its metabolites in the body has prompted the hypothesis that VD and/or its metabolites gets sequestered in the excess fat mass in obese persons[99]. However, the physiological mechanisms underlying this hypothesis have not been brought forward. Nevertheless, as pointed out in a recent study from Drincic *et al.*[100], it might just be that in individuals with a higher body mass, 25(OH)D is simply diluted in a higher volume, so they would require greater VD input than lean individuals to achieve a sufficient 25(OH)D status. Decreased plasma 25(OH)D levels could also result from a modification in VD metabolism occurring during obesity development. Indeed, modifications in the expression of genes encoding key enzymes of VD metabolism have been reported in the adipose tissue of obese people[38].

The relationship between obesity and $1,25(OH)_2D$ is less clear. Recent studies have reported an inverse relationship between $1,25(OH)_2D$ and BMI and fat mass[101,102] while an older study found a direct relationship[103]. The origin of these inconsistent results is unclear but could stem from methodological bias in calcitriol measurement or else indicate that serum $1,25(OH)_2D$ displays an inter-individual variability that is not adiposity-related. Also, no data are available on $1,25(OH)_2D$ concentration in adipose tissue, which could be the critical effector in relation to obesity.

Several recent prospective studies have reported that low 25(OH)D plasma levels were associated with higher prevalence of obesity in adults[104,105], children[106] and elderly women[107]. Low 25(OH)D was also associated with higher 5-year waist circumference[108]. Low VD intake has also been considered as predictor of obesity[109]. Mechanistic explanations to these prospective observations are scarce. Indeed, the impact of VD on the regulation of energy metabolism in human subjects is still unclear. Baseline 25(OH)D was positively correlated to diet-induced thermogenesis[110], which could at least partly explain why low 25(OH)D concentrations chronically modify energy balance. However, Boon *et al.* reported no effect of VD supplementation on energy expenditure and fat metabolism, but it should be noted that their supplementation regime was for 1 week only[111].

Intervention studies have been designed to study the causality between low plasma 25(OH)D levels and obesity. Except for Salehpour *et al.*[112] who reported that VD supplementation decreased body fat mass in healthy overweight and obese women, most randomised clinical trials have failed to demonstrate any benefit of VD supplementation in terms of weight loss[113–115]. These data were recently meta-analysed, and the lack of major effect of VD supplementation on weight loss was confirmed[116]. However, a well-designed randomised controlled trial combining a weight loss programme together with placebo or VD supplementation highlighted that VD supplementation was associated with a significantly higher reduction of BMI and waist circumference in subjects with 25(OH)D levels raised to 80 nM/l[117], suggesting that beneficial effects of the supplementation only kick in with elevated plasma 25(OH)D levels.

A recent study using Mendelian randomisation of several thousands of volunteers of different age, gender and geographical location demonstrated that an increase in BMI could cause a decrease in 25(OH)D status, whereas VD insufficiency would at most result in only very minor effects on obesity[118]. Note that the study only focused on genes related to 25(OH)D status (VDBP, DHCR7, CYP2R1 and CYP24A1) and chosen on the basis of a genome-wide association study[119] that explained 1–4 % of the variation in 25(OH)D concentrations, and so it cannot be ruled out that the use of polymorphisms present in other VD-related genes (such as VDR or CYP27B1) as instrumental variables of the Mendelian randomisation may lead to divergent results. Another large-scale study including Chinese women failed to show an association between obesity and genetic variants in genes in the pathway of VD metabolism[120], whereas

several other studies found associations, notably between VDR polymorphisms and adiposity[121]. The origin of these discrepancies has not been established but could stem from ethnic specificities, since it is well established that ethnicity is a significant determinant of plasma 25 (OH)D[122].

Taken together, these data suggest that VD may limit weight gain in human subjects even if it has no clear effect on weight reduction in obese or overweight people. However, clinical trials are needed to provide conclusive proof and to define the mechanisms governing this potential preventive effect of VD against obesity development. The question of the impact of VD supplementation and polymorphisms present in genes coding for key enzymes of VD metabolism remains unclear, and will require further investigations. Equally useful would be studies on the impact of VD supplementation on adipose tissue biology in well-designed randomised clinical trials that should follow several criteria, chiefly low baseline 25 (OH)D, use of doses necessary to raise 25(OH)D concentrations up to 75–80 nM/l, and genotyping subjects.

Conclusions

Recent data from different research groups are converging to highlight the impacts of VD on adipose tissue/adipocyte biology. One of the best-documented effects is the ability of VD to limit the expression for inflammatory markers in adipose tissue and adipocytes. However, several key points urgently warrant further investigation, chiefly VD metabolism and its regulation in adipose tissue, which needs to be clarified, especially in the context of physiopathological disorders such as obesity. The last 5 years have seen some very interesting data in transgenic mice and rodents subjected to VD supplementation or restriction, but still without convergent findings. It is urgent to identify the origin of these discrepancies, where a key factor could be the quantification of VD metabolites in adipose tissue. Randomised clinical trials have been performed, but again the results remain contrasted, leaving persistent uncertainty over a beneficial role of VD supplementation on weight management. However, results from a recent intervention study suggest that a minimum level of plasma 25(OH)D has to be reached in order to elicit a beneficial effect from supplementation, since only subjects that became replete showed improvements in several parameters[116]. This observation is consistent with successful investigations in mice where plasma 25(OH)D levels were inflated. This same study also demonstrates that it is important to stratify the data according to 25(OH)D status in order to explore and interpret differential physiological responses at the end of the supplementation period, an approach that should be used in future clinical studies. Recent prospective studies have presented low plasma 25(OH)D levels as a predictor of body weight gain, suggesting that VD may limit the prevalence of obesity. If these observations are confirmed in dedicated well-designed clinical studies, it could pave the way to the use of VD in preventive nutrition to limit the development of obesity and associated disorders, notably by reducing the inflammatory status.

To conclude, even if preclinical studies have provided strong support for beneficial impacts of VD supplementation, well-designed clinical studies are urgently needed to demonstrate real valuable utility for limiting obesity in human subjects.

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Conflict of Interest

None.

Authorship

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ARTICLE 2 : Vitamin D limits inflammation linked microRNA expression in adipocytes in vitro and in vivo: A new mechanism of the regulation of inflammation by vitamin D.

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Il est maintenant bien établi que la vitamine D présente un effet anti-inflammatoire au niveau adipocytaire. Cet effet a été évalué au niveau de l'expression des cytokines proinflammatoires et des chimiokines. A ce jour, aucune étude n'a décrit l'effet de cette vitamine sur l'expression des miRs au niveau des adipocytes soumis à un stress inflammatoire mimant l'obésité.

Au cours de cette étude, nous avons eu recours à une approche ciblée (miRNA PCR arrays) permettant de quantifier l'expression de 84 microARNs dans des primocultures d'adipocytes humains prétraités avec la forme active de la vitamine D ($1,25(OH)_2D$) et incubés ou non avec du TNFα. Ces effets ont été également étudiés dans des adipocytes murins 3T3-L1. Pour étudier l'effet de la vitamine D sur l'expression des miRs in vivo, nous avons eu recours à un modèle d'inflammation chronique (trois lots d'animaux ont été soumis soit à un régime témoin, soit à un régime High Fat soit à un régime High Fat supplémenté en vitamine D pendant 10 semaines).

Dans un dernier temps, les mécanismes moléculaires ont été décryptés dans les adipocytes 3T3-L1 et le modèle de souris transgéniques aP2-p65.

PRINCIPAUX RESULTATS DE L'ETUDE

L'analyse des miRNA PCR arrays réalisée sur des adipocytes humains prétraités avec la forme active de la vitamine D ($1,25(OH)_2D$) et incubés avec du TNFα a permis de montrer que trois miRs sont régulés positivement par le TNFα. Parmi ces miRs, on retrouve le miR155 mais également deux autres miRs. Il s'agit des miR-146a et miR-150. L'expression de ces trois miRs est fortement limitée par le prétraitement avec la 1,25(OH)2D. Ces résultats ont été reproduits sur des cultures d'adipocytes murins pour l'expression des miR-146a et miR155. De plus, l'effet de la vitamine D sur l'expression des miR-146a, miR-150 et miR-155 a été confirmé dans le modèle murin d'inflammation chronique supplémenté en vitamine D.

En ce qui concerne les mécanismes moléculaires mis en jeu dans la régulation de l'expression des miRs par la vitamine D au niveau des adipocytes murins, nous avons montré que la limitation de l'expression des miR-146a et miR-155 par la vitamine D est médiée par une inhibition de la voie de signalisation NF-κB.

Nous avons été en mesure de mettre en évidence que la vitamine D a la capacité de diminuer l'expression des miRs in vitro et in vivo, suggérant ainsi un nouveau niveau de régulation de l'inflammation par la vitamine D. Ces travaux apportent des connaissances nouvelles qui aideront à mieux comprendre les effets potentiellement bénéfiques de la vitamine D sur la prévention de l'obésité et des pathologies associées.

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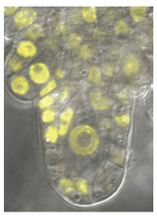

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Research Paper

Vitamin D limits inflammation-linked microRNA expression in adipocytes in vitro and in vivo: A new mechanism for the regulation of inflammation by vitamin D

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Abstract

Inflammation of adipose tissue is believed to be a contributing factor to many chronic diseases associated with obesity. Vitamin D (VD) is now known to limit this metabolic inflammation by decreasing inflammatory marker expression and leukocyte infiltration in adipose tissue. In this study, we investigated the impact of VD on microRNA (miR) expression in inflammatory conditions in human and mouse adipocytes, using high-throughput methodology (miRNA PCR arrays). Firstly, we identified three miRs (miR-146a, miR-150, and miR-155) positively regulated by TNFα in human adipocytes. Interestingly, the expression of these miRs was strongly prevented by $1,25(OH)_2D$ preincubation. These results were partly confirmed in 3T3-L1 adipocytes (for miR-146a and miR-150). The ability of VD to control the expression of these miRs was confirmed in diet-induced obese mice: the levels of the three miRs were increased following high fat (HF) diet in epididymal white adipose tissue and reduced in HF diet fed mice supplemented with VD. The involvement of NF-κB signaling in the induction of these miRs was confirmed in vitro and in vivo using aP2-p65 transgenic mice. Finally, the ability of VD to deactivate NF-κB signaling, via p65 and IκB phosphorylation inhibition in murine adipocyte, was observed and could constitute a driving molecular mechanism. This study

demonstrated for the first time that VD modulates the expression of miRs in adipocytes in vitro and in adipose tissue in vivo through its impact on NF-κB signaling pathway, which could represent a new mechanism of regulation of inflammation by VD.

Keywords

inflammation; vitamin D; adipocyte; miRs; aP2-p65 transgenic mice; NF-κB

Introduction

Adipose tissue was originally considered as a passive reservoir for energy storage, mechanical and heat insulation, and participating to the regulation of thermogenesis [1]. Now, this tissue, and especially visceral adipose tissue, is also known to synthesize and secrete a large variety of pro-inflammatory and anti-inflammatory adipokines, cytokines, and chemokines [2] that act at both autocrine/paracrine and endocrine level [3].

Other markers such as microRNAs (miRs) have been associated with inflammatory response in adipose tissue during obesity [4-6]. miRs are small noncoding RNAs that can modulate transcriptional networks, influencing most of biological processes. miRs regulate gene expression by degrading complementary mRNA targets or by inhibiting translation, and their function depends on the complementarity of sequence with the target mRNA [7]. Several miRs are involved in adipocyte differentiation [8], fat cell behavior, [9] and oxidative stress [4]. The relevance of miRs in adipose tissue biology is increasingly recognized, being intrinsically linked to different pathways, including obesity-related inflammation. This is notably the case for miR-155, which was reported to be increased in inflamed adipocytes [10], but also in subcutaneous adipose tissue biopsies of obese subjects [11], and which displays a strong impact on the regulation of inflammatory tone and adipocyte differentiation. [11]

Similar to the increased pro-inflammatory markers, a low vitamin D (VD) status is common and well documented in obesity [12]. Whether low VD status is the consequence of obesity, or whether this factor accelerates the development of obesity in humans is presently unknown. However, VD insufficiency could influence the development of obesity-related diseases, including inflammation and insulin resistance [12-16]. Indeed, it was shown that $1,25(OH)_2D$ significantly decreased the release of cytokines and chemokines by human preadipocytes [17] and adipocytes [18]. We also demonstrated the anti-inflammatory properties of $1,25(OH)_2D$ in murine and human adipocytes, in both basal and TNFα-stimulated conditions [19]. Similarly, $1,25(OH)_2D$ reduced the expression of cytokines in human adipose tissue biopsies submitted to IL-1β stimulation in vitro [20]. The molecular mechanisms have been investigated, and vitamin D receptor (VDR) and NF-κB signaling pathways and p38 MAP kinases were shown to be involved in 3T3-L1 adipocytes [19] and in human adipocytes [21]. Interestingly, Zoico et al. recently demonstrated that VD, similarly to $1,25(OH)_2D$, was able to blunt the LPS-mediated pro-inflammatory effect in human adipocytes [22]. Using a microarray approach, we recently demonstrated that $1,25(OH)_2D$ was able to downregulate a large set of chemokines in human and murine adipocytes, and

consequently reduced macrophage migration mediated by adipocyte-conditioned medium [23]. Finally, we demonstrated for the first time this effect in vivo, in acute and chronic models of adipose inflammation [23].

In the present study, we aimed to explore the impact of VD on adipose inflammation associated miRs. To this end, we evaluated the impact of $1,25(OH)_2D$ on miR expression in inflammatory conditions in human and mouse adipocytes, using a high-throughput methodology (miRNA PCR arrays). We also confirmed the anti-inflammatory effects of VD in vivo, in a chronic mice model of adipose tissue inflammation, and identified molecular mechanisms involved in miR regulation by VD.

Results

$1,25(OH)_2D$ modulates microRNA expression in human and murine adipocytes

To examine, in detail, the impact of $1,25(OH)_2D$ on the modulation of miRs in human adipocytes submitted to inflammatory stress, human adipocytes primary cell cultures were preincubated with or without $1,25(OH)_2D$ for 24 h followed by incubation with TNFα. Using a miRNA PCR array approach, we quantified 84 miRs and 6 housekeeping genes. Analysis of the results showed that 76 miRs were expressed in adipocytes in basal condition. Among these miRs, only 3 were positively regulated by TNFα in our conditions. As shown in Figure 1, the inflammatory response mediated by TNFα significantly increased the expression levels of miR-146a, miR-150, and miR-155 (4.3-, 1.9-, and 2.1-fold, respectively; Figure 1). Interestingly, their expression in adipocytes preincubated with $1,25(OH)_2D$ in inflammatory conditions only increased by 1.8-, 0.9-, and 1.3-fold, respectively, compared with control condition (Figure 1).

Our results were partly confirmed in 3T3-L1 adipocytes, where the levels of miR-146a and miR-155 increased in TNFα-treated cells (by 6.7- and 8-fold, respectively) and in the adipocytes pretreated with $1,25(OH)_2D$ and incubated with TNFα (by 2.1- and 6.5-fold, respectively; Figure 2). No effect on miR-150 was observed in this cell model.

Vitamin D_3 supplementation limits microRNA expression in epididymal white adipose tissue

To further examine the effect of VD on miR expression in vivo, a model of chronic inflammation induced by a high-fat (HF) diet was used. In this preclinical model, the induction of miR-146a, miR-150, and miR-155 levels was observed after 10 weeks of HF diet (by 2.4-, 1.4- and 1.5-fold, respectively) compared with control mice (Figure 3). Interestingly,

VD supplementation for 10 weeks strongly limited the expression of these three miRs in epididymal adipose tissue (1.1-, 1.1-, and 1.1-fold, respectively; Figure 3).

1,25(OH)$_2$D limits microRNA expression through NF-κB signaling in 3T3-L1 adipocytes.

To identify signaling pathways involved in miR-146a and miR-155 regulation by TNFα, 3T3-L1 cells were incubated with specific inhibitors of MAP kinases (JNK, p38) and NF-κB signaling, and then stimulated or not with TNFα. NF-κB signaling inhibition resulted in a significant decrease in miR-146a and miR-155 expression (by 2.7- and 1.6-fold compared with TNFα condition; Figure 4A), whereas no effects of JNK and p38 inhibitors were observed. To confirm the role of NF-κB in miR-146a and miR-155 regulation in vivo, we used a transgenic model overexpressing NF-κB p65 subunit in adipose tissue (aP2-p65 mice [24]). Results showed that miR-146a and miR-155 were significantly increased in epididymal adipose tissue of aP2-p65 mice compared with controls (by 2.2- and 10-fold, respectively; Figure 4B). All together, these data strongly support the specific role of NF-κB on the regulation of these two miRs.

Thus, we examined the impact on NF-κB signaling by 1,25(OH)$_2$D preincubation (10 nM and 100 nM) for 24 hours followed by a 24-hour incubation with TNFα. To this end, the phosphorylation levels of p65 and IκB were quantified by ELISA. Expectedly, the phosphorylation levels of p65 and IκB were significantly increased into 3T3-L1 adipocytes incubated with TNFα (by 4.1- and 3.6-fold, respectively), whereas incubation with 1,25(OH)$_2$D strongly limited the phosphorylation of p65 and IκB, suggesting that 1,25(OH)$_2$D reduced NF-κB activation in 3T3-L1 adipocytes (Figure 4C).

Discussion

Our results indicate that TNFα-mediated inflammation increased the production of miR-146a, miR-150, and miR-155 in adipocytes. We also reported a beneficial role of VD or its active form (1,25(OH)$_2$D) on the expression of these miRs in human and murine adipocytes, but also in inflamed adipose tissue of mice. Molecular mechanisms for these effects were investigated and the NF-κB signaling pathway is suggested to be involved in miR regulation and to be targeted by 1,25(OH)$_2$D. If the anti-inflammatory effect of VD has largely been reported based on cytokines and chemokines expression in adipocytes, as described in the introduction; its impact regarding its ability to modulate inflammatory-linked miRs has never been reported.

In this study, we identified miR-146a, miR-150, and miR-155 as adipocyte inflammatory stimulus modulated miR. These miRs have been largely suspected to be involved in inflammatory tone regulation; indeed, miR-155, which is described as a typical multifunctional microRNA [25], has been associated with the regulation of different immune-related processes, such as hematopoiesis [26], innate immunity [27], cancer, [28] and B-cell and T-cell differentiation [29]. In the context of obesity onset and/or obesity comorbidities, we have recently shown that miR-155 was induced in adipocytes submitted to inflammatory stress and in subcutaneous adipose tissue biopsies of obese subjects [11]. We also showed that this miR induced inflammatory response, chemokine expression, and macrophage migration in 3T3-L1 adipocytes [11]. A recent study also demonstrated that the deletion of miR-155 in female mice prevented diet-induced obesity, improved insulin sensitivity and energy uncoupling machinery, and abrogated HF diet-induced adipocyte hypertrophy and white adipose tissue inflammation [30]. Together, these data confirm the importance of miR-155 in adipose tissue function, notably in the control of inflammation.

To the best of our knowledge, no specific effect of miR-146a in adipocyte or adipose tissue biology has been reported. However, in inflammatory context, it has been reported that miR-146a is involved in the control of innate immunity. Indeed, miR-146a was found to be inducible upon stimulation with LPS in a NF-κB-dependent manner in human monocytes [31]. Such induction of miR-146a could contribute to the establishment of endotoxin tolerance in monocytes and to the regulation of TNFα production [32]. In addition, exposure of human lung alveolar epithelial cells resulted in a pronounced increase of miR-146a levels, that in turn induced a decrease of IL-8 and RANTES chemokines [33]. Finally, miR-146a deficiency during diabetes led to increased expression of M1 activation markers and pro-inflammatory cytokines and suppression of M2 markers in macrophages [34]. Together, these data strongly suggest that miR-146a displays an anti-inflammatory effect. However, its role in adipose tissue will require further investigation.

Regarding miR-150, inflammatory or anti-inflammatory properties are less clear. A recent study demonstrated that miR-150 could physiologically modulate metabolic activities and inflammatory response both in cells and animals by regulating lipid metabolism and inflammatory response. Indeed, overexpression of miR-150 in macrophages resulted in an increase in lipid accumulation, associated with an elevated expression of several pro-inflammatory cytokines [35]. Conversely, when miR-150 knockout mice were challenged with a HF diet, these mice presented reduced whole body weight with less fat accumulation, improved systemic glucose tolerance, and insulin sensitivity [35]. In contrast, another

study reported that miR-150 knockout mice showed exacerbated obesity-associated tissue inflammation and systemic insulin resistance [36]. Such versatility has already been suggested in human cancer, where the role of miR-150 is context-dependent, i.e., this miR can have either oncogenic or tumor suppressor activity in cells that originate from different tissues [37]. Thus, its specific role in adipose tissue and, notably, in the control of inflammatory tone will require further investigation.

To identify molecular mechanisms involved in miR regulation, several approaches were combined. First, we demonstrated that miR-146a and miR-155 regulations were primarily NF-κB-dependent in 3T3-L1 adipocytes, through the use of specific NF-κB inhibitor that blunted TNFα-mediated induction of these two miRs. The involvement of this signaling pathway was also confirmed in vivo through the use of aP2-p65 transgenic mice that display specific adipose tissue overexpression of p65 [24] and that presented a strong induction of these two miRs. Based on this putative role of NF-κB in the induction of miR-146a and miR-155, the effect of the VD on the phosphorylation of two intermediates of the NF-κB signaling pathway (p65 and IκB) has been investigated. As previously demonstrated [19, 23], we reported that $1,25(OH)_2D$ has a strong limiting effect on NF-κB signaling in 3T3-L1 adipocytes through the reduction of the phosphorylation levels of p65 and IκB. This effect could be related to the ability of $1,25(OH)_2D$ to bind VDR and to interact with IκB kinase (IKK) [38] or could result from the induction of phosphatases involved in the dephosphorylation of p65 and IκB. All together these data strongly suggested that the inhibition of miRs by VD is largely mediated by its ability to reduce NF-κB signaling, which is consistent with data generated in macrophages [39]

Interestingly, we observed both in vitro (in human and murine adipocytes) and in vivo (in mice) global upregulation of the miRs in pro-inflammatory conditions, suggesting that these three miRs, independently of their inflammatory effect (pro- or anti-), actively participated in inflammatory process in adipocytes and adipose tissue. We also observed that VD, through the downregulation of these three miRs, displayed an overall blocking effect of the inflammatory pathway in adipocytes. The modulation of miRs by VD is reported here for the first time in the context of obesity-associated comorbidities; similar ability has been widely described in cancer context. Indeed, differential regulation of miR expression profiles by $1,25(OH)_2D$ has been demonstrated in prostate cancer cells [40], bladder cancer cells, [41] and in lung cancer. [42]

These original observations not only reinforce the role of VD as an anti-inflammatory agent in adipose tissue and adipocytes, but also demonstrate the relevance of miR regulation as a new anti-inflammatory mechanism in adipose tissue/adipocytes, which could represent an interesting new target to limit metabolic inflammation.

Material and Methods

Reagents

Dulbecco's modified Eagle's medium (DMEM) was purchased from Life Technologies (Cergy Pontoise, France); fetal bovine serum (FBS) was obtained from PAA Laboratories (Les Mureaux, France). Isobutylmethylxanthine, dexamethasone, and insulin were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). TRIzol reagent was obtained from Life Technologies (Saint Aubin, France). QuantiTect SYBR Green PCR Master Mix, miScript Universal Primer and miScript Primer assays were purchased from Qiagen (Courtabœuf, France).

Cell culture

Adipocyte cells were grown at 37°C in a 5% CO_2 humidified atmosphere. The human preadipocytes (3 independent cultures) were obtained from Promocell and cultured with preadipocyte differentiation medium, according to the manufacturer's instructions. Mature adipocytes (day 12) were incubated with 1,25$(OH)_2$D (100 nM, 24 h) followed by a 24-hour incubation with TNFα (15 ng/mL). Experiments were performed in triplicate, on 3 independent cultures.

The 3T3-L1 cells (ATCC, Manassas, VA) were seeded in 3.5 cm-diameter dishes at a density of 15×10^4 cells/well, and grown in DMEM supplemented with 10% FBS, at 37°C, as previously reported [43]. To induce differentiation, two-day postconfluent 3T3-L1 preadipocytes (day 0) were stimulated for 72 h with 0.5 mM isobutylmethylxanthine, 0.25 µmol/L dexamethasone and 1 µg/mL insulin in DMEM supplemented with 10% FBS. The cultures were then treated with DMEM supplemented with 10% FBS and 1 µg/mL insulin. The mature adipocytes (day 8) were incubated with 1,25$(OH)_2$D (100 nM, 24 h) followed by a 24-hour incubation with TNFα (15 ng/mL). Experiments were performed in triplicate, on 3 independent cultures.

3T3-L1 adipocytes incubations

To identify signaling pathways involved in microRNA regulation, 3T3-L1 cells were treated with specific inhibitors of mitogen activated protein (MAP) kinases (JNK, p38) and NF-κB signaling [JNK inhibitor II (10 μM), SB 202190 (20 μM) and BAY 117082 (10 μM), respectively] for 1 h (all obtained from Calbiochem, Merck Millipore, Darmstadt, Germany) and then stimulated or not with TNFα (15 ng/mL) for 24 h. All the treatments were performed on day 8.

NF-κB activation

The levels of p65 (Ser536) and IκBα (Ser32/36) phosphorylation were quantified using the ELISA Instant One kit according to the manufacturer's instructions (eBiosciences SAS, Paris, France).

miRNA PCR arrays

miRNA PCR arrays (Qiagen, Courtabœuf, France) were used to quantify miRs extracted from human culture adipocytes, according to the manufacturer's instructions. Reactions were performed in a 12.5 μL volume containing 6.25 μL 2X QuantiTect SYBR Green PCR Master Mix (Qiagen, Courtabœuf, France), 1.25 μL 10X miScript Universal Primer (Qiagen, Courtabœuf, France). After an initial incubation step of 15 min at 95°C, amplification reaction was performed in 40 cycles comprising 3 steps (94°C, 15 sec, 55°C, 30 sec, and 70°C, 30 sec). For each condition, the expression was quantified in duplicate and the SNORD68 was used as the endogenous control in the comparative cycle threshold (C_T) method.

RNA isolation and qPCR

Total cellular RNA was extracted using TRIzol reagent, according to the manufacturer's instructions. To quantify miR-146a, miR-150, and miR-155, cDNAs were first synthesized from 1 μg of total RNA in 20 μL using 5X miScript Hiflex Buffer, 10X nucleic mix and miScript reverse transcriptase according to the manufacturer's instructions (Qiagen). Real-time quantitative RT-PCR analyses were performed using the Mx3005P Real-Time PCR System (Stratagene, La Jolla, CA), as previously described [44]. Reactions were performed in a 12.5 μL volume containing 6.25 μL of 2X QuantiTect SYBR Green PCR Master Mix (Qiagen), 1.25 μL of 10X miScript Universal Primer (Qiagen), 1.25 μL of 10X miScript Primer Assay [Mm_miR146_1 miScript Primer Assay, Mm_miR-150_1 miScript Primer Assay, Mm_miR-155_1

miScript Primer Assay and Hs_SNORD68 miScript Primer Assay (Qiagen)] and 2.5 µL of RNase-free water. For each condition, the expression was quantified in duplicate, and the SNORD68 was used as the endogenous control in the comparative cycle threshold (C_T) method.

Animal experiments

The protocol was approved through the local ethics committee. Six-week-old male C57BL/6J mice were obtained from Janvier (Le Genest Saint Isle, France). The mice were fed ad libitum (chow diet A04, Safe, Augy, France), with full access to drinking water. The animals were maintained at 22°C under a 12 h light: 12 h dark cycle with a 50% humidity level.

To assess the impact of vitamin D (cholecalciferol) on miRs expression, three experimental groups of mice were fed ad libitum with a low (10% of total energy as fat; TD06416; Harlan, Indianapolis, Indiana, USA) or HF diet, providing 45% of total energy as fat (TD06415, Harlan, Indianapolis, Indiana, USA), supplemented (3000 IU/kg of body weight, i.e., around 90 UI/j/mouse, Sigma-Aldrich, Saint Quentin Fallavier, France) or not (300 IU/kg of body weight, i.e., around 9 UI/j/mouse) with cholecalciferol. Mice (10 per group) were assigned into one of the three experimental groups depending on the diet, i.e., control, HF, and HF plus vitamin D (HFVD), for 10 weeks, as previously described [45]. Epidydimal adipose tissue was collected and stored at -80°C.

aP2-p65 mice were generated on the C57BL/6J background, as described elsewhere [24, 46]. Mice were housed in the animal facility at the Pennington Biomedical Research Center with a 12:12-h light-dark cycle and constant temperature (22-24°C). Male mice were fed with a chow diet (MF 5001, 11% calorie in fat) and the epididymal fat tissue was collected at 20 weeks. The mice were housed at four per cage with free access to water and diet.

Statistical analysis

Data are expressed as mean ± SEM. Significant differences between the control and treated group were determined using ANOVA, followed by the PLSD Fischer post hoc test using Statview software, and $P<0.05$ was considered statistically significant. Significant differences between two groups were determined using t test.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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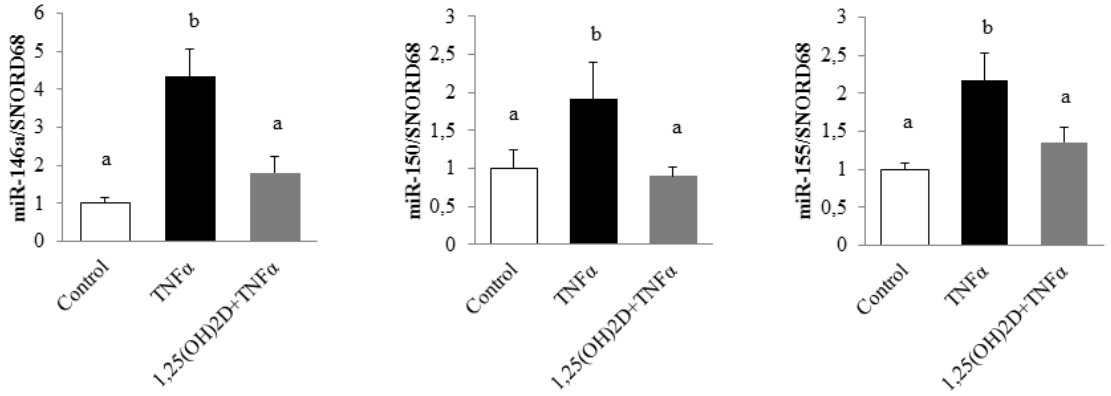


Figure 1. 1,25(OH)$_2$D limits microRNA expression levels in human adipocytes. Human adipocytes were preincubated with 1,25(OH)$_2$D (100 nM) for 24 h followed by a second 24-h incubation with TNFα (15 ng/mL). miR expression levels were quantified through miRNA PCR arrays approach. SNORD68 was used as the endogenous control. The values are presented as means ± SEM. Bars not sharing the same letters are significantly different ($P<0.05$). Experiments were performed in triplicate, at least 3 independent times.

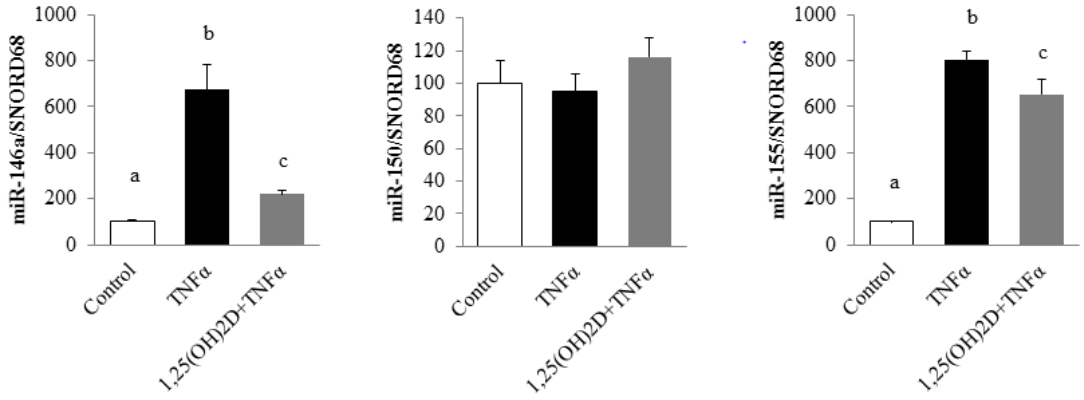


Figure 2. 1,25(OH)$_2$D limits microRNA expression levels in murine adipocytes. 3T3-L1 adipocytes were preincubated with 1,25(OH)$_2$D (100 nM) for 24 h followed by a second 24-h incubation with TNFα (15 ng/mL). Expression levels of miR-146a and miR-155 were quantified by qPCR in 3T3-L1 adipocytes. SNORD68 was used as the endogenous control. Values are presented as means ± SEM. Bars not sharing the same letters are significantly different ($P<0.05$). Experiments were performed in triplicate, at least 3 independent times.

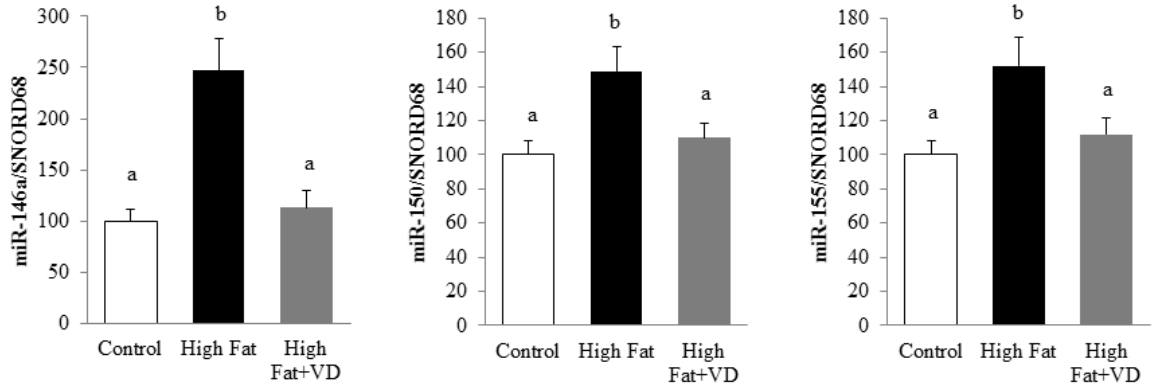
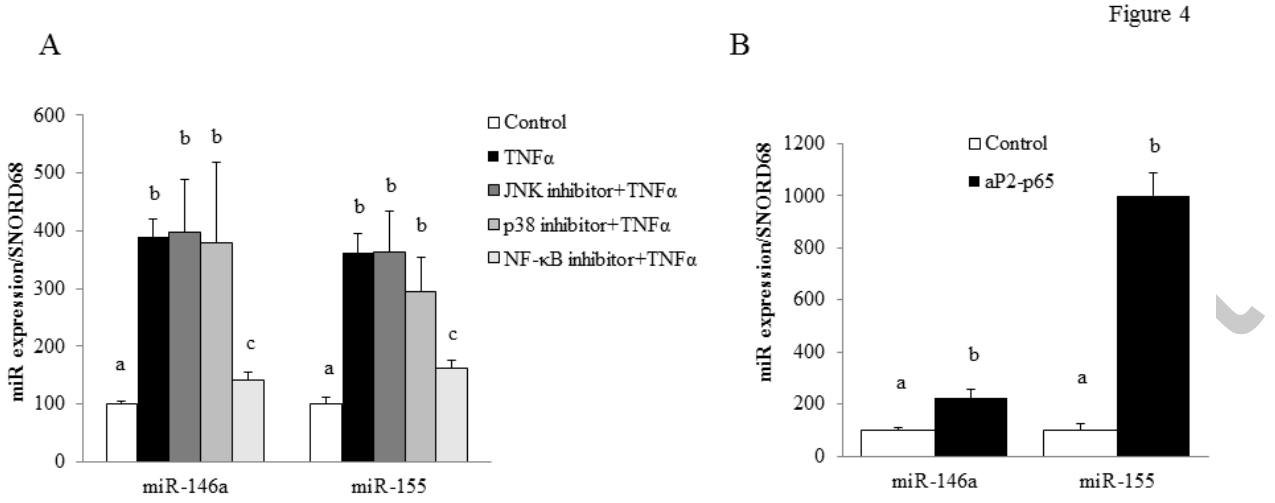


Figure 3. Vitamin D limits miR-146a, miR-150, and miR-155 expression in mice epididymal adipose tissue. The expression levels of miR-146a, miR-150, and miR-155 were quantified through qPCR in epididymal adipose tissue of mice fed standard, high fat (HF) diet or VD-supplemented HF diets for 10 weeks (n = 10) and expressed relative to SNORD68 in the white adipose tissue. Data are expressed as relative expression ratios. Values are presented as means ± SEM. Bars not sharing the same letters are significantly different ($P<0.05$).



C

	Control	1,25(OH)$_2$D (10 nM)	1,25(OH)$_2$D (100 nM)	TNFα	1,25(OH)$_2$D (10 nM) +TNFα	1,25(OH)$_2$D (100 nM) +TNFα
p65 phosphorylation	0.61±0.01 ^{a}	0.87±0.014 ^{a}	0.73±0.01 ^{a}	2.53±0.013 ^{b}	1.14±0.008 ^{a}	1.27±0.025 ^{a}
IκB phosphorylation	0.61±0.007 ^{a}	0.44±0.005 ^{a}	0.62 ±0.013 ^{a}	2.2±0.021 ^{b}	0.99±0.01 ^{a}	0.85±0.01 ^{a}

Figure 4. 1,25(OH)$_2$D limits NF-κB activation. A. 3T3-L1 cells were treated with specific inhibitors of MAP kinases (JNK, p38) or NF-κB signaling [(JNK inhibitor II (10 µM), SB 202190 (20 µM) and BAY 117082 (10 µM), respectively] for 1 h and then stimulated with TNFα (15 ng/mL) for 24 h, and miR-146a and miR-155 expression were measured by qPCR. SNORD68 was used as the endogenous control. The data are expressed as relative expression ratios. Values are presented as means ± SEM. Bars not sharing the same letters are significantly different ($P<0.05$). Experiments were performed in triplicate, at least 3 independent times. **B.** Expression of miR-146a and miR-155 were quantified by qPCR in epididymal adipose tissue of aP2-p65 transgenic mice. SNORD68 was used as the endogenous control. Data are expressed as relative expression ratios. The values are presented as means ± SEM. Bars not sharing the same letters are significantly different ($P<0.05$). **C.** 3T3-L1 cells were preincubated with 1,25(OH)$_2$D (10 and 100 nM) for 24 h in dose-dependent manner and incubated with TNFα (15 ng/mL) for 5 min. Phosphorylation levels of the NF-κB subunits (p65 and IκB) were evaluated using ELISA. Data are expressed as relative expression ratios. Values

are presented as means ± SEM. Values not sharing the same letters are significantly different ($P<0.05$). Experiments were performed in triplicate, at least 3 independent times.

ARTICLE 3 : Gene expression pattern in response to cholecalciferol supplementation highlights cubilin as a major protein of 25(OH)D uptake in adipocytes and male mice white adipose tissue.

En révision à Endocrinology.

Il est maintenant établi que la vitamine D, ou cholécalciférol, participe à la régulation transcriptionnelle des enzymes impliquées dans son propre métabolisme au niveau du rein et du foie dans une moindre mesure. Cependant, aucune donnée n'est disponible à ce jour concernant l'impact de la vitamine D sur cette régulation transcriptionnelle dans le tissu adipeux blanc, bien que celui-ci soit son site principal de stockage.

Dans un premier temps, nous avons analysé l'expression des ARNm des différents acteurs impliqués dans le métabolisme de la vitamine D au niveau du tissu adipeux de souris supplémentées avec du cholécalciférol pendant 4 jours.

Dans un deuxième temps, nous avons confirmé au niveau cellulaire la régulation de l'expression de la cubiline mise en évidence in vivo et en avons étudié le mécanisme moléculaire mise en jeu.

Enfin, l'implication de la cubiline dans l'absorption de la 25(OH)D au niveau des adipocytes a été étudiée. Pour ceci, nous avons eu recours à un modèle d'adipocytes 3T3-L1 transfectés avec des siARN dirigés contre la cubiline, ou traités avec un inhibiteur spécifique puis incubées avec de la 25(OH)D radiomarquée.

PRINCIPAUX RESULTATS DE L'ETUDE

La supplémentation des souris avec du cholécalciférol pendant 4 jours a permis de mettre en évidence que le niveau d'expression des ARNm de Cyp24a1, Cyp27a1 et cubiline était régulé négativement au niveau du tissu adipeux épidydimal. Ces résultats ont été validés dans des adipocytes murins 3T3-L1 incubés en présence de la forme active de la vitamine D.

Une attention particulière a été consacrée à la régulation de l'expression de la cubiline. La régulation négative de l'ARNm de la cubiline observée dans le tissu adipeux epidydimal et dans les adipocytes murins 3T3-L1 a été confirmée au niveau protéique dans le tissu adipeux epidydimal des souris supplémentées en cholécalciférol pendant 4 jours et au niveau de l'ARNm dans des adipocytes primaires humains traités avec la $1,25(OH)_2D$, ainsi que dans des adipocytes 3T3-L1 traités avec un agoniste de VDR (le récepteur nucléaire impliqué dans la signalisation de la vitamine D) (EB1089). De plus, l'utilisation d'ARN interférents ciblés contre VDR a démontré l'implication de ce dernier dans cette régulation.

Enfin, l'implication de la cubiline dans l'absorption de 25(OH)D au niveau des adipocytes murins 3T3-L1 a été démontrée par l'utilisation d'un modèle cellulaire traité avec de la 25(OH)D radiomarquée et co-traité avec soit un inhibiteur chimique (RAP) et soit des ARN interférents ciblés contre cubiline.

Gene expression pattern in response to cholecalciferol supplementation highlights cubilin as a major protein of 25(OH)D uptake in adipocytes and male mice white adipose tissue.

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Abbreviated title: Cholecalciferol impacts 25(OH) uptake in adipocytes.

Key terms: vitamin D, metabolism, adipose tissue, adipocyte, nutrition, cubilin, VDR

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Abstract

It is well established that the active form of vitamin D, i.e. $1,25(OH)_2D$, regulates the expression of genes involved in its own metabolism and transport in the kidney, and possibly in the liver. However, little is known about the transcriptional impact of cholecalciferol supplementation on white adipose tissue (WAT) and adipocytes, which are a major site of vitamin D and 25(OH)D storage in the organism. To fill this gap, we investigated the impact of cholecalciferol supplementation in WAT via a panel of genes coding for enzymes and proteins involved in vitamin D metabolism and uptake.

Mice supplemented with cholecalciferol (15 000 IU/kg of body weight/day) for 4 days showed decreased mRNA levels of proteins involved in cholecalciferol metabolism (Cyp24a1, Cyp27a1) and decreased cubilin mRNA levels in WAT. These data were partly confirmed in 3T3-L1 adipocytes incubated with $1,25(OH)_2D$. The down-regulation of cubilin mRNA observed in WAT and in 3T3-L1 was confirmed at protein level in WAT and at mRNA level in human primary adipocytes. VDR agonist (EB1089) and RNA interference approaches demonstrated that VDR was involved in this regulation. Furthermore, chemical inhibitor and by RNA inference analysis demonstrated that cubilin was involved in 25(OH)D uptake by adipocytes.

This study established an overall snapshot of the genes regulated by cholecalciferol in mouse WAT and cell-autonomously in adipocytes. We highlighted that the regulation of cubilin expression is mediated by a VDR-dependent mechanism, and we demonstrated that cubilin is involved in 25(OH)D uptake by adipocytes.

Introduction

Vitamin D, or calciferol, is a hormone that is synthesized in the epidermis after exposure to UVB radiation or can be obtained from eating fatty fish (1,2). After intestinal uptake (3), dietary vitamin D reaches the bloodstream. Both dietary and endogenous cholecalciferol undergo their first hydroxylation in the liver by 25-hydroxylases including Cyp2r1—although Cyp27a1, Cyp3a11 and Cyp2j6 are also involved (4)—to produce 25-hydroxyvitamin D (25(OH)D), the major circulating form of vitamin D (5). 25-hydroxylation is generally regarded as poorly regulated (6), even if 25-hydroxylase activity was decreased by $1,25(OH)_2D$ in rat liver (7). In plasma, calciferol and 25(OH)D are principally bound to the vitamin D-binding protein (DBP, encoded by the Gc gene) [87% of 25(OH)D], their transport protein, and albumin [13% of 25(OH)D], although a small quantity remains unbound [<1% of 25(OH)D] (8,9). 25(OH)D is taken up by the kidney, where a fraction is 1α-hydroxylated by Cyp27b1 to produce $1,25(OH)_2D$, the active form of vitamin D. This uptake is mediated by the megalin/cubilin complex (10), which involves other proteins such as disabled 2 (Dab2) (11) and amnionless (Amn) (12). CYP27B1 activity in the kidney is positively regulated by parathyroid hormone and low calcium levels and deactivated by fibroblast growth factor 23 and $1,25(OH)_2D$ itself through a negative feedback mechanism (13,14).

In target tissues, 25(OH)D and $1,25(OH)_2D$ can be catabolized by 24-hydroxylases (Cyp24a1) to generate inactive metabolites (15). In the kidney, this step is auto-regulated, and cholecalciferol supplementation induced Cyp24a1 expression (16,17). The molecular mechanisms have been unraveled and shown to involve transcriptional regulation mediated by the vitamin D receptor (VDR), which binds $1,25(OH)_2D$ with high affinity. After heterodimerization with the retinoic acid receptor, the resulting complex can bind to vitamin D

response elements (VDRE) in the promoter region of regulated genes, and induce their transcriptional activation or repression (18).

Vitamin D and 25(OH)D are stored mainly in white adipose tissue (WAT), plasma and skeletal muscle (19). The uptake of vitamin D and its metabolites in preadipocytes and skeletal muscle cells has recently been described (20), and involved megalin (21). In adipocytes, vitamin D and 25(OH)D are not only stored in lipid droplets (22) but could also be converted to active metabolites (23,24) that are able to modulate adipocyte biology (25-27). Indeed, adipocytes have been shown to express most of the genes involved in vitamin D metabolism, such as 25-hydroxylases (23,24), 1α-hydroxylase (24, 28), megalin (20), Cyp24a1 and Vdr (23,29). Given that in liver and in kidney, vitamin D regulates its own metabolism at a transcriptional level via its active metabolite $1,25(OH)_2D$, it is highly likely that similar regulations occur in WAT. Nevertheless, $1,25(OH)_2D$-mediated transcriptional regulation of genes involved in vitamin D metabolism in adipocytes and in WAT has only been only partly established so far.

The main goal of this work was to study the overall impact of cholecalciferol supplementation on the regulation of genes involved in its own metabolism and uptake in vivo in WAT and in vitro in adipocytes. This approach brought key insight into the gene expression pattern of cubilin, and we went on to investigate the underlying molecular mechanism involved. We also demonstrated that cubilin is involved in 25(OH)D uptake by adipocytes.

Materials and methods

Reagents – DMEM was obtained from Life Technologies, and fetal bovine serum (FBS) was obtained from PAA Laboratories. Isobutylmethylxanthine, dexamethasone, and insulin were bought from Sigma-Aldrich. TRIzol reagent, random primers, and Moloney murine leukemia virus reverse transcriptase were obtained from Life Technologies. SYBR Green reaction buffer was purchased from Eurogentec (Liege, Belgium). [3H]-25(OH)D (161 Ci/mmol for specific activity) was sourced from PerkinElmer (Waltham, MA).

Animal experiments – The protocol received approval from the local ethics committee. Six-week-old male C57BL/6J mice were obtained from Janvier Labs (Le Genest-Saint-Isle, France), fed ad libitum with control food (chow diet A04 from Safe-diets) and had full access to drinking water. Male mice were used to avoid the cyclic hormonal changes associated with the estrus cycle in female mice. Animals were maintained at 22°C under a 12h/12h light/dark cycle with a 20% humidity level. Mice were supplemented with cholecalciferol (15 000 IU/kg of body weight/day; Sigma-Aldrich, Saint-Quentin-Fallavier, France) for the cholecalciferol group (Cholecalciferol, n=6 mice) or with vehicle alone (olive oil) for control group (Control, n=8 mice) for 4 days, by gavage (total volume of 200 µL), as previously described (30,31). Weight gain was measured daily. After 4 days of treatment, the mice were fasted overnight and blood was collected by cardiac puncture under anesthesia. After euthanasia, tissues (kidney, liver and epididymal WAT) were collected, weighed, and stored at -80°C.

Cell culture and treatment – 3T3-L1 preadipocytes (American Type Culture Collection, VA) were seeded in 3.5 cm-diameter dishes at a density of 15×10^4 cells/well and grown in DMEM supplemented with 10% FBS at 37°C in a 5% CO_2-humidified atmosphere, as previously

111 described (32,33). After two-day confluence, 3T3-L1 (day 0) were stimulated for 48 h with 0.5
112 mM isobutylmethylxanthine, 0.25 µmol/L dexamethasone, and 1 µg/mL insulin in DMEM
113 supplemented with 10% FBS to induce differentiation. The cultures were successively treated
114 with DMEM supplemented with 10% FBS and 1 µg/mL insulin.
115 Human preadipocytes (isolated from female subcutaneous adipose tissue biopsies) supplied by
116 Promocell (Heidelberg, Germany) were cultured and differentiated into adipocytes according
117 to the company's instructions. Briefly, cells were seeded at a density of 5000 cells/cm² in
118 Preadipocyte Growth Medium and grown until confluence, then allowed to differentiate for 3
119 days in Preadipocyte Differentiation Medium. Mature adipocytes were cultivated in Adipocyte
120 Nutrition Medium for another 11 days, as previously reported (34).
121 To examine the regulation of genes coding for proteins involved in cholecalciferol metabolism,
122 both human and murine adipocytes were incubated with 1,25$(OH)_2$D (1, 10 and 100 nM)
123 dissolved in absolute ethanol for 24 hours or with EB1089 (10, or 100 nM), a VDR agonist, for
124 24 hours.
125
126 **RNA interference** – 3T3-L1 differentiated cells seeded in 24 or 12-well plates were transfected
127 with either targeted siRNA (against VDR or cubilin, respectively) or a non-targeting siRNA
128 according to the manufacturer's instructions (Dharmacon, Lafayette, CO) using INTERFERin
129 (Polyplus-transfection) for 24 h, as previously described (30).
130
131 **Uptake of 25(OH)D by adipocytes** –3T3-L1 adipocytes were incubated with [3H]-25(OH)D
132 at a concentration of 11.25 nCi/mL and with 50 nM of non-radiolabeled 25(OH)D (Sigma-
133 Aldrich, St Louis, MO) in DMEM supplemented with 1 µg/mL insulin in the presence of either
134 1.9 µM DBP, 0.125% bovine serum albumin (BSA) or ethanol (control condition). These 3T3-
135 L1 adipocytes were treated with 10 or 100 nM of 1,25$(OH)_2$D or with 100 or 500 nM of

receptor-associated protein (RAP), an inhibitor of the megalin-cubilin complex. After 16 h of incubation, cells were lysed and radioactivity was measured by liquid scintigraphy. The results were expressed as counts per minute (CPM) per well.

RNA extraction and real-time qPCR – Total RNA was extracted from the liver, kidney and epididymal WAT or from cells using TRIzol reagent (Life Technologies, Courtaboeuf, France). One µg of total RNA was used to synthesize cDNAs using random primers and Moloney murine leukemia virus reverse transcriptase (Life Technologies, Courtaboeuf, France). Real-time quantitative PCR analyses were performed using the Mx3005P Real-Time PCR System (Stratagene, La Jolla, CA), as previously described (35). For each condition, expression was quantified in duplicate, and 18S rRNA was used as the endogenous control in the comparative cycle threshold (CT) method (36). Sequences of the primers used in this study are reported in supplemental data (Supplemental Table 1).

Mouse cubilin protein quantification – Quantity of cubilin protein in WAT was determined using a specific ELISA kit (Mouse cubilin, Mybiosource, San Diego, CA) according to the manufacturer's protocol.

In-silico promoter analysis – The MatInspector software implemented in the Genomatix suite (www.genomatix.de) was used to perform in-silico identification of VDR response elements within human and mouse cubilin promoter regions. Briefly, human and murine cubilin promoters were extracted directly from the Genomatix ElDorado Database (www.genomatix.de). These promoter regions correspond to loci identified by their ElDorado reference ID. Response elements were identified on these promoter regions using the MatInspector software workflow (37).

Cholecalciferol, 25(OH)D and 1,25(OH)2D quantification in plasma and WAT – All quantifications were performed using LC-MS/MS as per the protocol below.

Preparation of analytical and deuterated standards – A working solution of deuterated analytes was prepared at 0.02 ng/mL of each internal standard (IS), i.e. d3-cholecalciferol, d3-25(OH)D and d3-1,25(OH)2D). Primary stock solutions of cholecalciferol, 25(OH)D and 1,25$(OH)_2$D standards were prepared at concentrations of 100, 50 and 10 ng/mL, respectively, in ethanol, and stored at -80°C in the dark. Calibration curves were prepared by serial dilution of the three analyte stock solutions to obtain calibration standards from 0 to 75 ng/mL, then adding 1.5 µL of the working solution of deuterated analytes to each dilution. After complete evaporation of solvent, derivatization was performed. A one-step derivatization was employed to improve the ionization efficiency of the metabolites using Amplifex diene as reagent (38). Amplifex (30 µL) was added to the dried sample above, vortexed for 15 s, and incubated for 30 min at ambient temperature. Next, 30 µL of deionized water was added, vortexed for 15 s, and transferred for LC injection. Calibration curves were plotted as peak area ratio of the vitamin D metabolite to the respective IS versus a range of analyte concentrations.

Preparation of plasma – Sample preparation was adapted from Wang et al. (39). As cholecalciferol and its metabolites are light-sensitive, the extraction procedure was conducted under low light. After thawing on ice, mice plasmas were centrifuged at 11,000 rpm for 15 min at 4°C, then 100 µL of each sample was transferred to a glass test tube containing 10 µL of deuterated standard working solution. Proteins were precipitated by adding acetonitrile (ACN), vortex-mixed, and centrifuged for at 3,000 g for 10 min. The supernatant was moved to another glass tube, and the volume was reduced to half under a nitrogen stream. Then, 5 mL of ethyl acetate was added to the solution for liquid-liquid extraction. After shaking vigorously, samples were centrifuged at 590 g for 20 min, and the upper organic layer was transferred to a fresh

186 glass tube and reduced under nitrogen stream. The samples were then derivatized as described
187 above.
188 Epididymal WAT preparation – Sample preparation was adapted from Lipkie et al. (40). First,
189 25 µL of deuterated standard working solution was added to tissue homogenates (50 mg of
190 tissue ground within 1 mL of PBS) in glass test tube. Acetonitrile (ACN) was added, vortex-
191 mixed for 5 min, and centrifuged at 6,000 g for 5 min. Then, methyl tert-butyl ether (MTBE)
192 was added, vortexed for 5 min, centrifuged, and the upper organic layer was collected into a
193 fresh glass tube. The extraction was repeated twice, and the combined supernatants were dried
194 under nitrogen. Oasis HLB SPE cartridges (Waters, Guyancourt, France) were conditioned with
195 ethyl acetate, methanol (MetOH) and H_2O. The sample was reconstituted with 1 mL of MetOH
196 and 1 mL of K_2HPO_4 (0.4 M), and added onto the cartridge. The cartridge was washed with
197 H_2O and 70% MetOH, and then dried for 2 min under vacuum. Tips were washed with ACN,
198 and analytes were eluted with ACN and dried under nitrogen. After complete evaporation of
199 solvent, the samples were derivatized as described above.
200 LC-MS/MS analysis – Accurate mass measurements were performed on the Q-Exactive Plus
201 mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a Heated
202 Electrospray Ionization (H-ESI II) probe. Thermo Xcalibur 3.0.63 software was used for
203 instrument setup, control of the LC-MS system during acquisition, and data treatment. Tune Q
204 Exactive Plus 2.5 software was used for direct control of the mass spectrometer.
205 Samples were injected onto a Hypersil GOLD C18 column (2.1 × 100 mm; Thermo Scientific,
206 Les Ulis, France). Flowrate was 0.4 mL/min, and injection volume was 5 µL. The mobile phase
207 was composed of A = ultrapure water with 0.1% formic acid (FA) (v/v), and B = ACN with
208 0.1% FA (v/v). Starting conditions were A = 70% and B = 30%, held for 4 min. A linear gradient
209 was applied until 10.0 min where A = 35% and B = 65% which was held until 12.0 min, at 14
210 min A=0% and B = 100% until 16 min. Starting conditions were re-implemented at 18 min.

211 The SRM transitions used for quantification for each analyte were: 716.5→657.5
212 (Cholecalciferol), 719.5→660.5 (d3-Cholecalciferol), 732.5→673.4 (25(OH)D), 735.5→676.4
213 (d3-25(OH)D), 751.5→692.4 (d3-1,25$(OH)_2$D) and 748.5→689.4 (1,25$(OH)_2$D).
214 Data was validated for linearity and repeatability data (Supplemental Table 2).
215
216 **Statistical analysis** – Data are presented as mean ± SEM. Significant differences between
217 control group and treatment group were determined using a Student's t-test or ANOVA
218 followed by the Tukey-Kramer post hoc test, all using Statview software (SAS Institute, Cary,
219 NC). $p<0.05$ was considered statistically significant.

Results

Cholecalciferol supplementation modifies expression of genes involved in cholecalciferol metabolism and uptake in WAT and adipocytes.

To examine the impact of cholecalciferol supplementation on the regulation of genes coding proteins involved in its own metabolism in WAT, wild-type C56BL/6J male mice were supplemented with cholecalciferol (15 000 IU/kg of body weight/day) for 4 days. Body weight and absolute and relative organ weights were not modified by the treatment (Table 1). As expected, supplemented mice showed higher plasma concentrations of cholecalciferol, 25(OH)D and 1,25$(OH)_2$D (26.22, 6.32 and 9.94-fold, respectively, Table 1) and increased cholecalciferol and 25(OH)D in WAT (4.87-fold and 3.41-fold, respectively; Table 1). WAT from supplemented mice also had a higher quantity of 1,25$(OH)_2$D, but the difference compared to control mice did not reach statistical significance (Table 1).

The expression of genes coding for vitamin D metabolism proteins (Supplemental Table 3) was measured by real-time PCR in liver, kidney and epididymal WAT (eWAT). In liver, no major difference in gene expression was observed between the two groups except a decrease of mRNA expression of cubilin (Cubn) in cholecalciferol-supplemented mice (0.44-fold, Figure 1A). In kidney, Cyp24a1 and Vdr mRNA levels were increased whereas Cyp27b1, Gc and Cubn mRNA were decreased in cholecalciferol-supplemented mice (20.68, 1.78, 0.96, 0.33 and 0.18-fold respectively, Figure 1B). Interestingly, in eWAT, a decrease of Cyp24a1, Cyp27a1 and Cubn gene expression was observed in cholecalciferol-supplemented mice compared to controls (0.59, 0.34 and 0.62-fold, respectively, Figure 1C). Note that genes not mentioned as regulated in the different tissues were not modified by cholecalciferol supplementation.

To study the effect of 1,25$(OH)_2$D-mediated cell-autonomous regulation on adipocyte gene expression, 3T3-L1 adipocytes were treated with different doses of 1,25$(OH)_2$D (1, 10 and 100

nM) for 24 hours (Figure 2 and Supplemental Table 4). Cyp27a1 and Cubn mRNA levels were decreased in cells treated with 10 and 100 nM of 1,25$(OH)_2$D compared to control cells (0.36 and 0.34-fold for Cyp27a1 and 0.34 and 0.56-fold for cubilin, respectively). Conversely, Cyp24a1 mRNA expression increased strongly with the higher dose (489.88-fold for 100 nM), similarly to Vdr mRNA (2.87 and 10.35-fold for 10 and 100 nM, respectively).

The regulation of cubilin expression in adipocytes is VDR-dependent.

To gain further insight into the mechanism of cholecalciferol uptake by WAT/adipocytes, we focused analysis on the regulation of cubilin. The down-regulation (0.28-fold) of Cubn mRNA was confirmed in human primary white adipocytes incubated with 1,25$(OH)_2$D (100 nM for 24 h; Figure 3A). The impact of the cholecalciferol supplementation on cubilin protein was confirmed by ELISA in mouse WAT, which showed 0.23-fold-lower cubilin protein in cholecalciferol-supplemented mice (Figure 3B). 3T3-L1 adipocytes were incubated with EB1089, a specific VDR agonist, for 24 h (Figure A4). mRNA level of Cubn was decreased in adipocytes treated with 10 and 100 nM of EB1089 (0.73 and 0.78-fold, respectively), thus supporting the putative role of VDR in this regulation. In addition, in silico analysis with MatInspector software unveiled the location of several VDRE in both human and mouse cubilin promoters (Table 2). To confirm the involvement of VDR in this regulation, 3T3-L1 adipocytes were transfected with either a small interfering RNA (siRNA) oligonucleotide directed against VDR or a non-silencing control for 24 h. qPCR confirmed that the RNA interference was efficient, and a significant decrease of VDR expression was observed (0.83-fold; Figure 4B). In addition, the transfection with siRNA-targeting VDR completely blunted the 1,25$(OH)_2$D-mediated inhibition of Cubn mRNA level (100 nM of 1,25$(OH)_2$D for 24 h; Figure 4C).

25(OH)D endocytosis is mediated by cubilin and regulated by 1,25(OH)$_2$D in 3T3-L1 adipocytes.

To highlight the functional role of cubilin for 25(OH)D uptake, we undertook a preliminary experiment to measure 25(OH)D uptake in 3T3-L1 adipocytes. Cells were incubated for 16 h without (control) or with [3H]-25(OH)D and non-labelled 25(OH)D (50 nM) under its free form (dissolved in ethanol) or complexed with different proteins: DBP or BSA. CPM served reflected the uptake of 25(OH)D. The highest uptake of 25(OH)D was obtained with BSA as vehicle (44-fold compared to control). A significant but lower uptake was obtained with free form and DBP conditions (22.8-fold and 4.4-fold respectively, Figure 5A).

To confirm the involvement of cubilin receptor in [3H]-25(OH)D endocytosis, cells were treated with RAP, a specific inhibitor of the megalin/cubilin complex. When [3H]-25(OH)D was solubilized in ethanol, there was no difference between RAP-treated conditions and controls (Figure 5B). However, when [3H]-25(OH)D was bound to BSA or DBP, there was a strong decrease of [3H]-25(OH)D uptake (0.23-fold and 0-49 fold, respectively; Figure 5C and 5F). In a second set of experiments, 3T3-L1 adipocytes were transfected with either a siRNA directed against Cubilin (siCubilin) or a non-targeted siRNA (siNT) used as control for 24 h. [3H]-25(OH)D uptake in complexes with ethanol, BSA or DBP was measured for 16 h. [3H]-25(OH)D uptake was decreased in the presence of siCubilin compared to siNT when the BSA was used as vehicle (0.63-fold, Figure 5E).

To study the effect of modulating cubilin expression on [3H]-25(OH)D uptake, we incubated the cells with 1,25(OH)$_2$D (10 or 100 nM) and measured the uptake of [3H]-25(OH)D solubilized in ethanol (Figure 5F) or bound to BSA (Figure 5G) or DBP (Figure 5H). In all conditions, incubation with 100 nM of 1,25(OH)$_2$D decreased uptake of [3H]-25(OH)D in adipocytes (0.04, 0.19 and 0.45-fold, respectively). Note that even if statistically significant,

293 the 1,25(OH)$_2$D-induced modification of ethanol-solubilized [3H]-25(OH)D uptake was
294 quantitatively negligible (Figure 5F).
295 .

Discussion

Here we used targeted gene profiling to show that cholecalciferol regulates the expression of several genes involved in cholecalciferol metabolism and uptake in WAT in response to a short-term cholecalciferol supplementation. Among regulated genes, we report, for the first time, the negative regulation of Cubn gene. Additional experiments in 3T3-L1 adipocytes demonstrated that Cubn regulation was VDR-dependent. Finally, we demonstrated that cubilin is involved in 25(OH)D uptake in adipocytes.

To analyze the regulatory effect of cholecalciferol supplementation on gene expression in WAT, mice received cholecalciferol for 4 days by oral gavage. Cholecalciferol was diluted in olive oil to assure better absorption of this lipophilic molecule (41). In this study, no difference of total body mass or organ mass (liver and AT) was detected between groups, but as expected, plasma concentrations of cholecalciferol, 25(OH)D and $1,25(OH)_2D$ in WAT concentrations of cholecalciferol and 25(OH)D were strongly increased in cholecalciferol-supplemented mice. Despite a clear tendency to increase, the quantity of $1,25(OH)_2D$ in WAT of supplemented mice was not statistically different from control mice. In agreement with previously published data (42), cholecalciferol supplementation strongly induced kidney mRNA expression of Cyp24a1 and Vdr and decreased mRNA levels of Cyp27b1, thus validating our experimental conditions. Interestingly, we also observed a decrease of Gc and Cubn gene expression in the kidney. This regulation, if confirmed at protein level, could result in a decrease in renal recycling of cholecalciferol and its metabolites, thus constituting a way to eliminate excess cholecalciferol from the plasma. In the liver, we only observed a decrease of Cubn expression decreased, but there was no change in the expression of mRNA coding for other enzymes of hepatic cholecalciferol metabolism. This decrease of Cubn expression suggests that the uptake of these

molecules could be regulated in the liver at a transcriptional level, through a negative feedback mechanism, thus limiting their hepatic uptake. We also gained an overview of the transcriptional effect of cholecalciferol supplementation in epididymal WAT. Our results showed a decrease of Cyp27a1 and Cyp24a1 mRNA levels, suggesting a putative decrease of 25-hydroxylation and inactivation of metabolites. Cyp24a1 induction has already been reported (28), but this is the first report of Cyp27a1 repression. This novel finding result will require further investigations, especially to confirm the real contribution of Cyp27a1 in the adipose metabolism of cholecalciferol. Interestingly, we also found a specific down-regulation of Cubn mRNA levels while other partners in the endocytosis complex (megalin, Dab2 and Amn) were not transcriptionally affected.

To demonstrate the direct effect of $1,25(OH)_2D$ on these regulations, we used murine 3T3-L1 adipocytes. In these cells, we observed an up-regulation of Vdr and Cyp24a1, both of which are well-known VDR target genes (43), thus validating our experimental model. The fact that Cyp24a1 was decreased in vivo but strongly increased in vitro is surprising, but could be due to indirect regulations that simultaneously occurred in vivo whereas induction in vitro only resulted from direct VDR-mediated induction m (43) Interestingly, the patterns of Cyp27a1 and Cubn regulation were reproduced in vitro, confirming the direct and cell-autonomous nature of the regulation. While it is well documented that most of the enzymes of cholecalciferol metabolism are expressed in adipocytes (25), including Vdr (29), 25-hydroxylation enzymes (23,24), 1α-hydroxylation enzyme (24,44) and megalin (20), here we report that certain putative actors of hepatic 25-hydroxylation (4) are not expressed in adipocytes. This is notably the case of Cyp2r1 and Cyp3a11, which were not detected in our conditions, in agreement with Zoico et al. who did not detect Cyp2r1 in 3T3-L1 cells (24). The ability of adipocytes to produce 25(OH)D has been demonstrated (23,24), but the enzyme involved has not yet been identified.

346 Based on our results, i.e. down-regulation of Cyp27a1, which could be considered as a negative
347 feed-back, we could posit that Cyp27a1 is a major contributor to 25(OH)D production in
348 adipocytes, but further investigations are needed.
349
350 The down-regulation of Cubn mRNA levels in response to cholecalciferol was confirmed not
351 only in 3T3-L1 adipocytes but also in human primary adipocytes and in mouse WAT. The
352 cubilin protein is known to play a crucial role in 25(OH)D uptake, since mutations causing
353 cubilin dysfunction lead to urinary excretion of 25(OH)D (10). Indeed, cubilin participates
354 together with megalin (45), Dab-2, an intracellular adaptor protein, and AMN, a transmembrane
355 protein (11,12), in the endocytosis of 25(OH)D, notably in proximal tubules of the glomerulus.
356 We identified mRNA coding for megalin, Dab-2 and AMN in adipocytes and WAT, but we did
357 not observe any modification in expression levels. The detection of megalin mRNA does not
358 fit with Abboud et al.'s report that megalin is expressed in preadipocytes but not in adipocytes
359 (20). This discrepancy could be due to cell culture model specifics, but it nevertheless clearly
360 demonstrates the existence of the megalin/cubilin complex in adipocytes.
361 To investigate the molecular mechanism involved in Cubn regulation, several approaches were
362 combined. First, the use of a specific VDR agonist (EB1089) led to similar down-regulation of
363 Cubn expression compared to 1,25$(OH)_2$D, suggesting that the regulation described in vitro is
364 mediated by VDR. This involvement was demonstrated by the RNA interference experiments
365 implemented here using siRNA targeted against VDR. Furthermore, an in silico analysis
366 (MatInspector in the Genomatix suite) confirmed the presence of putative VDREs within the
367 murine and human promoters of cubilin.
368 To study the involvement of cubilin in 25(OH)D uptake by adipocytes, experiments were
369 undertaken using radiolabeled 25(OH)D. In the physiological context, plasma 25(OH)D is
370 either bound to DBP (87% of total 25(OH)D) or albumin (13% of total 25(OH)D) or else

371 considered unbound (“free form”) (>1% of total 25(OH)D) (7). In preliminary experiments, the
372 ability of different vehicles to deliver 25(OH)D to adipocytes was tested. We observed that the
373 best vehicle for 25(OH)D was BSA, followed by ‘free form’ (mimicked here by an ethanolic
374 solution) then DBP. These data suggest that BSA-complexed 25(OH)D is easily absorbed by
375 cells. Note too that the free form can also be internalized in adipocytes. Finally, it appears that
376 the DBP is probably not the best way to deliver 25(OH)D to adipocytes but corresponds to a
377 25(OH)D storage site in plasma, as previously suggested (46).
378 To confirm the involvement of cubilin in 25(OH)D uptake by adipocytes, two strategies were
379 implemented. First, we used RAP (inhibitor of megalin/cubilin complex (47)), and second, we
380 used an RNA interference approach. Interestingly, 25(OH)D uptake in complex with BSA or
381 DBP was decreased by RAP and, to a lesser extent, siRNA directed against Cubn (especially
382 for 25(OH)D-DBP complexes that were not impacted by siRNA). Note that 25(OH)D uptake
383 of the free from (in ethanol) was not impacted by RAP or siRNA, suggesting that the uptake of
384 unbound 25(OH)D occurs independently of the megalin/cubilin pathway. Finally, to confirm
385 that cubilin regulation is involved in 25(OH)D uptake, adipocytes were incubated with
386 1,25$(OH)_2$D. Interestingly, this incubation led to a decrease of cubilin expression and was
387 associated with a decrease of 25(OH)D uptake. Taken together, these data provide strong
388 evidence that cubilin is involved in 25(OH)D uptake by adipocytes. From a physiological point
389 of view, these data suggest that a negative feedback regulation occurs in WAT to control the
390 uptake of cholecalciferol and its metabolites via a modulation of cubilin expression. This kind
391 of limitation of cholecalciferol and metabolite storage, which is generally assumed to be a
392 passive mechanism due to lipophilicity, suggests that cholecalciferol and 25(OH)D storage in
393 WAT is actually tightly controlled and regulated.
394 Here we demonstrate for the first time that there is a coordinated overall regulation of genes
395 coding for enzymes involved in the cholecalciferol metabolism in WAT and in adipocytes. Our

data also demonstrate that cubilin is involved in 25(OH)D uptake in adipocytes, and suggest that this mechanism is transcriptionally regulated, thus extending our knowledge of adipocyte cholecalciferol metabolism.

401 **Figure legends**

402

403 **Figure 1: Effect of cholecalciferol supplementation on the expression of cholecalciferol**
404 **metabolism genes in liver, kidney and adipose tissue.**

405 Expression of genes coding for proteins involved in cholecalciferol metabolism relative to 18S
406 ribosomal RNA in the liver (A), kidney (B) and epididymal white adipose tissue (WAT; C) of
407 control mice or cholecalciferol-supplemented mice (control n=9, cholecalciferol n=6). Values
408 are reported as means ± SEM. * $p < 0.05$ for an unpaired Student's t-test.

409

410 **Figure 2: Effect of 1,25(OH)$_2$D incubation on expression of cholecalciferol metabolism**
411 **genes in 3T3-L1 adipocytes.**

412 3T3-L1 adipocytes were incubated with 1,25(OH)$_2$D (1, 10 and 100 nM) for 24 h (A to E).
413 Expression of genes coding for proteins involved in cholecalciferol metabolism relative to 18S
414 ribosomal RNA. Values are reported as means ± SEM. Bars not sharing the same letter were
415 significantly different in a Tukey-Kramer post hoc test at $p < 0.05$.

416

417 **Figure 3: Cubilin mRNA level is down-regulated in human adipocytes and at protein level**
418 **in mice adipose tissue.**

419 (A) Human primary white adipocytes were incubated with 100 nM of 1,25(OH)$_2$D for 24 h.
420 Expression of cubilin relative to 18S ribosomal RNA. (B) Cubilin protein quantification
421 performed by ELISA tests in epididymal white adipose tissue (WAT) of mice (control n=9,
422 cholecalciferol n=6). Values are reported as means ± SEM. * $p < 0.05$ for an unpaired Student's
423 t-test.

Figure 4: The regulation of cubilin expression is VDR-dependent.

(A) 3T3-L1 adipocytes were incubated with EB1089, a vitamin D receptor agonist (VDR) (10 and 100 nM) for 24 h. (B) The 3T3-L1 adipocytes were transfected with either a small interfering RNA (siRNA) oligonucleotide for VDR or a non-silencing control for 24 h. The efficiency of RNA interference against VDR was determined by qPCR. (C) These cells were transfected with siRNA and incubated with 100 nM of $1,25(OH)_2D$ for 24h. Values are reported as means ± SEM. Bars not sharing the same letter were significantly different in a Tukey-Kramer post hoc test at $p < 0.05$. * $p < 0.05$ for an unpaired Student's t-test.

Figure 5: 25(OH)D uptake in 3T3-L1 adipocytes is mediated by cubilin.

3T3-L1 adipocytes were incubated with control (Ctrl) or with 25(OH)D solubilized in ethanol (free form) or complexed with bovine serum albumin (BSA) or vitamin D-binding protein (DBP) (A). Cells were incubated with receptor-associated protein (RAP) (100 or 500 nM) and 25(OH)D was solubilized in ethanol (B) or complexed to BSA (C) or DBP (D) for 16 h. 3T3-L1 cells were transfected with siRNA (non-targeted (siNT) or directed against VDR (siVDR)) and incubated with 25(OH)D solubilized in ethanol (free form) or complexed with BSA or DBP (E). Cells were incubated with $1,25(OH)_2D$ (10 and 100 nM) and 25(OH)D was solubilized into ethanol (F) or complexed to BSA (G) or DBP (H). In each experiment, 25(OH)D uptake was quantified by measuring the number of counts per minute per well by liquid scintillation. Values are reported as means ± SEM. Bars not sharing the same letter were significantly different in a Tukey-Kramer post hoc test at $p < 0.05$. * $p < 0.05$ for an unpaired Student's t-test.

Table 1: Morphological and biological parameters of mice.

	Control mice	Cholecalciferol-supplemented mice
Body weight (g)	21.9 ± 0.37	21.1 ± 0.90
Liver weight (mg)	976.9 ± 29.09	955.5 ± 57.74
Liver weight/body weight ratio	0.0446 ± 0.0007	0.0451 ± 0.0009
Adipose tissue weight (mg)	242.9 ±10.12	281.0 ± 32.63
Adipose tissue weight/body weight ratio	0.0111 ± 0.0005	0.0136 ± 0.002
Serum cholecalciferol (ng/mL)	2.58 ± 0.60	67.65 ± 9.66 *
Serum 25(OH)D (ng/mL)	16.79 ± 0.98	106.16 ± 18.60 *
Serum 1,25(OH)$_2$D (pg/mL)	68.6 ± 16.95	682.2 ± 122.11 *
AT cholecalciferol quantity (ng)	158 ± 22.01	770.24 ± 175.28 *
AT 25(OH)D quantity (ng)	27.3 ± 1.05	93.13 ± 30.91 *
AT 1,25(OH)$_2$D quantity (pg)	1.58 ± 0.31	2.77 ± 0.68

Values are reported as means ± SEM. Student's t-test. p values: *, $p < 0.05$.

Table 2: VDRE sequences and location identified with MatInspector software within human and mouse cubilin promoters.

	Sequence	ElDorado reference ID	Position of the VDRE
Human promoter	gtttcaaaGGTCaaatagataatga	GXP_271874 (-)	17171654_17172330
Mouse promoter	tcaagagGATTcaaaggcaacttca	GXP_425459 (-)	13491712_13492424

In silico analysis with MatInspector software of human and mouse cubilin promoters. The position of the VDRE (referred to by a start _ end number) corresponds to its location within the input sequence (ElDorado reference ID).

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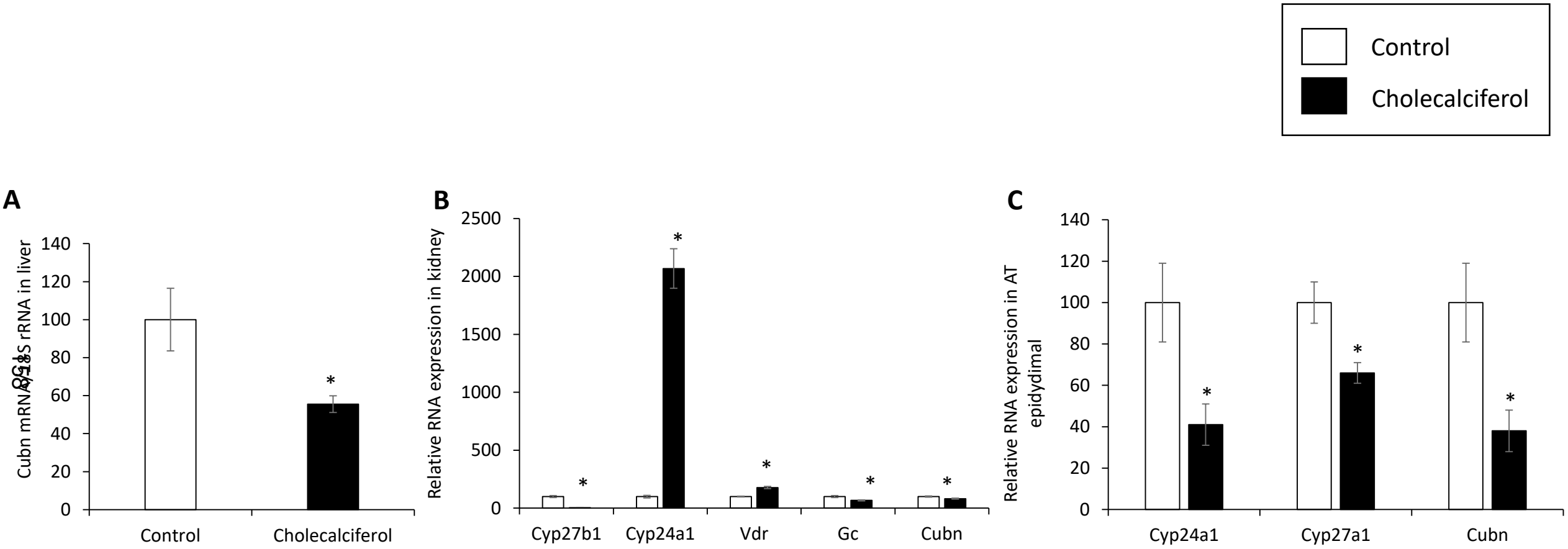
588

Figure 1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Cholecalciferol
A
Cubn mRNA/18S rRNA in liver
140
120
100
80
60
40
20
0
*
Control
Cholecalciferol
B
Relative RNA expression in kidney
2500
2000
1500
1000
500
0
*
*
*
*
*
Cyp27b1
Cyp24a1
Vdr
Gc
Cubn
C
Relative RNA expression in AT epididymal
140
120
100
80
60
40
20
0
*
*
*
Cyp24a1
Cyp27a1
Cubn

Figure 2

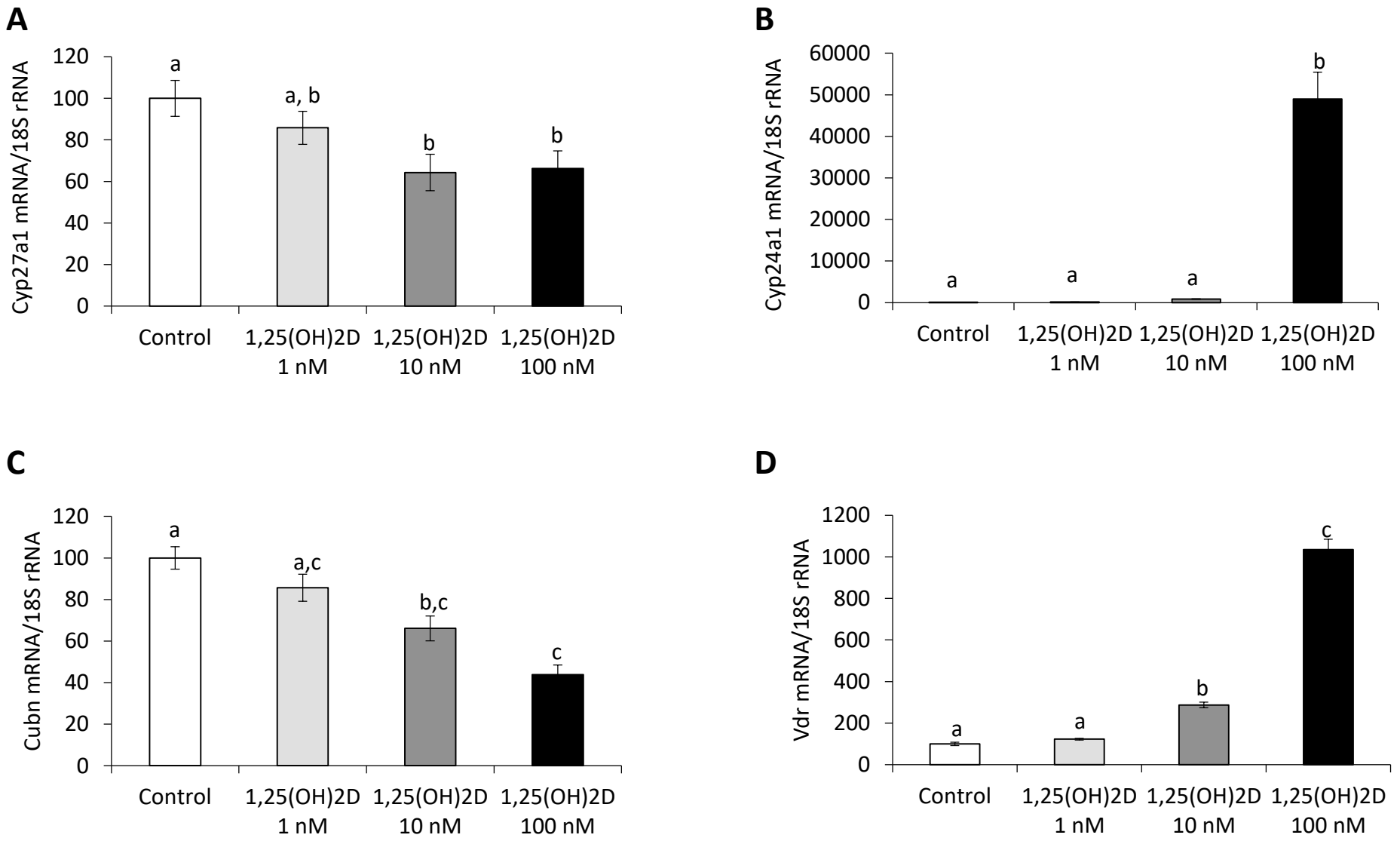
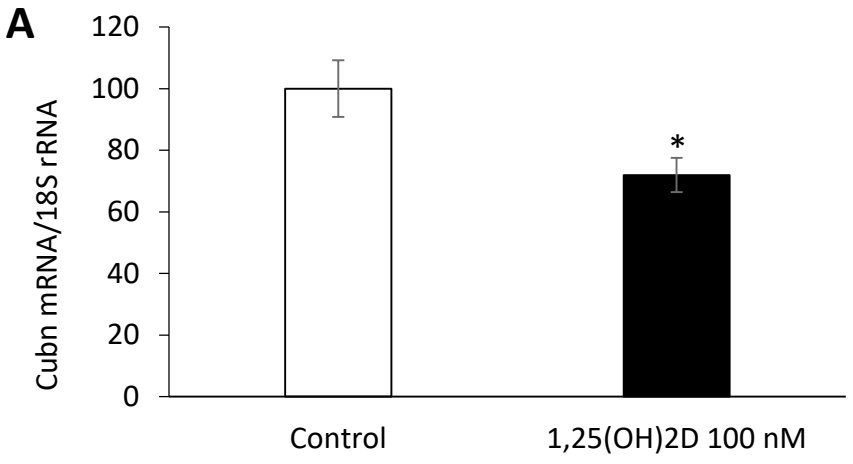

A
Cyp27a1 mRNA/18S rRNA
120
100
80
60
40
20
0
a
a, b
b
b
Control
1,25(OH)2D 1 nM
1,25(OH)2D 10 nM
1,25(OH)2D 100 nM
B
Cyp24a1 mRNA/18S rRNA
60000
50000
40000
30000
20000
10000
0
a
a
a
b
Control
1,25(OH)2D 1 nM
1,25(OH)2D 10 nM
1,25(OH)2D 100 nM
C
Cubn mRNA/18S rRNA
120
100
80
60
40
20
0
a
a,c
b,c
c
Control
1,25(OH)2D 1 nM
1,25(OH)2D 10 nM
1,25(OH)2D 100 nM
D
Vdr mRNA/18S rRNA
1200
1000
800
600
400
200
0
a
a
b
c
Control
1,25(OH)2D 1 nM
1,25(OH)2D 10 nM
1,25(OH)2D 100 nM

Figure 3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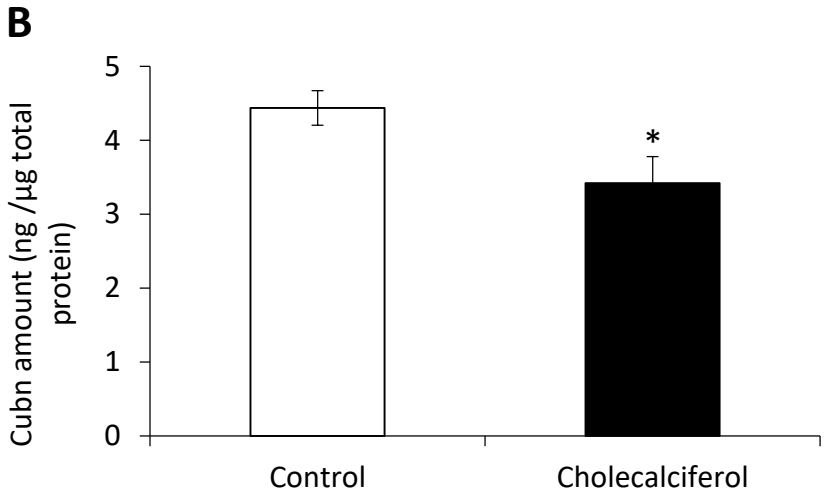
120
100
80
60
40
20
0
Cubn mRNA/18S rRNA
*
Control
1,25(OH)2D 100 nM


B
5
4
3
2
1
0
Cubn amount (ng /µg total protein)
*
Control
Cholecalciferol

160

Figure 4

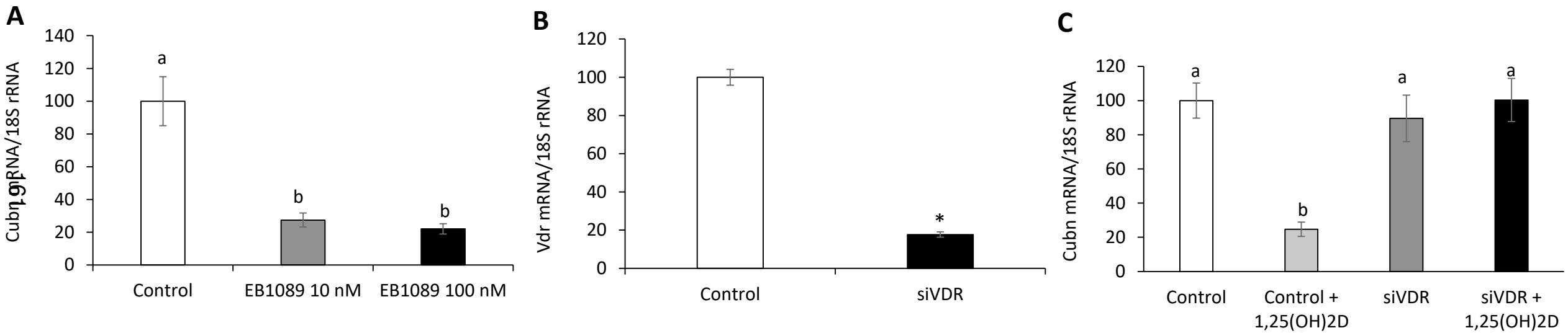
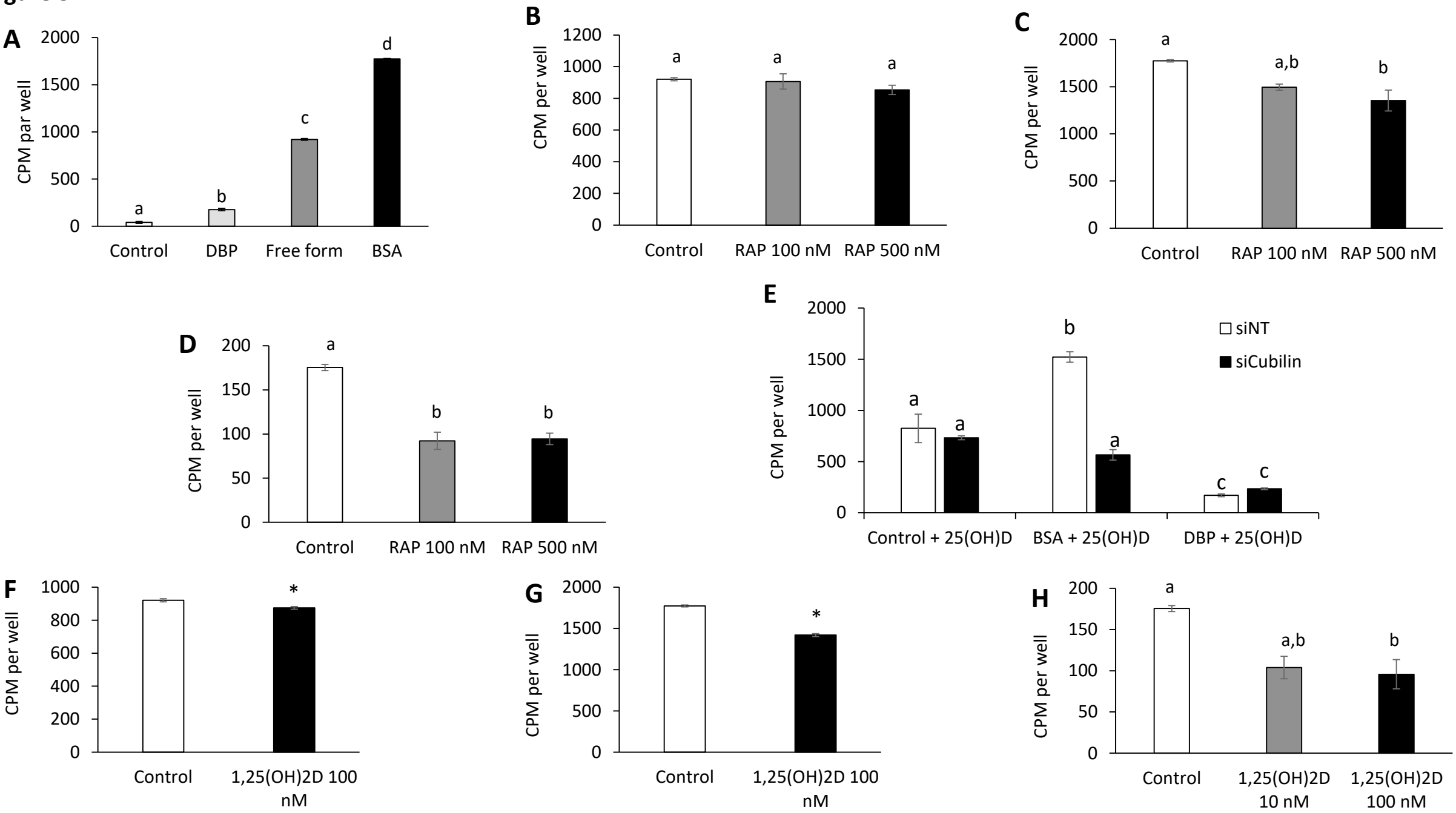

A
Cubn mRNA/18S rRNA
140
120
100
80
60
40
20
0
a
b
b
Control
EB1089 10 nM
EB1089 100 nM
B
Vdr mRNA/18S rRNA
120
100
80
60
40
20
0
*
Control
siVDR
C
Cubn mRNA/18S rRNA
120
100
80
60
40
20
0
a
b
a
a
Control
Control + 1,25(OH)2D
siVDR
siVDR + 1,25(OH)2D

Figure 5


A
CPM par well
2000
1500
1000
500
0
a
b
c
d
Control
DBP
Free form
BSA
B
CPM per well
1200
1000
800
600
400
200
0
a
a
a
Control
RAP 100 nM
RAP 500 nM
C
CPM per well
2000
1500
1000
500
0
a
a,b
b
Control
RAP 100 nM
RAP 500 nM
D
CPM per well
200
150
100
50
0
a
b
b
Control
RAP 100 nM
RAP 500 nM
E
CPM per well
2000
1500
1000
500
0
a
a
b
a
c
c
siNT
siCubilin
Control + 25(OH)D
BSA + 25(OH)D
DBP + 25(OH)D
F
CPM per well
1000
800
600
400
200
0
*
Control
1,25(OH)2D 100 nM
G
CPM per well
2000
1500
1000
500
0
*
Control
1,25(OH)2D 100 nM
H
CPM per well
200
150
100
50
0
a
a,b
b
Control
1,25(OH)2D 10 nM
1,25(OH)2D 100 nM

Supplemented tables

Supplemental table 1: Primers sequences

Gene	Forward sequence	Reverse sequence
Cyp2r1	TTTGTCGGCAACATCTGCT	TGCCTCCAAGATCTAAACTGAAA
Cyp3a11	TGAATATGAAACTTGCTCTCACTAAAA	CCTTGTCTGCTTAATTTCAGAGCT
Cyp27a1	CCTCACCTATGGGATCTTCATC	TTTAAGGCATCCGTGTAGAGC
Cyp2j6	CCCTCTACCCAGAAGTCCAA	TTCTGGCCAATCACCCTATC
Cyp24a1	AAGCCTACGCGCTGATGAT	CACGGGCTTCATGAGTTTC
Cyp27b1	AGTGGGGAATGTGACAGAGC	GGAGAGCGTATTGGATACCG
Megalin	GATGGATTAGCCGTGGACTG	TCCGTTGACTCTTAGCATCTGA
Cubilin	GCCATCCAGATGCAACCT	GGTGCAGACAGGCAACAAG
Vdr	AACCCCTCATAAAGTTCCAGGT	CTGTACCCCAGGTCGGTCT
Gc	CTACCTCAGAGGATTGCATGG	CTTTTTGGATAAGTTTTGACAGATTTT
Dab2	GCAGTCGAACTTTCTGCATCTC	GGTGTTACTGGGACCGTACCT
Amn	AGACAGTCACGCCATCTCG	GAGGCCAGGACCAACTCC.
18S rRNA	CGCCGCTAGAGGTGAAATTCT	CATTCTTGGCAAATGCTTTCG

Supplemental table 2: Method validation for LC-MS/MS analysis of vitamin D metabolites after Amplifex derivatization.

Analytes	Linear range ng/ml	LOD ng/ml	LOQ ng/ml	Inter-assay CV%	Intra-assay CV%
Vitamin D3	0.78-75	0.049	0.78	14.4	6.0
25OH D3	0.2-75	0.049	0.2	12.5	11.0
1,25(OH)$_2$D3	0.2-75	0.049	0.2	5.5	11.6

Supplemental table 3: Effect of vitamin D supplementation on gene expression of vitamin D metabolism in liver, kidney and epididymal white adipose tissue.

	Liver		**Kidney**		**Epididymal WAT**	
	Control	VD	Control	VD	Control	VD
Cyp27a1	100 ± 6	109 ± 6			100 ± 10	66 ± 5 *
Cyp2r1	100 ± 8	92 ± 5			100 ± 8	83 ± 16
Cyp2j6	100 ± 6	109 ± 8			100 ± 8	96 ± 8
Cyp3a11	100 ± 9	76 ± 9			100 ± 33	103 ± 38
Cyp24a1			100 ± 11	2068 ± 171 *	100 ± 19	41 ± 10 *
Cyp27b1			100 ± 9	4 ± 0 *	100 ± 25	78 ± 22
Vdr	100 ± 10	79 ± 6	100 ± 4	178 ± 10 *	100 ± 16	77 ± 14
Gc	100 ± 6	115 ± 6	100 ± 9	67 ± 4 *	100 ± 44	36 ± 11
Megalin	100 ± 7	93 ± 7	100 ± 5	98 ± 3	100 ± 25	50 ± 9
Cubilin	100 ± 16	56 ± 4 *	100 ± 5	82 ± 4 *	100 ± 19	38 ± 10 *
Dab2	100 ± 8	106 ± 5	100 ± 4	82 ± 5	100 ± 5	73 ± 9
Amn	100 ± 10	93 ± 8	100 ± 8	75 ± 5	100 ± 21	119 ± 32

Expression of genes coding for proteins involved in vitamin D metabolism relative to 18S ribosomal RNA in liver, kidney and epidydimal white adipose tissue (WAT) of control mice or vitamin D supplemented mice (control n=9, VD n=6). Values are presented as means ± SEM. For unpaired Student's t-test, p values: *, $p < 0,05$.

Supplemental table 4: Dose effect of $1,25(OH)_2D$ incubation on gene expression of vitamin D metabolism in 3T3-L1 adipocytes.

	3T3-L1 adipocytes			
	Control	$1,25(OH)_2D$ 1 nM	$1,25(OH)_2D$ 10 nM	$1,25(OH)_2D$ 100 nM
Cyp27a1	100 ± 9 [a]	100 ± 9 [a]	64 ± 9 [b]	66 ± 9 [b]
Cyp2j6	100 ± 9 [a]	103 ± 9 [a]	105 ± 4 [a]	91 ± 4 [a]
Cyp3a11	100 ± 37 [a]	80 ± 13 [a]	86 ± 22 [a]	97 ± 34 [a]
Cyp24a1	100 ± 10 [a]	138 ± 22 [a]	856 ± 46 [a]	48 988 ± 6453 [b]
Cyp27b1	100 ± 5 [a]	95 ± 13 [a]	80 ± 16 [a]	85 ± 9 [a]
Vdr	100 ± 8 [a]	123 ± 4 [a]	287 ± 13 [b]	1035 ± 50 [c]
Gc	100 ± 17 [a]	60 ± 5 [b]	70 ± 10 [a,b]	101 ± 6 [a]
Megalin	100 ± 10 [a]	86 ± 10 [a]	93 ± 10 [a]	97 ± 9 [a]
Cubilin	100 ± 5 [a]	86 ± 7 [a,c]	66 ± 6 [b,c]	44 ± 5 [b]

ARTICLE 4 : Short time effect of high fat diet on expression of vitamin D metabolism enzymes in male mice.

Article en préparation.

Plusieurs études montrent l'existence d'un lien inverse entre obésité et taux plasmatique en 25-hydroxyvitamine D (25(OH)D), la forme circulante de la vitamine D. En effet, ce taux est plus bas chez les patients obèses que chez les individus normo pondéraux. Plusieurs mécanismes pourraient expliquer cette différence. Parmi ceux-ci, le plus souvent avancé est la possible séquestration passive du cholécalciférol et/ou de ses métabolites dans le tissu adipeux, qui constitue un réservoir physiologique pour ces molécules lipophiles. Récemment, il a été proposé que le métabolisme de la vitamine D pourrait être modifié au cours de l'obésité ce qui expliquerait les taux plasmatiques faibles retrouvés au cours de cette maladie. Afin d'identifier des éléments causaux dans cette association, il nous a semblé pertinent d'étudier les effets très précoces d'une alimentation riche en graisse sur le métabolisme de la vitamine D.

Dans cet article, nous avons eu recours à un modèle in vivo, de souris nourries soit avec des croquettes classiques (groupe contrôle) soit par une alimentation riche en graisse avec 60% d'énergie sous forme de lipides pendant 4 jours.

Nous avons mesuré les taux plasmatiques et adipeux de la vitamine D et de ses métabolites par test ELISA ou par la méthode LC-MS/MS mise au point dans le laboratoire (**Article 7**). Nous avons ensuite analysé l'expression génique des différentes enzymes impliquées dans le métabolisme de la vitamine D au niveau du foie, du rein et du tissu adipeux par qPCR.

PRINCIPAUX RESULTATS DE L'ETUDE

Les résultats de cette étude montrent qu'un régime riche en graisse, apportant la même quantité de vitamine D que le régime contrôle durant 4 jours, entraine une modification de l'expression des gènes codant des enzymes du métabolisme de la vitamine D au niveau du foie, du rein et surtout du tissu adipeux.

Bien que la masse corporelle des souris ne soit pas modifiée, nous observons une augmentation de l'index d'adiposité chez les souris nourries avec une alimentation riche en graisse. De plus, nous observons dans le plasma de ces souris : 1) une diminution de la concentration en cholécalciférol, 2) pas de modification de la concentration de la 25(OH)D totale, 3) une diminution des concentrations en 25(OH)D libre qui pourrait être expliquée par la diminution de l'expression de l'enzyme Cyp3a11 impliquée dans la 25-hydroylation retrouvée au niveau hépatique de ces souris et 4) une augmentation de la concentration en $1,25(OH)_2D$ qui est peut être due à la diminution de l'expression de l'enzyme Cyp24a1 au niveau rénal impliquée dans la dégradation des métabolites de la vitamine D. De plus, les taux de $1,25(OH)_2D$ pourraient expliquer les modifications géniques retrouvées au niveau du rein c'est-à-dire une augmentation de l'expression de Vdr et une diminution de l'expression de Cyp27b1, par une régulation négative relativement bien connue [12].

Au niveau du tissu adipeux, les résultats suggèrent un possible stockage de la vitamine D sous forme de 25(OH)D. En effet, on observe une augmentation de l'expression des enzymes Cyp27a1 et Cyp2r1, impliquées dans la 25-hydroxylation, et diminution de l'expression de l'enzyme Cyp27b1, impliquée dans la 1-hydroxylation.

Short time effect of high fat diet on expression of vitamin D metabolism enzymes in male mice.

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Abbreviated title: High fat diet for 4 days regulates vitamin D metabolism

Key terms: vitamin D, metabolism, adipose tissue, nutrition, high fat diet

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Disclosure statement: The authors have nothing to disclose.

Abstract

Serum total and free form of 25(OH)D is lower in obese people. Hypotheses have been advanced to explain this observation but no mechanism have been elucidated to explain if vitamin D (VD) status is a cause or a consequence of obesity. We investigated VD status during the start of development of high fat diet-induced obesity with control of VD consumption, VD metabolism and VD storage (in white adipose tissue, WAT).

Male C57BL6 mice were fed with control diet (control group) and high fat diet containing 60% energy from fat (High Fat group) for 4 days without difference of VD intake for mice.

For 4 days of diet, HF mice have the same body weight of control mice but we observed an significantly increased of fat mass which are associated with, in serum, 1) a decrease of cholecalciferol concentration, 2) a decrease of free 25(OH)D concentration which could be explain by a decrease of Cyp3a11 expression in the liver, 3) no modification of total 25(OH)D concentration due to the same VD intake and 4) an increase of $1.25(OH)_2D$ concentration which could be explain by a decrease of Cyp24a1 expression observed in the kidney. Indeed, this increase of $1.25(OH)_2D$ level could explain the increase of mRNA levels of Vdr and the decrease of mRNA of Cyp27b1, found in the kidney, by a negative feedback.

In WAT, after validation of our model by the increase of mRNA levels of Tnfα as reported in low grade-inflammation associated with obesity, we found no modification of VD metabolites quantity detected by liquid chromatography-tandem mass spectrometry (LCMS/MS). Otherwise, we observed an increase of Cyp2r1 and Cyp27a1 mRNA expression and a decrease of Cyp27b1 mRNA expression suggested a possible storage of VD in WAT under 25(OH)D form during obesity.

Keywords: vitamin D, metabolism, adipose tissue, nutrition, high fat diet

Introduction

Vitamin D (VD) or cholecalciferol have two origins, dietary and endogenous production [1, 2], and the VD is taken to the liver via the bloodstream where 4 enzymes (Cyp2r1, Cyp27a1, Cyp2j6 and Cyp3a11) catalyse the synthesis of 25-hydroxyvitamin D (25(OH)D) [3]. Total 25(OH)D is the major circulating form of VD and represent plasma status. In plasma, VD and 25(OH)D are mainly bound to vitamin D binding protein (DBP, encoded by Gc gene) (87% of 25(OH)D), their transport protein, and albumin (13% of 25(OH)D) but a small quantity remains unbound (<1% of 25(OH)D) [4, 5]. In the kidney, 25(OH)D is converted to 1α-dihydroxyvitamin D ($1.25(OH)_2D$), by the Cyp27b1. This $1.25(OH)_2D$ corresponds to the active form of VD, also called calcitriol and is a potent activator of the vitamin D receptor (VDR) [6]. 25(OH)D and $1.25(OH)_2D$ can be catabolized by 24-hydroxylase, Cyp24a1, to inactive metabolites [7]. The uptake of VD and its metabolites has been described in kidney and adipocytes and involved the complex megalin-cubilin [8, 9]. Moreover, VD and in a least extend 25(OH)D are stored in WAT [10], where they can be metabolized, since adipocytes contain all enzymatic equipment involved in VD metabolism [11].

Low levels of total circulating 25(OH)D are strongly associated with obesity and more specially with increased of fat mass and body mass index [11-13]. Moreover, it has recently been shown that the free form of 25(OH)D were also low in obesity [14]. Several hypotheses have been emitted to explain these observations. First, Wortsman and a1. in 2000, suggested a possible sequestration of VD in white adipose tissue (WAT) caused by a passive phenomenon due to the hydrophobic nature of VD [15]. Drincic and a1. proposed that 25(OH)D was simply diluted in a higher volume in obese and hypothesized a volumetric dilution [16]. Indeed, these authors showed an inverse correlation between plasma 25(OH)D concentration and total volume (lean and fat mass), which

could be a best predictive marker than fat mass. In addition, Wamberg and al. suggested that VD metabolism in WAT is modified by obesity and reported a modification of Cyp2j2 expression in biopsies of obese compared to lean patients [17]. More recently, Park and al. reported that high fat diet-induced obesity for 18 weeks influenced VD metabolizing enzymes expression in liver, kidney and WAT, which could provide a possible mechanism for altered VD metabolism in obesity [18]. However, the precise mechanism initiating the decrease of free or total 25(OH)D during obesity remains elusive.

The objective of this paper was to identify early events that could explain the low 25(OH)D level observed in obesity. To this aim, ashort term high fat diet (4 days) was implemented to initiate obesity and to study early modifications of VD status. In parallel, a profile of expression of genes coding for enzymes involved in VD metabolism was established in the liver, kidney and WAT in order to bring mechanistic keys.

Materials and methods

Reagents - TRIzol reagent, random primers, and Moloney murine leukemia virus reverse transcriptase were obtained from Life Technologies (Courtaboeuf, France). SYBR Green reaction buffer was purchased from Eurogentec (Liege, Belgium).

Animal Experiments – The protocol received the agreement of the local ethics committee. Six week-old male C57BL/6J mice were obtained from Janvier (Le Genest Saint Isle, France), fed ad libitum with control food (chow diet A04 from Safe-diets) during the 1-week acclimation period and with full access to drinking water. The animals were maintained at 22°C under a 12-hour light, 12-hour dark cycle a 20% humidity level. Mice were divided into control diet group (control: chow diet A04 from Safe-diets, n = 10) or high fat diet group (HF group: 60% energy from lipids of Test Diet, n = 10). Weight gain was measured once a week and dietary intake, every two weeks. After 4 days, mice were fasted overnight and blood was collected by cardiac puncture anesthesia, serum was isolated by centrifugation at 3000 rpm for 15 min at 4°C and was stored at -80°C. Cervical dislocation was used to scarify animals and tissue (kidney, liver and epididymal white adipose tissue) were collected, weighted and stored at 80°C.

Free 25(OH)D quantification – To determinate the free 25(OH)D quantity in mice serum, a specific ELISA kit was used (Mice free form of 25(OH)D, DIAsources, Louvain-La-Neuve, Belgique) according to manufacturer protocol.

RNA extraction and real-time PCR – Total RNA was extracted from the liver, kidney and epididymal AT or from cells using TRIzol reagent (Life technologies, Courtaboeuf, France). One µg of total RNA was used to synthetize cDNAs using random primers and Moloney murine leukemia virus reverse transcriptase (Life technologies, Courtaboeuf, France). Real-time quantitative PCR analyses were performed using the Mx3005P Real-Time PCR System (Stratagene, La Jolla, USA) as previously described [19]. For each condition, expression was quantified in duplicate, and 18S rRNA was used as the endogenous control in the comparative cycle threshold (CT) method [20]. The sequences of the primers used in this study are reported in supplemental data (Supplemental Table 1).

Cholecalciferol, 25(OH)D and 1.25(OH)2D quantification in plasma and WAT - All quantifications were performed using LC-MS/MS according to the following protocol.

Preparation of analytical and deuterated standards – A working solution of deuterated analytes was prepared at 0,02 ng/mL of each internal standards (IS) (d3-cholecalciferol, d3-25(OH)D and d3-1.25(OH)2D). A primary stock solution of cholecalciferol, 25OHD and 1.25$(OH)_2$D standards were prepared at concentrations respectively of 100, 50 and 10 ng/mL in ethanol and stored at -80°C in the dark. Calibration curves were prepared by serial dilution of the 3 analytes stock solution to obtain calibration standards from 0 to 75ng/mL and addition of 1.5 µl of the working solution of deuterated analytes to each dilution. After complete evaporation of solvent, derivatization was proceeded. A one-step derivatization was employed to improve the ionization efficiency of the metabolites using Amplifex diene as reagent [21]. 30 µL of the Amplifex was added to the dried sample above, vortexed for 15 s and incubated for 30 min at ambient temperature. Next, 30 µL of deionized water was added, vortexed for 15 s and transferred for LC

injection. Calibration curves were plotted with peak area ratio of the vitamin D metabolite to the respective internal standard versus a range of concentrations of the analyte.

Preparation of plasma - Sample preparation was adapted from Wang et al. [22]. The extraction procedure was conducted under low light because cholecalciferol and its metabolites are light sensitive. After thawing on ice, mice plasmas were centrifuged at 4°C for 15 min, at 11 000 rpm. 100 µL of each sample was transferred to a glass test tube containing 10 µL of deuterated standards working solution. Proteins were precipitated by adding acetonitrile, vortex-mixed, and centrifuged for 10 min at 3 000 g. The supernatant was moved to another glass tube, and the volume was reduced to a half under a nitrogen stream. 5 mL of ethyl acetate was adding to the solution for liquid-liquid extraction. After shaking vigorously, samples were centrifuged for 20 min at 590 g and the upper organic layer was transferred into a new glass tube and was reduced under nitrogen stream. The samples were derivatized as described above.

Epididymal WAT preparation – Sample preparation was adapted from Lipkie et al. [23]. 25 µL of deuterated standards working solution were added to tissue homogenates (50 mg of tissue grinded within 1 mL of PBS) in glass test tube. Acetonitrile (ACN) was added, vortex mixed for 5 min and centrifuged at 6 000 g for 5 min. Then, methyl tert-butyl ether (MTBE) was added, vortexed for 5 min, centrifuged, and the upper organic layer was collected in another glass tube. The extraction was repeated twice and the combined supernatants were dried under nitrogen. Oasis HLB SPE cartridges (Waters, Guyancourt, France) were conditioned with ethyl acetate, methanol (MetOH) and H_2O. The sample was reconstituted with 1 mL of MetOH and 1 mL of K_2HPO_4 (0.4 M) and added on the cartridge. The cartridge was washed with H_2O and 70% MetOH and dried for 2 min under vacuum. Tips were washed with ACN and analytes were eluted with ACN and dried under nitrogen. After complete evaporation of solvent, the samples were derivatized as described above.

LC-MS/MS analysis – Accurate mass measurements were performed on the Q-Exactive Plus mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a Heated Electrospray Ionization (H-ESI II) probe. Thermo Xcalibur 3.0.63 software was used for the instrument setup, control of the LC-MS system during acquisition and data treatment. Tune Q Exactive Plus 2.5 application was used for the direct control of the mass spectrometer.

Samples were injected onto a Hypersil GOLD C18 column (2.1 x 100 mm; Thermo Scientific, Les Ulis, France). Flow rate was 0.4 mL/min and injection volume was 5 µL. The mobile phase was composed of A= ultrapure water with 0.1% formic acid (FA) (v/v), and B = acetonitrile with 0.1 % FA (v/v). Starting conditions were A= 70% and B = 30% and were held for 4 min. A linear gradient was applied until 10.0 min where A= 35% and B = 65%, these conditions were held until 12.0 min, at 14 min A=0% and B = 100% until 16 min. Starting conditions were re-implemented at 18 min.

PRM (Parallel Reaction Monitoring) method was set up as MS/MS acquisition mode and full higher-energy collision dissociation spectra (HCD) spectra were acquired. For each compound, the most abundant ion was selected as precursor ion and isolated in a 2 uma window in a specified time segment and fragmented under 20 % of normalized collision energy (m/z 716.5 for cholecalciferol, m/z 719.5 for d3-Cholecalciferol, m/z 732.5 for 25OH-D3, m/z 735.5 for d3-25OH-D3, m/z 751.5 for d3-1,25OH-D3 and m/z 748.5 for 1,25OH-D3). In the data analysis, specific fragment ion were extracted within 5 ppm masse error limit for each compound analysed: m/z 657.4368 for cholecalciferol, m/z 660.4552 for d3-Cholecalciferol, m/z 673.4311 for 25OH-D3, m/z 676.4534 for d3-25OH-D3, m/z 689.4297 for 1,25OH-D3 and m/z 692.4458 for d3-1,25OH-D3.

Calibration curves were plotted with peak area ratio of analyte and internal standard versus concentration of analyte.

Validations were performed for linearity and repeatability data (Supplemental Table 2).

Statistical analysis - The data are communicated as the mean ± SEM. Significant differences between the control and treated group were determined using Student test with $p < 0.05$ was considered statistically significant.

Results

Morphological data of 4 days high fat diet fed mice.

The effect of a high fat diet (60% energy from fat, HF group) compared to control diet (control group) was evaluated in wild-type C57BL/6J male mice for 4 days. Body weight (start and the end of diet) and liver weigh were not modified by the diet (Table 1). Adiposity index (sum of epididymal, inguinal and perirenal adipose tissue mass relative to total body mass) as well as organ weight of subcutaneous, epididymal and peritoneal AT and ratio of organ weigh on body weight of these 3 organs, were increased in HF group (1.58-fold for adiposity index, Table 1). Energy intake was increased in HF group (1.2 -fold, Table 1). No difference was observed in VD intake between groups (Table 1).

Serum concentrations of cholecalciferol and metabolites parameters of 4 days high fat diet fed mice.

Serum cholecalciferol concentration was decreased in HF group compared to control group (0.68-fold, Table 1) as well as serum free form of 25(OH)D concentration (0.31-fold, Table 1). Conversely, no difference was observed in serum total of 25(OH)D concentrations between control group (27.9 ± 0.9 ng/mL) and HF group (29.9 ± 0.9 ng/mL). Serum $1.25(OH)_2D$ concentration was increased in HF group (2.78-fold, Table 1). Interestingly, we observed a negative correlation between serum free 25(OH)D concentrations and adiposity index ($r = -0.43$, $p \leq 0.01$) and serum $1.25(OH)_2D$ concentrations ($r = -0,77$, $p \leq 0.01$) and a positive correlation between adiposity index and serum 1.25(OH)2D concentrations ($r = 0.57$, $p \leq 0.01$).

Effect of a 4 days high fat diet on expression of genes coding for enzymes of the vitamin D metabolism in mice.

Expression of genes coding major actors of VD metabolism were studied in the liver, kidney and epididymal white adipose tissue (WAT) of mice (all data presented in supplemental Table 3). We observed a significant decrease of mRNA expression of Cyp3a11, involved in 25-hydroxylation (0.76-fold, Figure 2A), in the liver. In kidney, a decrease of Cyp24a1 and Cyp27b1 expression decreased in HF group (0.42 and 0.34-fold respectively, Figure 2B) whereas Vdr expression was significantly higher in HF group compared to control group (1.15-fold, Figure 2B). In WAT, relative RNA expression of Tnfα, Cyp27a1 and Cyp2r1 were significantly increased in HF group compared to control group (2.23, 1.48 and 1.98-fold respectively, Figure 2C). Conversely, expression of Cyp27b1 and Vdr were decreased in HF group (0.78 and 0.67-fold respectively, Figure 2C).

Quantification of cholecalciferol and metabolites in epididymal WAT of 4 days high fat diet fed mice.

Cholecalciferol and metabolites were quantified in WAT by LCMS/MS. No difference of $1.25(OH)_2D$ concentration was observed whereas concentrations of cholecalciferol and 25(OH)D were decreased in HF group (0.5 and 0.25-fold respectively, Table 2). Quantities of cholecalciferol, 25(OH)D and 1.25(OH)2D in WAT were not modified by the short term high fat diet (Table 2).

Discussion

In this present study, we report for the first time the effect of a shirt term high fat diet on cholecalciferol and metabolites plasma and WAT levels as well as expression of genes involved in VD metabolism in the liver, kidney and WAT.

In order to mimic a food imbalance state associated with weight gain, we have used a short-time (4 days) high-fat diet. This model has the advantage of being able to understand the early effects of an unbalanced diet and the implementation of weight gain by fat mass gain. This model is thus used to study the causal evens that are concomitant with the initiation of weight gain, in contrast to a classical model of high fat diet-induced (generally 10 weeks or more) which mainly reflect consequences of weight gain. As expected, this diet did not affect body weight but increased adiposity index of HF fed mice. Note that the two diets brought the same quantity of VD in both control mice and HF mice, which is a crucial point for this experiment.

Modulation of different parameters were observed in serum of mice. Firstly, we observed no difference in serum total 25(OH)D concentration between the 2 groups. This observation is not in agreement with the passive sequestration hypothesis proposed by Wortsman and al. [15] which suggests that the 25(OH)D plasma decrease observed during obesity is the direct result of adipose tissue expansion. In fact, we observed an increase of adiposity index without modification of total 25(OH)D concentration.

Conversely and in agreement with previously published data [14], we quantified a decrease of serum free 25(OH)D in HF group compared to control group. These results do not support the dilution volumetric hypothesis as reported by Drincic and al. [16] which suggests that plasma

content is more correlated to total volume than fat mass. Indeed, the adiposity index was more correlated than body weight with serum free 25(OH)D ($r = -0.4332$, $p < 0.01$ for adiposity index and $r = -0.1404$, $p = 0.278$ for body weight (data not show)). It is somehow import to remind that all the volumetric dilution theory has been emitted regarding total 25(OH)D and not free 25(OH)D, thus the extrapolability to free 25(OH)D remains questionable and will require further investigations.

In HF group, we observed an increase of serum $1.25(OH)_2D$ concentration compared to control group. Such increase could be linked to the down-regulation of Cyp24a1 in kidney in HF group, and could lead to the decrease of Cyp27b1 mRNA levels of in kidney of HF group through a well-established negative feedback mechanism [4, 24]. In addition, the increase of $1.25(OH)_2D$ is associated to an increase of Vdr mRNA expression [2].

Interestingly, we reported in WAT of HF fed mice, an increase of mRNA levels of 2 enzymes involved in 25-hydroxylation, Cyp2r1 and Cyp27a1,. This data related to the expression of Cyp2r1, which encodes a major enzyme of 25-hydroxylation [25] are innovative and suggest an increased ability of adipose tissue to store VD as 25(OH)D due to the enhanced expression of these 2 enzymes. Park and a1. previously reported an increase of mRNA expression of Cyp27a1 in WAT of mice [18], similarly to Wamberg and a1. in subcutaneous AT of obese patient compared to control patient [17]. In addition, mRNA expression of Cyp27b1 and Vdr were decreased in HF group. Based on these regulations we could speculate that at term $1.25(OH)_2D$ will decrease locally, even if not yet observed (no modification of quantity and concentration of 1.25(OH)2D in WAT). In summary, these data suggested that high fat diet for 4 days is associated with a putative

induction of the ability of adipose tissue to synthesis and to store 25(OH)D. This possible production could participate to decrease of serum cholecalciferol concentration observed in HF group and due to the direct trapping of this hydrophobic molecule in expanded adipose tissue. Interestingly the low cholecalciferol plasma level could be responsible of the low free 25(OH)D. To test these hypothesizes, we quantified VD metabolites in epididymal WAT by LC-MS/MS and we found a decrease of cholecalciferol and 25(OH)D concentration in HF group while no difference was observed between groups in term of cholecalciferol and metabolites quantity, suggesting that the observed gene expression are not yet accompanied of modulation of metabolites quantities. Such modulation of genes expression could thus be considered as a causal even in the modulation of cholecalciferol and metabolites accumulations in WAT that we have recently depicted in long term high fat diet [26]. Altogether theses data support the involvement of an active mechanism mediated by the regulation of gene expression, leading to the storage of cholecalciferol and 25(OH)D in WAT.

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Figure 1: Relationship between morphological data and serum concentration of vitamin D metabolites in mice fed with high fat diet.

Relationship between adiposity index and serum free 25(OH)D (A) and 1,25$(OH)_2$D (B) and relation between serum free 25(OH)D and 1.25$(OH)_2$D (C) in mice fed with control or high fat diet. For unpaired Student's t-test, one-tailed probability, p values: *, $p < 0,05$.

Figure 2: Effect of high fat diet for four days on gene expression of vitamin D metabolism in epididymal WAT in mice.

Expression of genes coding proteins of vitamin D metabolism relative to 18S ribosomal RNA in liver (A), kidney (B) and epidydimal white adipose tissue (WAT; C) of mice fed with control diet (control) or high fat diet (HF) for 4 days (n=10 per group). Values are presented as means ± SEM. For unpaired Student's t-test, p values: *, $p < 0,05$.

Table 1: Mice morphologic, food intake and serum concentration of vitamin D metabolites parameters.

		Control	HF
Body weight (g)		21.45 ± 0.28	22.06 ± 0.45
Liver	Organ weight (g)	875 ± 28.7	850 ± 29.59
	Ratio organ weight / body weight ($x10^{-3}$)	40.73 ± 1.02	38.44 ± 0.68
subcutaneous AT	Organ weight (g)	36.3 ± 5.06	71 ± 3.7 *****
	Ratio organ weight / body weight ($x10^{-3}$)	1.67 ± 0.21	3.21 ± 0.15 *****
epididymal AT	Organ weight (g)	187.4 ± 17.7	282.2 ± 18.2 *****
	Ratio organ weight / body weight ($x10^{-3}$)	8.69 ± 0.77	12.71 ± 0.66 *
peritoneal AT	Organ weight (g)	31.7 ± 3.9	63.7 ± 5.5 *
	Ratio organ weight / body weight ($x10^{-3}$)	1.47 ± 0.19	2.85 ± 0.20 *****
Adiposity index		1.185 ± 0.10	1.87 ± 0.09 *
Energy intake (kcal/g)		14.38 ± 0.44	17.28 ± 0.40 *
Vitamin D intake (UI/g/day)		4.46 ± 0.24	4.52 ± 0.08
Cholecalciferol (ng/mL)		1.196 ± 0.148	0.387 ± 0.098 *
Total 25(OH)D (ng/mL)		27.986 ± 0.998	29.987 ± 0.925
Free form 25(OH)D (pg/mL)		10.879 ± 0.48	7.46 ± 0.60 *
$1.25(OH)_2D$ (ng/mL)		0.548 ± 0.051	1.524 ± 0.137 *

Values are presented as means ± SEM. For unpaired Student's t-test, p values: *****, $p < 0,05$ between control and high fat group (HF) for each condition.

Table 2: Concentration and quantity of vitamin D metabolites in epididymal white adipose tissue quantified by liquid chromatography-tandem mass spectrometry (LC-MS/MS)

	Cholecalciferol (ng/g of tissue)	25(OH)D (ng/g of tissue)	1.25$(OH)_2$D (ng/g of tissue)	Cholecalciferol (ng)	25(OH)D (ng)	1.25$(OH)_2$D (ng)
Control	949.2 ± 193.3	76 ± 6.1	68.4 ± 6.8	174.1 ± 28.6	13.1 ± 1	14.1 ± 1.4
HF	471.9 ± 73.9 *	57.1 ± 3.5 *	63.1 ± 7.8	122.8 ± 12.5	15.3 ± 0.8	17.7 ± 2.5

Quantity of vitamin D metabolites was calculated with concentration X mass of epidydimal adipose tissue. Values are presented as means ± SEM. For unpaired Student's t-test, p values: *, p < 0,05 between control and high fat group (HF) for each condition.

Figure 1

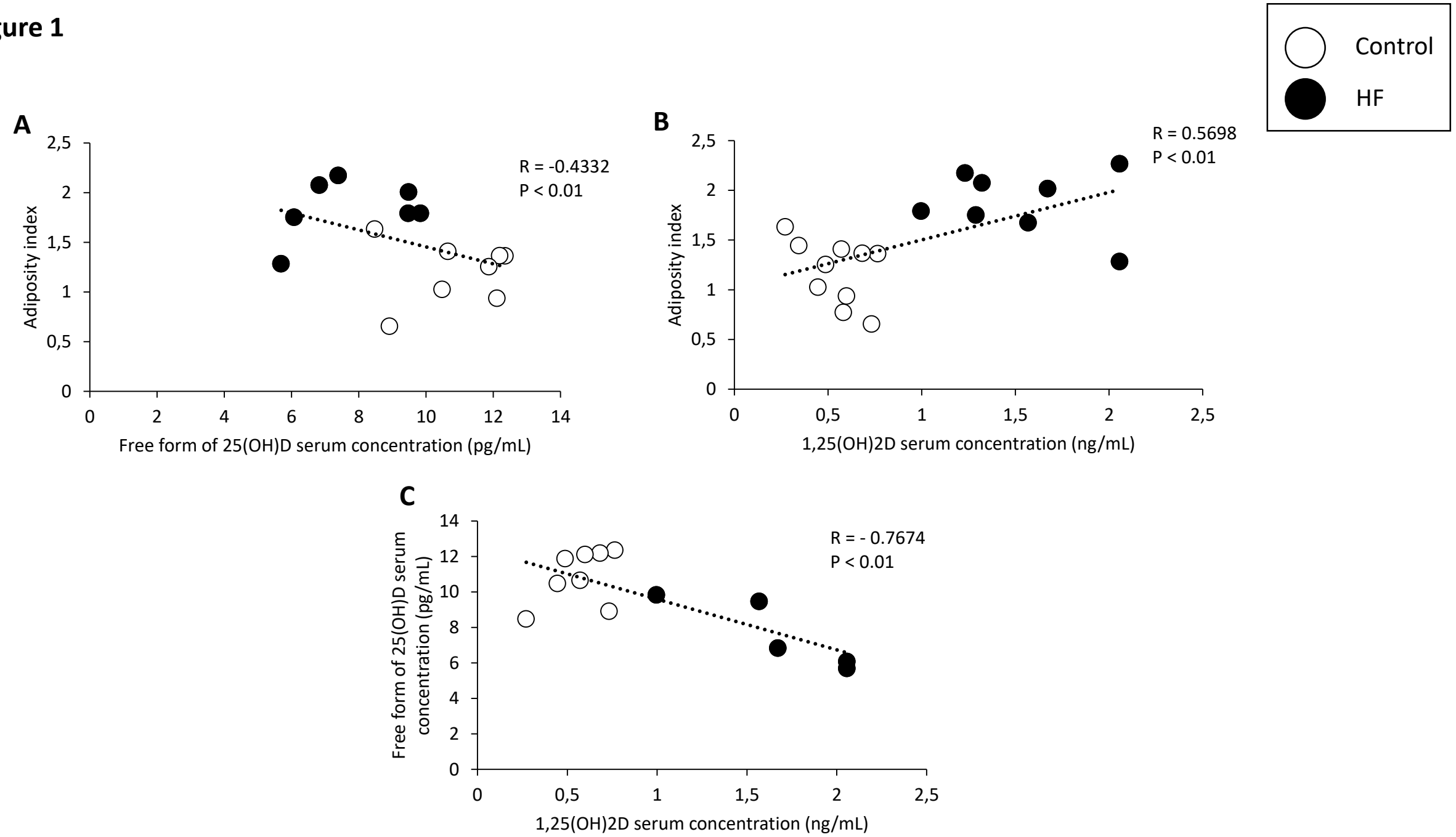
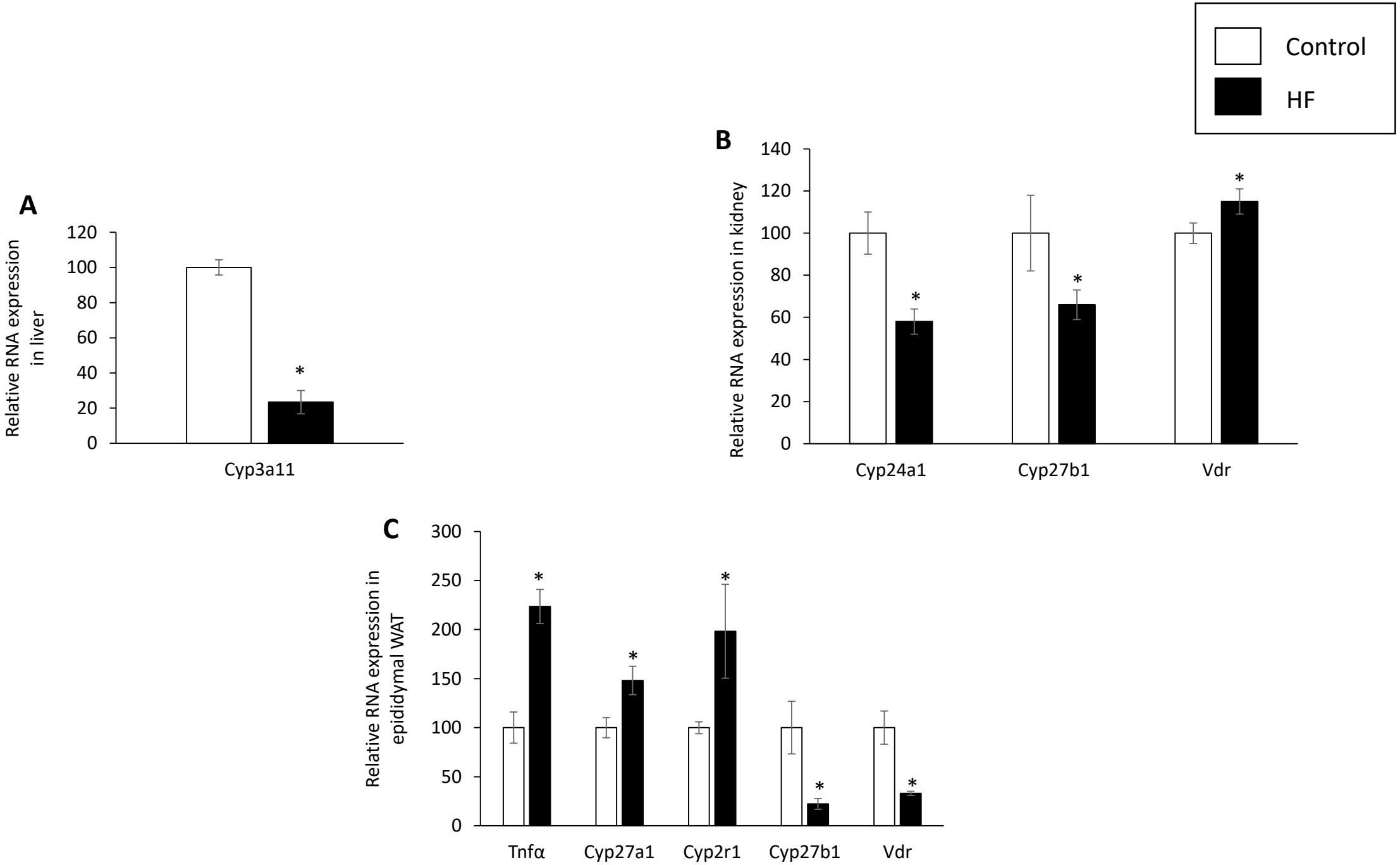

Control
HF
A
Adiposity index
Free form of 25(OH)D serum concentration (pg/mL)
R = -0.4332
P < 0.01
B
Adiposity index
1,25(OH)2D serum concentration (ng/mL)
R = 0.5698
P < 0.01
C
Free form of 25(OH)D serum concentration (pg/mL)
1,25(OH)2D serum concentration (ng/mL)
R = - 0.7674
P < 0.01

Figure 2


Control
HF
A
Relative RNA expression in liver
120
100
80
60
40
20
0
*
Cyp3a11
B
Relative RNA expression in kidney
140
120
100
80
60
40
20
0
*
*
*
Cyp24a1
Cyp27b1
Vdr
C
Relative RNA expression in epididymal WAT
300
250
200
150
100
50
0
*
*
*
*
*
Tnfα
Cyp27a1
Cyp2r1
Cyp27b1
Vdr

Supplemental data

Supplemental table 1: Primers sequences

Gene	Forward sequence	Reverse sequence
Cyp2r1	TTTGTCGGCAACATCTGCT	TGCCTCCAAGATCTAAACTGAAA
Cyp3a11	TGAATATGAAACTTGCTCTCACTAAAA	CCTTGTCTGCTTAATTTCAGAGCT
Cyp27a1	CCTCACCTATGGGATCTTCATC	TTTAAGGCATCCGTGTAGAGC
Cyp2j6	CCCTCTACCCAGAAGTCCAA	TTCTGGCCAATCACCCTATC
Cyp24a1	AAGCCTACGCGCTGATGAT	CACGGGCTTCATGAGTTTC
Cyp27b1	AGTGGGGAATGTGACAGAGC	GGAGAGCGTATTGGATACCG
Megalin	GATGGATTAGCCGTGGACTG	TCCGTTGACTCTTAGCATCTGA
Cubilin	GCCATCCAGATGCAACCT	GGTGCAGACAGGCAACAAG
Vdr	AACCCCTCATAAAGTTCCAGGT	CTGTACCCCAGGTCGGTCT
Gc	CTACCTCAGAGGATTGCATGG	CTTTTTGGATAAGTTTTGACAGATTTT
TNFα	CATCTTCTCAAAATTCGAGTGACAA	TGGGAGTAGACAAGGTACAACCC
18S rRNA	CGCCGCTAGAGGTGAAATTCT	CATTCTTGGCAAATGCTTTCG

Supplemental table 2: Method validation for LC-MS/MS analysis of vitamin D metabolites after Amplifex derivatization.

Analytes	Linear range ng/ml	LOD ng/ml	LOQ ng/ml	Inter-assay CV%	Intra-assay CV%
Vitamin D3	0.78-75	0.049	0.78	14.4	6.0
25OH D3	0.2-75	0.049	0.2	12.5	11.0
1,25(OH)$_2$D3	0.2-75	0.049	0.2	5.5	11.6

Supplemental table 3: Effect of high fat diet on gene expression of vitamin D metabolism in liver, kidney and adipose tissue for 4 days.

	Liver		Kidney		Epididymal AT	
	Control	**HF**	**Control**	**HF**	**Control**	**HF**
Cyp27a1	100 ± 4	99 ± 5	Nm		100 ± 10	148 ± 14 *
Cyp2r1	100 ± 6	89 ± 8	Nm		100 ± 6	131 ± 8 *
Cyp2j6	100 ± 4	116 ± 6	Nm		100 ± 10	117 ± 8
Cyp3a11	100 ± 4	23 ± 2 *	Nm		100 ± 27	51 ± 16
Cyp24a1	Nm		100 ± 10	58 ± 6 *	Nd	
Cyp27b1	Nm		100 ± 18	66 ± 7 *	100 ± 27	22 ± 5 *
Vdr	100 ± 13	83 ± 7	100 ± 5	115 ± 6 *	100 ± 17	33 ± 2 *
Gc	100 ± 4	87 ± 5 *	100 ± 8	63 ± 6	100 ± 20	55 ± 14
Megalin	100 ± 9	140 ± 18 *	100 ± 4	108 ± 4	100 ± 22	188 ± 75
Cubilin	No expression		100 ± 5	95 ± 3	100 ± 21	52 ± 4 *
TNFα	Nm		Nm		100 ± 16	223 ± 17 *

Expression of genes coding proteins of vitamin D metabolism relative to 18S ribosomal RNA in liver, kidney and epidydimal adipose tissue (AT) of mice fed with control diet (control) or high fat diet with 60% energy from lipids (HF60) for 4 days (n=10 per groups). Values are presented as means ± SEM. Nm is gene no measured and Nd is gene no detected. For unpaired Student's t-test, p values: *, $p < 0,05$.

ARTICLE 5 : Evolution of vitamin D metabolism and storage in AT during the development of high-fat diet induced obesity in mice.

Article soumis à Journal of Steroid Biochemistry and Molecular Biology.

Comme nous l'avons montré précédemment (**Article 5**), un régime riche en graisse apporté à des souris pendant 4 jours, mimant des évènements précoces de la survenue de l'obésité, modifie les concentrations plasmatiques de certains métabolites de la vitamine D ce qui peut être expliqué par la régulation des gènes impliqués dans ce métabolisme au niveau du foie, du rein et du tissu adipeux. Au vu de ces résultats, nous avons voulu étudier l'effet d'un régime riche en graisse à 7 et 11 semaines, mimant le développement de l'obésité, chez des souris, sur la modification de l'expression génique des acteurs impliqués dans le métabolisme de la vitamine D, principalement au niveau du tissu adipeux, afin d'apporter une explication à la modification du statut en vitamine D retrouvée au cours de l'obésité.

Nous avons eu recours à un modèle murin expérimental constitué de deux groupes de souris, l'un nourri avec une alimentation riche en graisse avec 10% d'énergie sous forme de lipides (groupe contrôle) et l'autre avec 60% d'énergie sous forme de lipides (groupe obèse), pendant 7 et 11 semaines.

Nous avons mesuré les taux plasmatiques et adipeux des différents paramètres biochimiques du métabolisme de la vitamine D soit par test ELISA soit par la méthode LC-MS/MS mis au point dans le laboratoire (**Article 7**). Nous avons ensuite analysé l'expression génique des différents acteurs impliqués dans le métabolisme de la vitamine D au niveau du foie, du rein et du tissu adipeux par qPCR.

PRINCIPAUX RESULTATS DE L'ETUDE

Au cours de cette étude, nous avons pu mettre en évidence qu'un régime riche en graisse, apportant les mêmes quantités en vitamine D alimentaire entre les groupes, pendant 7 et 11 semaines, était associé avec une modification du métabolisme de la vitamine D au niveau plasmatique ainsi qu'à une modification de l'expression des gènes du métabolisme de la vitamine D au niveau du foie, du rein et du tissu adipeux.

En effet, les souris nourries avec une alimentation riche en graisse pendant 7 et 11 semaines avaient une augmentation du poids et de l'index d'adiposité. Ces modifications morphologiques étaient associées à une modification des concentration plasmatiques des différents paramètres biochimiques en lien avec le métabolisme de la vitamine D : 1) diminution du cholécalciférol et augmentation de la 25(OH)D totale à 7 semaines qui peuvent être expliquées par l'augmentation de l'expression génique des enzymes impliquées dans la 25-hydroxylation (Cyp27a1, Cyp2r1 et Cyp2j6), 2) augmentation de la PTH sans modification de la $1,25(OH)_2D$ à 7 et 11 semaines, et 3) diminution de la 25(OH)D libre à 11 semaines seulement. Dans ce modèle, nous ne retrouvons pas la modification attendue du statut en 25(OH)D totale ce qui peut être expliquer par l'apport alimentaire en vitamine D équivalent retrouvé entre les groupes.

Les résultats obtenus au niveau du tissu adipeux suggèrent une production et un stockage actif de la 25(OH)D au niveau du tissu adipeux chez les souris nourries avec une alimentation riche en graisse pendant 11semaines. En effet, il a été reporté une augmentation de l'expression des ARNm codants pour la Cyp2r1, enzyme impliquée dans le 25-hydroxylation, et une diminution de ceux codants pour la Cyp27b1, enzyme impliquée dans la 1-hydroxylation, ainsi qu'une augmentation de la quantité de cholécalciférol et de 25(OH)D dans le tissu adipeux des souris HF. En conclusion, le tissu adipeux joue un rôle actif dans la modification du métabolisme vitamine D observée pendant l'obésité.

Evolution of vitamin D metabolism and storage in adipose tissue during the development of high-fat diet induced obesity in mice.

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Abbreviated title : Obesity impacts vitamin D metabolism.

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Abstract

Low circulating levels of total and free 25(OH)D, indicative of vitamin D (VD) status, have been associated with obesity in human. Moreover, obesity is supposed to display a causal role on the reduction of 25(OH)D and several theories have been emitted to explain this relationship. In the present study, we hypothesized that obesity disrupted VD homeostasis in key organs of its metabolism. To this aim male C57BL6 mice were fed for 7 or 11 weeks with either a control diet (LF, 10% energy from fat) or a high fat diet (HF, 60% energy from fat), and diets were formulated to provide equivalent VD intake in both groups. After 7 weeks, we observed a transient increase of total 25(OH)D together with a strong decrease of cholecalciferol in plasma, that could be related to the induction of hepatic genes involved in 25-hydroxylation. After 11 weeks of regimen, no modification of total 25(OH)D was observed but a significant decrease of free 25(OH)D and cholecalciferol plasma levels. In adipose tissue an increase of 25(OH)D was also quantified, and this parameter was inversely correlated to the free 25(OH)D. Interestingly this accumulation of 25(OH)D in AT was highly correlated to the induction of Cyp2r1, which could actively participate to the trapping of cholecalciferol and subsequent conversion to 25(OH)D in adipose tissue. Together, our data strongly suggest that the enzymes involved in VD metabolism, notably in adipose tissue, are transcriptionally modified under high diet, contributing to the reduction of the free 25(OH)D associated to obesity.

Keywords : obesity, high fat diet, vitamin D, metabolism, adipose tissue, free 25(OH)D

Introduction

Vitamin D (VD) is a lipophilic hormone playing key roles in phosphocalcic homeostasis and bone metabolism [1], but also displays many other biological functions [2]. Two main origins have been depicted for VD, one part provided by the diet, mainly as cholecalciferol, the other part resulting of endogenous production [1, 3, 4]. To become active, the native cholecalciferol must be converted in the liver into 25(OH)D, this hydroxylation step is catalysed by 4 enzymes (CYP2R1, CYP27A1, CYP2J6 and CYP3A11) [5]. A second hydroxylation step occurs in the kidney by CYP2B1 to produce $1,25(OH)_2D$, the active cholecalciferol form, which is a potent activator of the vitamin D receptor (VDR) [6]. 25(OH)D and $1,25(OH)_2D$ can be catabolized by 24-hydroxylase, CYP24A1, to generate inactive metabolites [7].

The VD status is classically reflected by the total plasma 25(OH)D concentration, which represents the sum of free 25(OH)D and 25(OH)D bound to vitamin D binding protein (DBP, encoded by Gc gene) and albumin [8, 9]. Interestingly, this status is impacted by many physio-pathological parameters including obesity, which is classically associated to a decrease of total 25(OH)D [10, 11]. Indeed, 25(OH)D plasma levels are inversely correlated to all the parameters of obesity including: BMI, fat mass and waist circumference [12, 13]. In addition, it has recently been shown that the free forms of 25(OH)D and $1,25(OH)_2D$ were also decreased during obesity [14]. Concerning the low total 25(OH)D level observed in obese people, several hypotheses have been emitted: 1) impaired hepatic 25-hydroxylation linked to high levels of $1,25(OH)_2D$ and parathyroid hormone (PTH) [15]; 2) sequestration of VD in adipose tissue (AT) caused by a passive phenomenon due to the hydrophobic nature of VD [16]; 3) dilution of 25(OH)D in a higher volume in obese subjects (volumetric dilution concept [17]). More recently, Wamberg suggested that VD

64 metabolism in AT is modified by obesity. Indeed, they reported a modification of Cyp2j2
65 expression in biopsies of obese compared to lean patients [18]. Consistently, Park et al. depicted
66 the effect of a high fat diet on the expression of VD metabolizing enzyme in mice [19].
67
68 To go further in the identification of obesity impact on VD status and metabolism in mice and
69 notably its consequences on free 25(OH)D, a new parameter to consider, we implemented a
70 longitudinal study of high fat diet induced-obesity. Several biochemical parameters and expression
71 of genes coding for VD metabolizing enzymes were analysed in the main organs of the VD
72 metabolism and storage, i.e. in the liver, in the kidney and in white adipose tissue.

Materials and methods

Reagents - TRIzol reagent, random primers, and Moloney murine leukemia virus reverse transcriptase were obtained from Life Technologies (Courtaboeuf, France). SYBR Green reaction buffer was purchased from Eurogentec (Liege, Belgium).

Animal Experiments – The protocol received the agreement of the local ethics committee (APAFIS#2595-2016091911217758). Six week-old male C57BL/6J mice were obtained from Janvier Labs (Le Genest Saint Isle, France), fed ad libitum with control food (maintenance diet A04 from Safe-diets, Augy France) during the 1-week acclimation period and with full access to drinking water. The animals were maintained at 22°C under a 12-hour light, 12-hour dark cycle a 20% humidity level. Mice were divided into control diet group (LF: 10% energy from lipids, n = 10) or high fat diet group (HF: 60% energy from lipids, n = 10) (TestDiet, London, UK). The experimental diet composition is detailed in supplemental table 1. Weight gain was measured once a week and dietary intake, every two weeks. After 7 weeks or 11 weeks of diet, mice were fasted overnight and blood was collected by cardiac puncture under anesthesia, plasma was obtained by centrifugation at 3000 rpm for 15 min at 4°C and was stored at -80°C. Animals were sacrificed by cervical dislocation and tissues (kidney, liver and epididymal white adipose tissue) were collected, weighted, snap frozen in liquid nitrogen and stored at -80°C.

RNA extraction and real-time PCR – Total RNA was extracted from the liver, kidney and epididymal white adipose tissue using TRIzol reagent (Thermofisher, Courtaboeuf, France). One µg of total RNA was used to synthetized cDNAs using random primers and Moloney murine

leukemia virus reverse transcriptase (Thermofisher). Real-time quantitative PCR analyses were performed using the Mx3005P Real-Time PCR System (Stratagene, La Jolla, CA) as previously described [20]. For each condition, expression was quantified in duplicate, and 18S rRNA was used as the endogenous control in the comparative cycle threshold (CT) method [21]. Primers sequences are reported in supplemental table 2.

Protein quantification by ELISA – Parathyroid hormone (PTH) concentration in mice plasma was quantified using ELISA (Parathyroid hormone PTH, Euromedex, Strasbourg, France). Free form of 25(OH)D was also quantified by ELISA (DIAsources, Louvain-La-Neuve, Belgique). Colorimetric assay kits were used to quantify calcium and phosphate protein concentrations in mice plasma (Clinisciences, Nanterre, France).

Cholecalciferol, 25(OH)D and 1,25(OH)2D quantification in plasma and WAT - All quantifications were performed using LC-MS/MS according to the following protocol.

Preparation of analytical and deuterated standards – A working solution of deuterated analytes was prepared at 0,02 ng/mL of each internal standards (IS) (d3-cholecalciferol, d3-25(OH)D and d3-1,25(OH)2D). A primary stock solution of cholecalciferol, 25OHD and 1,25$(OH)_2$D standards were prepared at concentrations respectively of 100, 50 and 10 ng/ml in ethanol and stored at -80°C in the dark. Calibration curves were prepared by serial dilution of the 3 analytes stock solution to obtain calibration standards from 0 to 75ng/mL and addition of 1.5 µl of the working solution of deuterated analytes to each dilution. After complete evaporation of solvent, derivatization was proceeded. A one-step derivatization was employed to improve the ionization efficiency of the metabolites using Amplifex diene (Amplifex TM Diene Reagent, Sciex

Chemistry and consumables R&D, Framingham, MA) as reagent [22]. 30 µL of the Amplifex was added to the dried sample above, vortexed for 15 s and incubated for 30 min at ambient temperature. Next, 30 µL of deionized water was added, vortexed for 15 s and transferred for LC injection. Calibration curves were plotted with peak area ratio of the vitamin D metabolite to the respective internal standard versus a range of concentrations of the analyte.

Preparation of plasma - Sample preparation was adapted from Wang et al. [23]. The extraction procedure was conducted under low light because cholecalciferol and its metabolites are light sensitive. After thawing on ice, mice plasmas were centrifuged at 4°C for 15 min, at 11 000 rpm. 100 µL of each sample was transferred to a glass test tube containing 10 µL of deuterated standards working solution. Proteins were precipitated by adding acetonitrile, vortex-mixed, and centrifuged for 10 min at 3 000 g. The supernatant was moved to another glass tube, and the volume was reduced to a half under a nitrogen stream. 5 mL of ethyl acetate was adding to the solution for liquid-liquid extraction. After shaking vigorously, samples were centrifuged for 20 min at 590 g and the upper organic layer was transferred into a new glass tube and was reduced under nitrogen stream. The samples were derivatized as described above.

Epididymal WAT preparation – Sample preparation was adapted from Lipkie et al. [24]. 25 µL of deuterated standards working solution were added to tissue homogenates (50 mg of tissue grinded within 1 mL of PBS) in glass test tube. Acetonitrile (ACN) was added, vortex mixed for 5 min and centrifuged at 6 000 g for 5 min. Then, methyl tert-butyl ether (MTBE) was added, vortexed for 5 min, centrifuged, and the upper organic layer was collected in another glass tube. The extraction was repeated twice and the combined supernatants were dried under nitrogen. Oasis HLB SPE cartridges (Waters, Guyancourt, France) were conditioned with ethyl acetate, methanol (MetOH) and H_2O. The sample was reconstituted with 1 mL of MetOH and 1 mL of K_2HPO_4 (0.4 M) and

added on the cartridge. The cartridge was washed with H_2O and 70% MetOH and dried for 2 min under vacuum. Tips were washed with ACN and analytes were eluted with ACN and dried under nitrogen. After complete evaporation of solvent, the samples were derivatized as described above.

LC-MS/MS analysis – Accurate mass measurements were performed on the Q-Exactive Plus mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a Heated Electrospray Ionization (H-ESI II) probe. Thermo Xcalibur 3.0.63 software was used for the instrument setup, control of the LC-MS system during acquisition and data treatment. Tune Q Exactive Plus 2.5 application was used for the direct control of the mass spectrometer.

Samples were injected onto a Hypersil GOLD C18 column (2.1 x 100 mm; Thermo Scientific, Les Ulis, France). Flow rate was 0.4 mL/min and injection volume was 5 µL. The mobile phase was composed of A= ultrapure water with 0.1% formic acid (FA) (v/v), and B = acetonitrile with 0.1 % FA (v/v). Starting conditions were A= 70% and B = 30% and were held for 4 min. A linear gradient was applied until 10.0 min where A= 35% and B = 65%, these conditions were held until 12.0 min, at 14 min A=0% and B = 100% until 16 min. Starting conditions were re-implemented at 18 min.

The SRM transitions used for quantification for each analyte were: 716.5→657.5 (Cholecalciferol), 719.5→660.5 (d3-Cholecalciferol), 732.5→673.4 (25OH-D), 735.5→676.4 (d3-25OH-D), 751.5→692.4 (d3-1,25OH2-D) and 748.5→689.4 (1,25OH2-D).

Validations were performed for linearity and repeatability data (Supplemental Table 2).

Statistical analysis - The data are communicated as the mean ± SEM. Significant differences were determined using ANOVA, followed by the Tukey-Kramer post hoc test, and two-way ANOVA

using Statview software (SAS Institute, Cary, NC). $p < 0.05$ was considered statistically significant.

Results

Impact of high fat diet on morphological parameters of mice.

Mice were fed for 7 or 11 weeks with low fat (LF) or high fat (HF) diet. As expected, this diet led to an increase of body weight, liver weight and adiposity index in HF group at both 7 weeks and 11 weeks, compared to LF diet (Table 1). Concerning body weight, we observed a time and diet effect as well as a statistical interaction between time and diet, whereas in the case of liver weight and adiposity index, only time and diet effect were observed (Table 1). Food intakes were quantified. Energy intake was similar between HF and LF group. Cholecalciferol intake was calculated and no difference between groups was observed (Table 2).

Impact of high fat diet on plasma parameters related to VD metabolism of mice and quantities of cholecalciferol and metabolites in adipose tissue.

Plasma concentration of various parameters related to VD metabolism were quantified in mice submitted to LF or HF diet. After 7 weeks of HF diet, serum cholecalciferol concentration was decreased while plasma total 25(OH)D and parathyroid hormone (PTH) concentration were increased compared to LF group (Table 2). No difference was observed on plasma free form of 25(OH)D, $1,25(OH)_2D$, calcium and phosphate concentrations between groups. After 11 weeks of HF diet, we observed an increase of PTH concentration and no modification of cholecalciferol, total 25(OH)D, $1,25(OH)_2D$, calcium and phosphate plasma concentrations in HF group (Table 2). Interestingly, a decrease of the plasma free form of 25(OH)D was measured in HF group compared to control group (from 6.77 ± 0.21 pg/ml to 5.94 ± 0.26 pg/ml; $p<0.05$). This plasma free form of

25(OH)D appeared to be inversely correlated with body weight of mice (Fig. 1A), adiposity index (Fig. 1B) and plasma PTH (Fig. 1C).

Since adipose tissue (AT) is considered as a major cholecalciferol and metabolites storage site, its concentration and concentrations of 25(OH)D and $1,25(OH)_2D$ were quantified by LC-MS/MS in epididymal white adipose tissue (eWAT; Supplemental Table 4), and quantities were calculated (concentration X mass of epidydimal adipose tissue; Table 3). After 7 weeks of diet, we observed a significant increase of 25(OH)D quantity in HF group compared to LF group (Table 3). After 11 weeks of diet, a significant increase of cholecalciferol and 25(OH)D quantity was observed in eWAT of HF fed mice (Table 3). In the case of 25(OH)D eWAT quantity, the two-way analysis revealed a time and diet effect as well as a significant interaction of the 2 parameters. Interestingly, this 25(OH)D quantity in eWAT at 7 and 11 weeks was inversely correlated to plasma free form 25(OH)D ($p<0.01$, Fig. 1). No difference between groups was noted of $1,25(OH)_2D$ quantity in eWAT at both times (Table 3). In terms of concentration in eWAT, a decrease was observed for cholecalciferol at both times under HF effect, as well as a time effect, but no effect was observed concerning 25(OH)D and $1;25(OH)_2D$ (Supplemental Table 4).

Impact of high fat diet on gene expression in liver, kidney and eWAT of mice.

The expression of genes coding for VD metabolism proteins was measured by real-time PCR in liver, kidney and eWAT (all data presented in supplemental Table 5 and 6). After 7 weeks of regimen, the expression of genes coding for 3 hepatic enzymes involved in 25-hydroxylation, Cyp2r1, Cyp27a1 and Cyp2j6 were significantly upregulated in HF group (Fig. 2), whereas after 11 weeks of HF diet, only Cyp2r1 gene expression remained higher compared to LF diet (Fig. 2). In two-way ANOVA analyses, the expression of Cyp27a1, and Cyp2j6 was dependent of time,

212 diet and an interaction between the 2 factors was observed whereas Cyp2r1 was only dependent
213 of time and diet. In kidney, after 7 weeks of HF diet, mRNA expression of Cyp24a1 was decreased
214 compared to LF group (Supplemental table 5). After 11 weeks, the expression of Cyp27b1 was
215 increased and the expression of Cyp24a1 was decreased in HF group (Supplemental table 6). In
216 eWAT, after 7 weeks, the expression of Cyp2r1 and Vdr were induced in HF group. At 11 weeks
217 of regimen, Cyp2r1, Cubilin and Vdr were induced whereas Cyp27a1, Cyp2j6 and Cyp27b1 were
218 down-regulated in HF group compared to LF group (Fig. 3). Interestingly the induction of Cyp2r1
219 observed at 7 weeks (2-fold) was more pronounced at 11 weeks (3-fold) in HF vs. LF group. In
220 addition, the two-way ANOVA analysis revealed a time and diet effect as well as a significant
221 interaction of the 2 parameters only for this gene (Fig. 3).
222 Correlations were established between the expression of Cyp2r1 gene in eWAT and the body
223 weight of mice, weight of eWAT and free 25(OH)D plasma levels (Fig. 4). Cyp2r1 expression was
224 strongly correlated with eWAT mass ($r = 0.75$, $p < 0.01$) and mice body weight ($r = 0.78$, $p < 0.001$).
225 On the other hand, a negative correlation was found between the expression of Cyp2r1 and the
226 concentration of free 25(OH)D ($r = -0.62$; $p < 0.001$).
227
228 .

Discussion

The primary objective of our study was to demonstrate in a murine model the impact of obesity on VD metabolism and notably the effect on free 25(OH)D plasma level as recently described in humans [14] and to study gene expression in the main organs involved in VD metabolizing to bring mechanistic elements to these observations.

As expected, the HF regimen used in our study of weight gain led to a significant increase in the total mass of the animals as well as in the amount of adipose tissue and the adiposity index at the 2 times studied, i.e. 7 and 11 weeks. These morphological changes were not accompanied by major changes in energy intake or in the consumption of cholecalciferol. This latter parameter was particularly important to control in our case, since a modification of the cholecalciferol intake induced by a poor equilibration of the diet alone could have led to a VD deficiency. However, despite this cholecalciferol equilibrium throughout the diet, there was a 7-week decrease in plasma cholecalciferol content and an increase total 25(OH)D and PTH concentration. To our knowledge, no other study reported this observation apart from PTH. Indeed, PTH concentration is known to be positively correlated with fat mass [25] and body mass index [14]. If the reduction of cholecalciferol is probably due to the direct trapping of this hydrophobic molecule in expanded adipose tissue, the fact that cholecalciferol decreased whereas total 25(OH)D was increased could be linked to the overall induction of mRNA coding for the hepatic 25-hydroxylation. Indeed, even if more 25(OH)D is stored in adipose tissue due its enlargement, the fact that 25-hydroxylation is induced explain at least in part the reduced cholecalciferol levels. It is somehow surprising that these 25-hydroxylation enzymes were induced in our conditions since it has already been reported

252 a down-regulation in similar experimental condition [19], the origin of such discrepancy is
253 presently unknown, but could results from an adaptive process to high fat diet that occurs in this
254 window of time. In agreement, the increase of total 25(OH)D was not reported at 11 weeks in our
255 study and after 18 weeks of HF diet [19].
256
257 At 11 weeks, results related to free 25(OH)D, detected with ELISA kit, are consistent with a recent
258 study depicting similar results in obese subjects [14], however this clinical study also reported a
259 decrease of total 25(OH)D and an increase of $1,25(OH)_2D$, which was not the case in our study.
260 These discrepancies are not presently well understood but could be due to the fact that mice
261 received equal doses of cholecalciferol contrary to humans whose VD intake and endogenous
262 production are difficult to control. This is also noteworthy that the decrease of free 25(OH)D was
263 observed only for long term HF diet (11 weeks), after 7 weeks no modifications was observed,
264 suggesting a combined effect of aging and HF diet. Concerning $1,25(OH)_2D$, despite the secondary
265 hyperparathyroidism linked to obesity observed at 7 and 11 weeks, no modification of $1,25(OH)_2D$
266 was detected, even in presence of a strong decrease of Cyp24a1 observed in kidney (Supplemental
267 Tables 5 and 6), and an increase of Cyp27b1 at 11 weeks, similarly to recently reported by Park et
268 al. [19]. Discrepancies related to $1,25(OH)_2D$ quantification methodologies could be envisioned
269 (LC-MS/MS in our case vs. ELISA kit in Park et al. study).
270
271 In a quite interesting manner, no decrease in plasma levels of free 25(OH)D or total 25(OH)D at
272 7 weeks of HF diet was observed, although mice had already taken a lot of fat mass. This
273 observation does not fit with the hypothesis of Wortsmann et al. [16] who suggested that the
274 25(OH)D plasma decrease observed during obesity is the direct result of adipose tissue expansion.

However, it should be pointed out that the difference in fat mass gain between LF mice and HF mice is greater at 7 weeks than at 11 weeks (adiposity index increased by 2.74 at 7 weeks and by 2.09 at 11 weeks), whereas the difference in total body weight gain was greater at 11 weeks than 7 (total body weight increased by 1.21 at 7 weeks and by 1.33 at 11 weeks), suggesting that the decrease in the free 25(OH)D content at 11 weeks is better correlated with weight gain than with fat mass gain. These observations are therefore more in line with the volumetric dilution hypothesis of Drincic et al. [17] which suggests that plasma content is more correlated to total volume than fat mass. Indeed, in our paper, correlation is better between free 25(OH)D and body weight ($r = -0.53$) than free 25(OH)D and adiposity index ($r = -0.38$). It is somehow important to remind that these hypotheses have been emitted regarding total 25(OH)D and not free 25(OH)D, thus the extrapolability to free 25(OH)D remains questionable and will require further investigations.

We reported strong correlations between free 25(OH)D concentration and the various morphological parameters tested (total body weight, PTH level, adiposity index), while these relationships were non-existent at 7 weeks (data not shown). In this way, we could speculate that free 25(OH)D in plasma could a better marker of VD status than total 25(OH)D during obesity in agreement with the "free hormone hypothesis" [26]. Interestingly, we also reported that the plasma level of free 25(OH)D was inversely correlated to the quantity of 25(OH)D in adipose tissue, suggesting that the more adipose tissue expands during obesity, the more 25(OH)D is stored in adipose tissue thus reducing the free part of 25(OH)D in the plasma. In addition to a potential dilution effect, these data suggest that the stability of free 25(OH)D at 7 weeks could be the result of a balance between its clearance and synthesis. Indeed, the decrease in free 25(OH)D does not appear only as a result of an increase in the volume of the animals but also due to a blunting of an

298 adaptive process leading to a loss of homeostasis. However, this kinetic evolution of the plasma
299 level of free 25(OH)D during obesity between 7 weeks and 11 weeks clearly supports a causal role
300 of obesity in this decrease, as previously mentioned [10, 27]. Nevertheless, this decrease in free
301 25(OH)D content could in turn contribute to amplify obesity, since we have previously shown that
302 cholecalciferol supplementation limits the occurrence of obesity induced by a HF diet by
303 decreasing lipid oxidation [28, 29].
304
305 In order to provide some mechanistic explanations for our observations, we undertook a study of
306 gene expression notably in adipose tissue. The results shown an increase in Cyp2r1 expression in
307 7-week and 11-week HF mice with an expression level of 2.4-fold higher at 11 weeks than at 7
308 weeks, as well as a decrease of the expression of Cyp27b1 which encodes the enzyme involved in
309 the 1,25 hydroxylation at 11 weeks. A decrease in the expression of Cyp27b1 in the subcutaneous
310 AT of obese subjects compared to normal subjects was already reported by Wamberg et al. [18],
311 and in mice [19]. Nevertheless, data on the expression of Cyp2r1 encoding a major enzyme of 25-
312 hydroxylation [30] are innovative and suggest an increased ability of adipose tissue to store VD as
313 25(OH)D. In addition, this Cyp2r1 is inversely correlated with total body weight and epididymal
314 fat mass, suggesting that obesity is associated with an induction of the ability of adipose tissue
315 production of 25(OH)D, and a subsequent reduction of free 25(OH)D as previously evoqued. To
316 validate this hypothesis, we quantified cholecalciferol and 25(OH)D in adipose tissue. In the case
317 of cholecalciferol, we observed that the HF diet decreased cholecalciferol concentration but
318 increased the AT quantity in a similar way at 7 and 11 weeks (1.93 and 2-fold respectively). In
319 agreement, Carrelli and al. quantified ergocalciferol and cholecalciferol in subcutaneous and
320 omental AT of lean and obese women by LC-MS/MS [31]. Their data demonstrated that obese

subjects have greater adipose stores of ergocalciferol and cholecalciferol and supported the hypothesis that the large amount of adipose tissue in obese individuals serves as a reservoir for VD.

Concerning 25(OH)D in eWAT, no modification of concentration in eWAT were observed whereas the AT quantity was more increased at 11 weeks than at 7 weeks (3.2 and 2.7-fold respectively) suggesting that increase of 25(OH)D quantity in eWAT resulted of an induction of production between 11 and 7 weeks, consistent with the induction of Cyp2r1.

Despite a decreased expression of Cyp27b1 and increased expression of Vdr which suggested a local activation of VD signaling, we observed no modification of the concentration and quantity of $1,25(OH)_2D$ in eWAT which could be due to the very low level of 1,25(OH)2D in adipose tissue which is really near of the quantification limit.

To conclude, in our model of obesity induced by HF diet but with equal cholecalciferol intake, we observed a transient increase of total 25(OH)D together with a decrease of cholecalciferol in plasma that could be due to the up-regulation of 25-hydroxylases genes in the liver. On longer period, we reported a decrease of free 25(OH)D, strongly associated to the induction of Cyp2r1 in AT, which could be responsible of an active production and storage of 25(OH)D in AT as highlighted by direct quantification in AT. Together our data suggest that AT display an important and active role in the VD metabolism modification observed during obesity.

342 **Legends for Figures**

343

344 **Figure 1: Relationship between biochemical and morphological parameters and free**
345 **25(OH)D in high fat diet fed mice.**

346 Correlation between plasma free 25(OH)D concentration and body weight at the end (A) and
347 adiposity index (B) at 7 and 11 weeks of diet, and plasma PTH concentration (C) at 11 weeks.
348 Correlation between the quantity of 25(OH)D in eWAT and plasma free 25(OH)D.

349

350 **Figure 2: Effect of high fat diet on hepatic vitamin D metabolism of mice.**

351 Expression of genes coding proteins of vitamin D metabolism relative to 18S ribosomal RNA in
352 liver of mice fed with control diet (low fat, LF) or high fat diet (HF) for 7 weeks (LF n=10, HF
353 n=10). Values are presented as means ± SEM. Bars not sharing the same letter were significantly
354 different in Tukey-Kramer post hoc test $p < 0,05$. T, time effect in two-way ANOVA analysis (p
355 $< 0,05$); D, diet effect in two-way ANOVA analysis ($p < 0,05$); TxD, interaction between time and
356 diet in two-way ANOVA analysis ($p < 0,05$).

357

358 **Figure 3: Effect of high fat diet on adipose tissue vitamin D metabolism of mice.**

359 Expression of genes coding for proteins involved in vitamin D metabolism relative to 18S
360 ribosomal RNA in epididymal adipose tissue (AT) of mice fed with control diet (low fat, LF) or
361 high fat diet (HF) for 7 weeks (LF n=10, HF n=10). Values are presented as means ± SEM. Bars
362 not sharing the same letter were significantly different in Tukey-Kramer post hoc test $p < 0,05$. T,

363 time effect in two-way ANOVA analysis ($p < 0,05$); D, diet effect in two-way ANOVA analysis
364 ($p < 0,05$); TxD, interaction between time and diet in two-way ANOVA analysis ($p < 0,05$).
365
366 **Figure 4: Correlation between Cyp2r1 expression in adipose tissue and biochemical and**
367 **morphological parameters in mice.**
368 Correlation between adipose tissue Cyp2r1 mRNA levels and mice body weight (A), epididymal
369 white adipose tissue mass (B) and plasma free 25(OH)D (C).
370
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372

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Table 1: Mice morphologic parameters

	7 weeks		11 weeks		
	LF	**HF**	**LF**	**HF**	**Two-way ANOVA**
Body weight at start (g)	22.4 ± 0.22 [a]	22.2 ± 0.13 [a]	22.4 ± 0.22 [a]	22.2 ± 0.13 [a]	
Body weight at the end (g)	29.2 ± 0.44 [a]	35.4 ± 1 [b]	31.5 ± 0.5 [b]	42.2 ± 1.32 [c]	T, D and TxD
Liver weight (mg)	0.87 ± 0.03 [a]	0.97 ± 0.02 [a]	0.96 ± 0.02 [a]	1.21 ± 0.09 [b]	T, D
Adiposity index	3.12 ± 1.02 [a]	8.56 ± 2.9 [b]	4.9 ± 1.35 [a]	10.25 ± 0.86 [b]	T, D

Values are presented as means ± SEM. n = 10 for each group. Bars not sharing the same letter were significantly different in Tukey-Kramer post hoc test $p < 0.05$ between control group (low fat, LF) and high fat group (HF) for 7 and 11 weeks and each condition. T, time effect in two-way ANOVA analysis ($p < 0.05$); D, diet effect in two-way ANOVA analysis ($p < 0.05$); TxD, interaction between time and diet in two-way ANOVA analysis ($p < 0.05$).

Table 2: Food intake parameters

		Energy intake (kcal/g)	Vitamin D intake (UI/g/day)
7 and 11 weeks	**LF**	14.4 ± 0.81	3.44 ± 0.19
	HF	14.17 ± 0.46	3.61 ± 0.18

Values are presented as means ± SEM. Student's t-test was used, p values: *. $p < 0.05$ between control group (low fat, LF) and high fat group (HF) for 7 and 11 weeks and each condition.

Table 3: Biochemical parameters

	7 weeks		11 weeks		
	LF	**HF**	**LF**	**HF**	**Two-way ANOVA**
Plasma Cholecalciferol (ng/mL)	2.05 ± 0.36 [a]	0.60 ± 0.10 [b]	2.58 ± 0.13 [a]	1.27 ± 0.12 [a,b]	D
Plasma total 25(OH)D (ng/mL)	34.80 ± 1.17 [a]	40.56 ± 1.07 [b]	38.40 ± 0.89 [a,b]	42.70 ± 1.42 [b]	T, D
Plasma free 25(OH)D (pg/mL)	6.62 ± 0.15 [a,b]	6.37 ± 0.17 [a,b]	6.77 ± 0.21 [a]	5.94 ± 0.26 [b]	D
Plasma 1.25(OH)$_2$D (pg/mL)	374 ± 20 [a]	376 ± 27 [a]	240 ± 21 [b]	239 ± 9 [b]	T
Plasma PTH (pg/mL)	75.54 ± 7.16 [a]	127.61 ± 6.91 [b]	122.76 ± 11.74 [a,b]	196.766 ± 19.20 [c]	T, D
Plasma Calcium (mM)	0.093 ± 0.002 [a]	0.097 ± 0.002 [a]	0.099 ± 0.003 [a]	0.1 ± 0.002 [a]	
Plasma Phosphate (mM)	0.045 ± 0.003 [a]	0.042 ± 0.002 [a]	0.045 ± 0.003 [a]	0.046 ± 0.002 [a]	
eWAT Cholecalciferol (ng)	127.1 ± 10.1 [a]	263.6 ± 50 [a]	283.9 ± 28.5 [a]	547.5 ± 60.7 [b]	T, D
eWAT 25(OH)D (ng)	27.6 ± 2.5 [a]	74.9 ± 9 [b]	43.9 ± 5.2 [a]	140.4 ± 11.9 [c]	T, D and TxD
eWAT 1.25(OH)$_2$D (ng)	16.4 ± 3.7 [a]	37.3 ± 8.3 [a]	40.2 ± 6.1 [a,b]	60.6 ± 13.6 [b]	T, D

Values are presented as means ± SEM Bars not sharing the same letter were significantly different in Tukey-Kramer post hoc test $p < 0.05$ between control group (low fat, LF) and high fat group (HF) for 7 and 11 weeks and each condition. eWAT: epididymal white adipose tissue. T, time effect in two-way ANOVA analysis ($p < 0.05$); D, diet effect in two-way ANOVA analysis ($p < 0.05$); TxD, interaction between time and diet in two-way ANOVA analysis ($p < 0.05$).

Figure 1

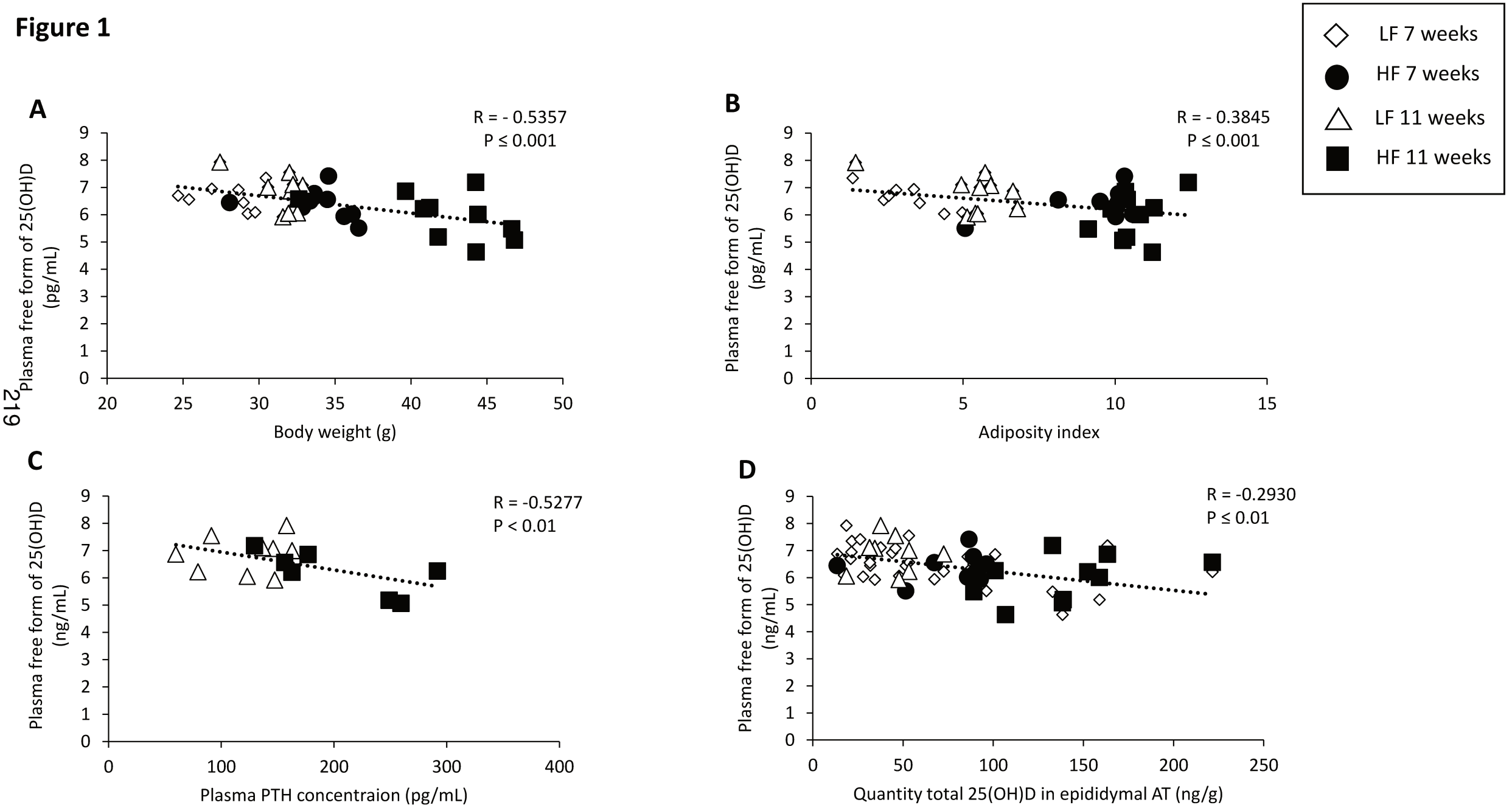
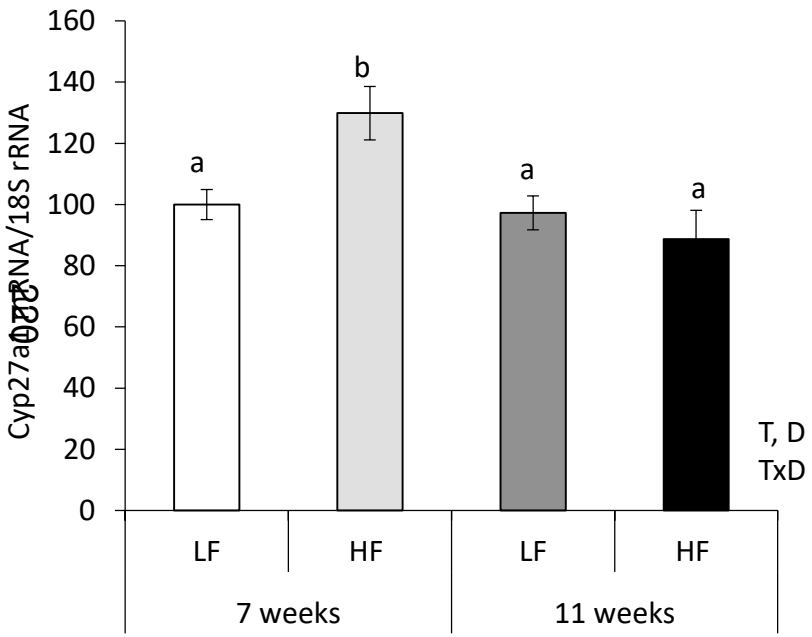
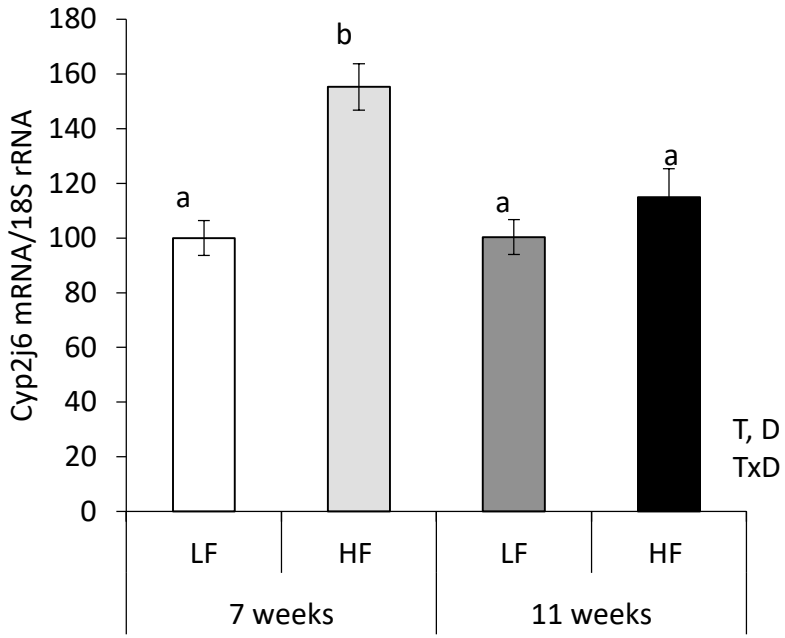

LF 7 weeks
HF 7 weeks
LF 11 weeks
HF 11 weeks
A
R = - 0.5357
P ≤ 0.001
Plasma free form of 25(OH)D (pg/mL)
Body weight (g)
B
R = - 0.3845
P ≤ 0.001
Plasma free form of 25(OH)D (pg/mL)
Adiposity index
C
R = -0.5277
P < 0.01
Plasma free form of 25(OH)D (ng/mL)
Plasma PTH concentraion (pg/mL)
D
R = -0.2930
P ≤ 0.01
Plasma free form of 25(OH)D (ng/mL)
Quantity total 25(OH)D in epididymal AT (ng/g)

Figure 2


Cyp27a1 mRNA/18S rRNA
160
140
120
100
80
60
40
20
0
a
b
a
a
T, D
TxD
LF
HF
LF
HF
7 weeks
11 weeks


Cyp2j6 mRNA/18S rRNA
180
160
140
120
100
80
60
40
20
0
a
b
a
a
T, D
TxD
LF
HF
LF
HF
7 weeks
11 weeks

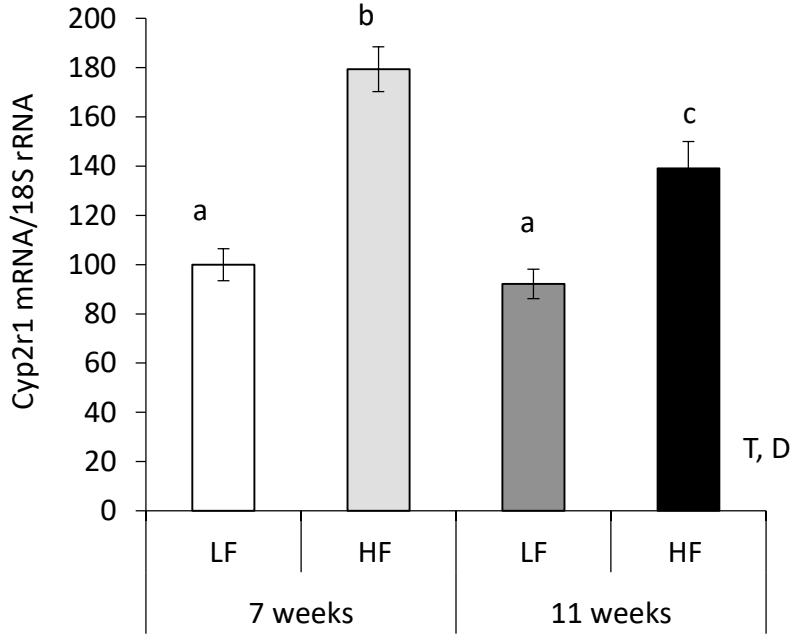

Cyp2r1 mRNA/18S rRNA
200
180
160
140
120
100
80
60
40
20
0
a
b
a
c
T, D
LF
HF
LF
HF
7 weeks
11 weeks

Figure 3

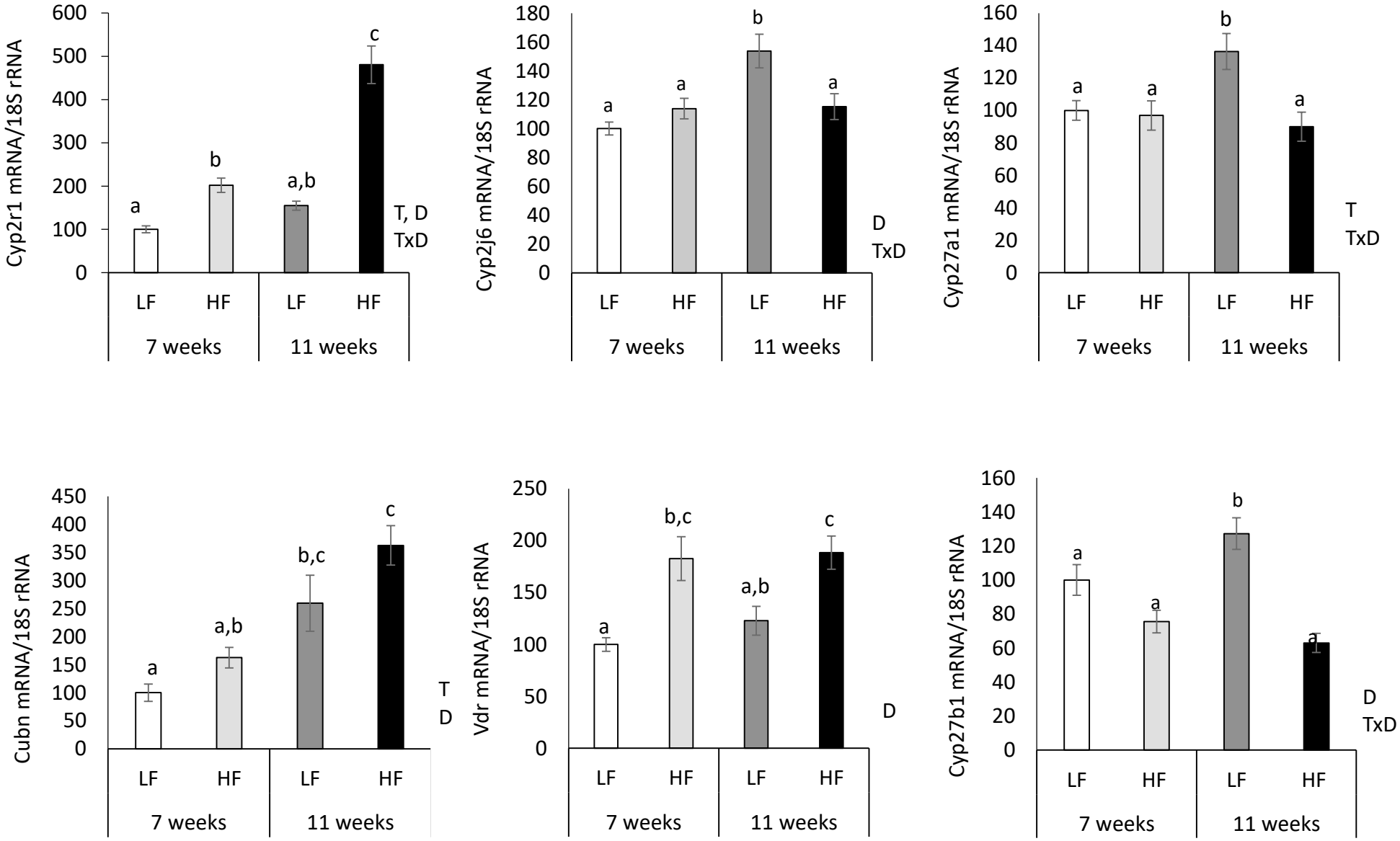
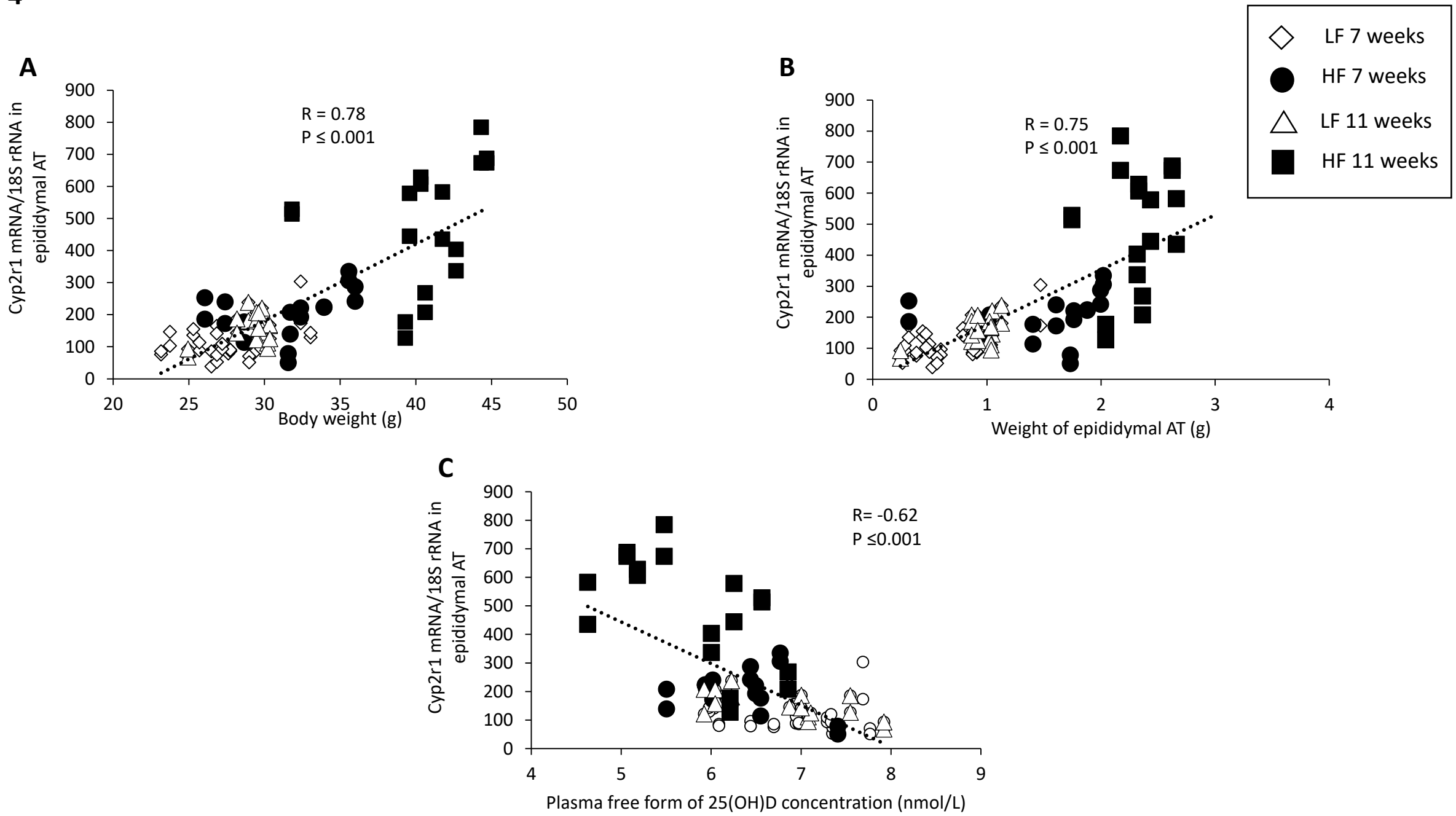

Cyp2r1 mRNA/18S rRNA
600
500
400
300
200
100
0
a
b
a,b
c
T, D
TxD
LF
HF
LF
HF
7 weeks
11 weeks
Cyp2j6 mRNA/18S rRNA
180
160
140
120
100
80
60
40
20
0
a
a
b
a
D
TxD
LF
HF
LF
HF
7 weeks
11 weeks
Cyp27a1 mRNA/18S rRNA
160
140
120
100
80
60
40
20
0
a
a
b
a
T
TxD
LF
HF
LF
HF
7 weeks
11 weeks
Cubn mRNA/18S rRNA
450
400
350
300
250
200
150
100
50
0
a
a,b
b,c
c
T
D
LF
HF
LF
HF
7 weeks
11 weeks
Vdr mRNA/18S rRNA
250
200
150
100
50
0
a
b,c
a,b
c
D
LF
HF
LF
HF
7 weeks
11 weeks
Cyp27b1 mRNA/18S rRNA
160
140
120
100
80
60
40
20
0
a
a
b
a
D
TxD
LF
HF
LF
HF
7 weeks
11 weeks

Figure 4


A
Cyp2r1 mRNA/18S rRNA in epididymal AT
R = 0.78
P ≤ 0.001
Body weight (g)
B
Cyp2r1 mRNA/18S rRNA in epididymal AT
R = 0.75
P ≤ 0.001
Weight of epididymal AT (g)
LF 7 weeks
HF 7 weeks
LF 11 weeks
HF 11 weeks
C
Cyp2r1 mRNA/18S rRNA in epididymal AT
R= -0.62
P ≤0.001
Plasma free form of 25(OH)D concentration (nmol/L)

Supplemented table 1: Experimental diets composition

Item (g)	Control diet	High fat diet
Sucrose	33.1290	8.8470
Dextrin	29.8560	0
Casein –Vitamin, Tested	18.9560	25.8450
Powdered Cellulose	4.7390	6.40610
Maltodextrin	3.3170	16.1530
Soybean oil	2.3700	3.2310
Lard	1.8960	31.6600
Potassium Citrate, Tribasic Monohydrate	1.5640	2.1320
Calcium Phosphate	1.2320	1.6800
DIO Mineral Mix	0.9480	1.2920
AIN-76A Vitamin mic	0.9480	1.2920
Calcium Carbonate	0.5210	0.7110
L-Cystine	0.2840	0.3880
Choline Bitartrate	0.1900	0.2580
FD&C Yellow 5 Lake	0.0500	0.0500
Total	100	100

Supplemental table 2: Primers sequences

Gene	Forward sequence	Reverse sequence
Cyp2r1	TTTGTCGGCAACATCTGCT	TGCCTCCAAGATCTAAACTGAAA
Cyp3a11	TGAATATGAAACTTGCTCTCACTAAAA	CCTTGTCTGCTTAATTTCAGAGCT
Cyp27a1	CCTCACCTATGGGATCTTCATC	TTTAAGGCATCCGTGTAGAGC
Cyp2j6	CCCTCTACCCAGAAGTCCAA	TTCTGGCCAATCACCCTATC
Cyp24a1	AAGCCTACGCGCTGATGAT	CACGGGCTTCATGAGTTTC
Cyp27b1	AGTGGGGAATGTGACAGAGC	GGAGAGCGTATTGGATACCG
Megalin	GATGGATTAGCCGTGGACTG	TCCGTTGACTCTTAGCATCTGA
Cubilin	GCCATCCAGATGCAACCT	GGTGCAGACAGGCAACAAG
Vdr	AACCCCTCATAAAGTTCCAGGT	CTGTACCCCAGGTCGGTCT
Gc	CTACCTCAGAGGATTGCATGG	CTTTTTGGATAAGTTTTGACAGATTTT
Dab2	GCAGTCGAACTTTCTGCATCTC	GGTGTTACTGGGACCGTACCT
Amn	AGACAGTCACGCCATCTCG	GAGGCCAGGACCAACTCC.
18S rRNA	CGCCGCTAGAGGTGAAATTCT	CATTCTTGGCAAATGCTTTCG

Supplemental table 3: Method validation for LC-MS/MS analysis of vitamin D metabolites after Amplifex derivatization.

Analytes	Linear range ng/ml	LOD ng/ml	LOQ ng/ml	Inter-assay CV%	Intra-assay CV%
Vitamin D3	0.78-75	0.049	0.78	14.4	6.0
25OH D3	0.2-75	0.049	0.2	12.5	11.0
1,25(OH)$_2$D3	0.2-75	0.049	0.2	5.5	11.6

Supplemental table 4: Concentration of vitamin D metabolites in epididymal adipose tissue quantified by liquid chromatography-tandem mass spectrometry (LC-MS/MS)

		Cholecalciferol (ng/g of tissue)	25(OH)D (ng/g of tissue)	$1,25(OH)_2D$ (ng/g of tissue)
7 weeks	**LF**	267.6 ± 14.9 [a,b]	54.5 ± 2.4 [a]	32.3 ± 4.9 [a]
	HF	190.6 ± 43.3 [a]	48.5 ± 1.4 [a]	28.1 ± 6.2 [a]
11 weeks	**LF**	331.6 ± 25.4 [b]	50.9 ± 4.4 [a]	45.4 ± 5.9 [a]
	HF	236 ± 31.3 [a,b]	61.9 ± 8.3 [a]	26 ± 5.9 [a]
Two-way ANOVA		T, D		D

Values are presented as means ± SEM Bars not sharing the same letter were significantly different in Tukey-Kramer post hoc test $p < 0,05$ between control group (low fat, LF) and high fat group (HF) for 7 and 11 weeks and each condition. T, time effect in two-way ANOVA analysis ($p < 0,05$); D, diet effect in two-way ANOVA analysis ($p < 0,05$); TxD, interaction between time and diet in two-way ANOVA analysis ($p < 0,05$).

Supplemental table 5: Effect of high fat diet on gene expression of vitamin D metabolism in liver, kidney and adipose tissue for 7 weeks.

	Liver		Kidney		Epididymal AT	
	LF	HF	LF	HF	LF	HF
Cyp27a1	100 ± 5	130 ± 9 *	Nm		100 ± 6	97 ± 9
Cyp2r1	100 ± 6	179 ± 9 *	Nm		100 ± 7	202 ± 7 *
Cyp2j6	100 ± 6	155 ± 8 *	Nm		100 ± 4	114 ± 7
Cyp3a11	Nm		Nm		Nd	
Cyp24a1	Nm		100 ± 8	72 ± 5 *	Nd	
Cyp27b1	Nm		100 ± 6	111 ± 7	100 ± 9	76 ± 7 *
Vdr	Nm		100 ± 4	88 ± 6	100 ± 6	182 ± 21 *
Gc	Nm		100 ± 7	77 ± 6	100 ± 20	63 ± 7
Megalin	Nm		100 ± 6	100 ± 5	100 ± 9	88 ± 9
Cubilin	Nm		100 ± 3	88 ± 5 *	100 ± 15	162 ± 18

Expression of genes coding proteins of vitamin D metabolism relative to 18S ribosomal RNA in liver, kidney and epidydimal adipose tissue (AT) of mice fed with control diet (low fat, LF) or high fat diet (HF) for 7 weeks (LF n=10, HF n=10). Values are presented as means ± SEM. For unpaired Student's t-test, p values: *, $p < 0,05$. Nm, data no measured, Nd, data no detected.

Supplemental table 6: Effect of high fat diet on gene expression of vitamin D metabolism in liver, kidney and adipose tissue for 11 weeks.

	Liver		**Kidney**		**Epididymal AT**	
	LF	HF	LF	HF	LF	HF
Cyp27a1	100 ± 4	128 ± 8 *	Nm		100 ± 8	66 ± 6 *
Cyp2r1	100 ± 6	219 ± 9 *	Nm		100 ± 7	344 ± 36 *
Cyp2j6	100 ± 4	143 ± 10 *	Nm		100 ± 8	75 ± 6
Cyp3a11	100 ± 8	39 ± 4	Nm		Nd	
Cyp24a1	Nd		100 ± 9	72 ± 8 *	Nd	
Cyp27b1	Nm		100 ± 9	264 ± 32 *	100 ± 9	48 ± 4 *
Vdr	100 ± 14	171 ± 23 *	100 ± 3	93 ± 6	100 ± 12	138 ± 11 *
Gc	100 ± 4	94 ± 7	100 ± 8	113 ± 11	100 ± 11	340 ± 85
Megalin	100 ± 6	61 ± 7	100 ± 3	33 ± 4	100 ± 32	20 ± 2
Cubilin	Nd		100 ± 4	78 ± 4	100 ± 19	140 ± 13

Expression of genes coding proteins of vitamin D metabolism relative to 18S ribosomal RNA in liver, kidney and epidydimal adipose tissue (AT) of mice fed with control diet (low fat, LF) or high fat diet (HF) for 11 weeks (LF n=10, HF n=10). Values are presented as means ± SEM. For unpaired Student's t-test, p values: *, $p < 0,05$. Nm, data no measured, Nd, data no detected.

ARTICLE 6 : Weight loss normalizes plasma free 25(OH) vitamin D in diet induced obese male mice.

Article en préparation.

Au cours des 2 derniers papiers (**Article 4 et 5**), nous avons pu mettre en évidence qu'un régime riche en graisse à différents temps entraînait une modification de l'expression des enzymes impliquées dans le métabolisme de la vitamine D au niveau de différents tissus, notamment le tissu adipeux. Au vu de ces résultats, il nous a semblé pertinent d'étudier la régulation du métabolisme de la vitamine D lors d'une perte de poids médiée par l'alimentation chez la souris, afin d'étudier la réversibilité des phénotypes mis en évidence.

Les souris sont réparties en 2 groupes et sont nourries soit avec un régime contrôle (10% d'énergie sous forme de lipides, groupe LF, n=10), soit avec un régime riche en graisse (60% d'énergie sous forme de lipides, groupe HF, n=20). Après 7 semaines de régime, 10 souris nourries avec une alimentation riche en graisse changent de groupe et sont nourries avec le régime contrôle pendant 4 semaines (groupe HF/LF). La durée de l'expérience est de 11 semaines (**Figure 23**). Les régimes ont été formulés pour fournir une consommation en vitamine D équivalente entre les groupes.

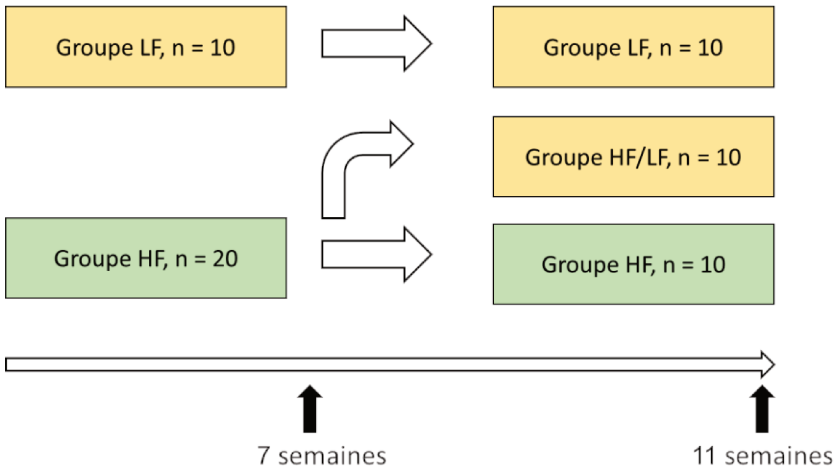


Figure 23 : Schéma expérimental du protocole in vivo de l'Article 6.

Les taux plasmatiques et adipeux des différents paramètres biochimiques du métabolisme de la vitamine D ont été mesurés soit par test ELISA soit par la méthode LC-MS/MS mis au point au

laboratoire (**Article 7**). L'expression génique des différents acteurs impliqués dans le métabolisme de la vitamine D a ensuite été analysée au niveau du foie, du rein et du tissu adipeux par qPCR.

PRINCIPAUX RESULTATS DE L'ETUDE

Les résultats de cette étude ont montré que la prise de poids induite par un régime riche en graisse puis un retour à un régime contrôle chez les souris entraine un retour à l'état basal en ce qui concerne les niveaux d'expressions géniques au niveau du fois, du rein et du tissu adipeux des différents acteurs mis en jeu dans le métabolisme de la vitamine D ainsi que les dosages plasmatiques et adipocytaires de la vitamine D et de ses métabolites.

En effet, nous observons une perte de masse corporelle progressive au cours du temps pour le groupe HF/LF qui entraine aussi une diminution de la masse du foie et du tissu adipeux (index d'adiposité) jusqu'à un retour à l'état basal (valeurs identiques à celles du groupe LF). Comme nous l'avons vu précédemment dans l'**Article 6**, un régime riche en graisse pendant 11 semaines chez les souris entraine, des modifications des concentrations plasmatiques (augmentation de la PTH et diminution du cholécalciferol) et adipocytaires (augmentation de la quantité de cholécalciférol et de 25(OH)D) ainsi que l'expression de certains acteurs du métabolisme de la vitamine D au niveau du foie (augmentation de l'expression génique de Cyp2r1, Cyp27a1 et Cyp2j6), du rein (augmentation de l'expression génique de Cyp27b1) et du tissu adipeux (augmentation de l'expression génique de Cyp2r1 et diminution de celle de Cyp27b1). Tous ces paramètres reviennent à l'état basal lors d'une perte de poids de 4 semaines (sauf pour la concentration plasmatique du cholécalciférol). De plus, nous avons observé une diminution des niveaux d'expression des ARNm codants pour la mégaline au niveau rénal ainsi qu'une régulation négative du gène codant pour la cubiline dans le tissu adipeux épididymal chez les souris HF/LF comparées aux animaux LF, ce qui suggère une altération de la réabsorption rénale ainsi que l'absorption dans tissu adipeux de la vitamine D liée à ses protéines de transport.

En conclusion, ces données innovantes démontrent ainsi la réversibilité des modifications induites par un régime obésogène sur le métabolisme de la vitamine D.

Weight loss normalizes plasma free 25(OH) vitamin D in diet induced obese male mice

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Abstract

Obesity has been consistently associated with lower circulating total and free 25-OH cholecalciferol (25(OH)D), and weight loss has been shown to improve 25OH levels in human obese subjects. We decided to test whether a weight loss would increase total and free 25(OH)D in a mouse model of diet induced obesity, with controlled cholecalciferol intake. Mice were fed with either a low fat (LF, n=10) or a high fat diet (HF, n=20). After 7 weeks, half of the HF group was switched to a low fat diet (HF/LF) for 5 weeks, while the other animals were maintained on the same diet.

Dietary intervention led to a decrease in body weight, and at the end of the experiment HF/LF mice were similar to LF animals in terms of total body weight, adiposity and plasma parameters linked to vitamin D status (PTH, calcium and phosphate levels). Surprisingly, weight loss decreased plasma total 25(OH)D compared to obese animals, but free 25(OH)D was increased. Cholecalciferol concentration was decreased in WAT and plasma of HF/LF mice compared to LF mice.

Expression of genes related to vitamin D metabolism indicated that renal and WAT uptake of protein-bound vitamin D was altered in HF/LF, which might explain the diminution of plasma total 25(OH)D after weight loss.

In conclusion, data demonstrate the reversibility of the changes induced by a diet induced obesity on the vitamin D metabolism.

Introduction

Cholecalciferol (vitamin D, VD) is a lipophilic hormone involved in the regulation of calcium and phosphate metabolism, but also in other physiological processes such as inflammation. Cholecalciferol can be synthesized endogenously from 7OH cholesterol in the skin after UVB exposure or through dietary intake. Once it has reached the bloodstream, cholecalciferol can be hydroxylated by hepatic 25-hydroxylases (encoded by Cyp2r1, Cyp27a1, Cyp2j6 and Cyp3a11 in mice) to produce 25-OH cholecalciferol (25(OH)D) [1]. 25(OH)D is the main circulating form of vitamin D in the fasting state and its concentration is generally considered as the best marker for an individual's VD status. 25(OH)D is transported in the plasma mainly bound to vitamin D binding protein (VDBP, encoded by the Gc gene), but a substantial part (around 15%) of 25(OH)D is also bound to albumin [2]. A very limited fraction (free 25(OH)D, < 1% of circulating 25(OH)D) remains unbound and is considered as the active fraction [3]. Plasma 25(OH)D concentration is maintained via the cubilin/megalin complex reabsorption in the kidney [4]. A fraction of 25(OH)D is also converted to $1.25(OH)_2D$, the active form of VD, by the 1-hydroxylase CYP27B1, and both metabolites are secreted to the bloodstream.

Besides its roles in calcium and phosphate metabolism, VD participates to numerous physiological processes, most of which are mediated by its nuclear receptor of VD (VDR), and are dependent on local (paracrine, autocrine or even intracrine) production of $1.25(OH)_2D$ [5]. Specifically, VD supplementation is able to limit diet induced obesity by increasing fatty acid oxidation and limiting inflammation in vivo an in vitro [6-8].

Epidemiological data indicate that weight loss correlates with increase in plasma 25(OH)D (ref), and previous studies have highlighted the fact obesity causes a modulation in the expression of genes involved in the metabolism in humans [9] and mice [10, 11].

62 In this study, we investigated whether weight loss in obese mice would improve VD status and
63 restore gene expression of proteins involved in the metabolism of VD.

Material and Methods

Reagents - TRIzol reagent, random primers, and Moloney murine leukemia virus reverse transcriptase were obtained from Life Technologies (Courtaboeuf, France). SYBR Green reaction buffer was purchased from Eurogentec (Liege, Belgium).

Animal Experiments – The protocol received the agreement of the local ethics committee (APAFIS#2595-2016091911217758). Six week-old male C57BL/6J mice were obtained from Janvier Labs (Le Genest Saint Isle, France), fed ad libitum with control food (A04 diet ,Safe-diets, Augy France) during the 1-week acclimation period and with full access to drinking water. The animals were maintained at 22°C under a 12-hour light, 12-hour dark cycle a 20% humidity level. Mice were divided into control diet group (LF: 10% energy from lipids, n=10) or high fat diet group (HF : 60% energy from lipids, n = 20) (TestDiet, London, UK). The experimental diet composition is detailed in supplemental table 1. Animals were weighed weekly and food intake was measured every two weeks. After 7 weeks, half of the HF mice (HF/LF group, n=10) were switched to a LF diet-induced until the end of the experiment (11 weeks), whereas the other animals maintained the same diet. After 11 weeks, mice were fasted overnight and blood was collected by cardiac puncture under anesthesia in heparin tubes (S-monovette, Sartstedt, Marnay, France), plasma was isolated by centrifugation at 3000 rpm for 15 min at 4°C and was stored at -80°C. Animals were sacrificed by cervical dislocation was used to euthanasia of animals and tissues (kidney, liver and epididymal white adipose tissue) were collected, weighted, snap frozen in liquid nitrogen and stored at -80°C.

RNA extraction and real-time PCR – Total RNA was extracted from the liver, kidney and epididymal white adipose tissue using TRIzol reagent (Thermofisher, Courtaboeuf, France). One µg of total RNA was used to synthetized cDNAs using random primers and Moloney murine leukemia virus reverse transcriptase (Thermofisher). Real-time quantitative PCR analyses were performed using the Mx3005P Real-Time PCR System (Stratagene, La Jolla, CA) as previously described

[12]. For each condition, expression was quantified in duplicate, and 18S rRNA was used as the endogenous control in the comparative cycle threshold (CT) method [13]. Primers sequences are reported in supplemental table 2.

Biochemical assays – Parathyroid hormone (PTH) concentration in mice plasma was quantified using ELISA (Parathyroid hormone PTH, Euromedex, Strasbourg, France). Free form of 25(OH)D was alsos quantified by ELISA (DIAsources, Louvain-La-Neuve, Belgique). Colorimetric assay kits were used to quantify calcium and phosphate concentrations in mice plasma (Clinisciences, Nanterre, France).

Preparation of standards and samples for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis – The 3 reference standards cholecalciferol, 25(OH)D3, $1.25(OH)_2D3$ were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). Ethanolic stock solution of all analytes were prepared at concentrations of 100 ng/mL for each standard and stored at -80°C in the dark. A working solution of deuterated analytes was prepared at 0.02 ng/mL of d3-cholecalciferol, d3-25-hydroxyvitamin D3 and d3-1.25-dihydroxyvitamin D3, (Cambridge Isotope Laboratories, MA, USA) and stored at -80°C in the dark.

Sample preparation - Sample preparation was adapted from Wang et al. [14]. The extraction procedure was conducted under low light because VD and its metabolites are light sensitive. After thawing on ice, mice plasmas were centrifuged at 4°C for 15 min, at 13 600 g. 100 µL of each sample was transferred to a glass test tube containing 10 µL of deuterated standards stock solution. Proteins were precipitated by adding acetonitrile, vortex-mixed, and centrifuged for 10 min at 3 000 g. The supernatant was moved to another glass tube, and the volume was reduced by a half under a nitrogen stream. 5 mL of ethyl acetate were added and after vigorous shaking, samples were

115 centrifuged for 20 min at 590 g, and the upper organic layer was transferred into a new glass tube
116 and was reduced under nitrogen stream.
117 A one-step derivatization was employed to improve the ionization efficiency of the metabolites and
118 Amplifex diene (Amplifex TM Diene Reagent, Sciex Chemistry and consumables R&D,
119 Framingham, MA) was used as reagent [15]. After complete evaporation of the solvent, a total of 30
120 µL of the Amplifex diene was added to the dried sample above, vortexed for 15 s and incubated for
121 30 min at room temperature. Next, 30 µL of deionized water wasere added to each tube, vortexed
122 for 15 s and transferred in vials for LC injection.
123 Calibration curves (0-50 ng/mL) for the 3 analytes were prepared by serial dilution of the stock
124 solution. As for plasma samples, standards were derivatized using Amplifex reagent.
125
126 **Epididymal adipose tissue preparation** – Sample preparation was adapted from Lipkie et al. [16].
127 25 µL of 0.02 ng/mL of deuterated stock solution) were added to tissue homogenates (50 mg of
128 tissue ground in 1 mL of PBS) in a glass test tube. Acetonitrile (ACN) was added, vortex mixed for
129 5 min and centrifuged at 6 000 g for 5 min. After addition of methyl tert-butyl ether (MTBE)
130 samples were vortexed for 5 min, centrifuged, and the upper organic layer was collected in another
131 glass tube. The extraction was repeated twice and the combined supernatants were dried under
132 nitrogen. Oasis HLB SPE cartridges (Waters, Guyancourt, France) were conditioned with ethyl
133 acetate, methanol (MeOH) and H_2O. The sample was reconstituted with 1 mL of MetOH and 1 mL
134 of K_2HPO_4 (0.4 M) and added on the cartridge. The cartridge was washed with H_2O and 70%
135 MeOH and dried for 2 min under vacuum. Tips were washed with ACN and analytes were eluted
136 with ACN and dried under nitrogen. After complete evaporation of the solvent, a total of 30 µL of
137 the Amplifex diene wasere added to the dried sample above, vortexed for 15 s and incubated for 30
138 min at ambient temperature. Next, 30 µL of deionized water were added to each tube, vortexed for
139 15 s and transferred for LC injection.
140

LC-MS/MS analysis – Accurate mass measurements were performed on the Q-Exactive Plus mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a Heated Electrospray Ionization (H-ESI II) probe. Thermo Xcalibur 3.0.63 software was used for the instrument setup, control of the LC-MS system during acquisition and data treatment. Tune Q Exactive Plus 2.5 application was used for the direct control of the mass spectrometer.

Samples were injected onto a Hypersil GOLD C_{18} column (2.1 x 100 mm; Thermo Scientific, Les Ulis, France). Flow rate was 0.4 mL/min and injection volume was 5 µL. The mobile phase was composed of A= ultrapure water with 0.1% formic acid (FA) (v/v), and B = acetonitrile with 0.1 % FA (v/v). Starting conditions were A= 70% and B = 30% and were held for 4 min. A linear gradient was applied until 10.0 min where A= 35% and B = 65%, these conditions were held until 12.0 min, at 14 min A=0% and B = 100% until 16 min. Starting conditions were re-implemented at 18 min.

PRM (Parallel Reaction Monitoring) method was set up as MS/MS acquisition mode and full higher-energy collision dissociation spectra (HCD) spectra were acquired. For each compound, the most abundant ion was selected as precursor ion and isolated in a 2 uma window in a specified time segment and fragmented under 20 % of normalized collision energy (m/z 716.5 for cholecalciferol, m/z 719.5 for d3-Cholecalciferol, m/z 732.5 for 25OH-D3, m/z 735.5 for d3-25OH-D3, m/z 751.5 for d3-1,25OH-D3 and m/z 748.5 for 1,25OH-D3). In the data analysis, specific fragment ion were extracted within 5 ppm masse error limit for each compound analysed: m/z 657.4368 for cholecalciferol, m/z 660.4552 for d3-Cholecalciferol, m/z 673.4311 for 25OH-D3, m/z 676.4534 for d3-25OH-D3, m/z 689.4297 for 1,25OH-D3 and m/z 692.4458 for d3-1,25OH-D3.

Calibration curves were plotted with peak area ratio of analyte and internal standard versus concentration of analyte. Validations were performed for linearity and repeatability data (Supplemental Table 3).

Statistical analysis - Data are presented as means ± SEM. Significant differences were determined using ANOVA, followed by the least significant difference post hoc test, using GNU PSPP software (https://www.gnu.org/software/pspp/). $p < 0.05$ was considered statistically significant.

Results

A switch from HF to LF diet abolishes obesity in mice

After 11 weeks, HF fed animals had a higher body weight, liver weight and adiposity index (Table 1) than LF group. Weight loss was triggered in HF animals whose diet was switched to LF at week 7 (Fig. 1): body weight decreased regularly in the HF/LF group from 7 to 11, despite similar energy intake in all three groups, and became equivalent to LF group at week 11. Other morphological parameters (adiposity and liver weight) were also diminished in the HF/LF group compared to the HF group and were similar to LF group at the end of the experiment (Table 1). Cholecalciferol intake was calculated based on food intake, and was not different between the three groups throughout the study (Table 2).

Effect of weight loss on adipose tissue and plasma vitamin D status

Plasma concentration of various parameters related to VD metabolism were examined in the three groups. After 11 weeks, total plasma 25(OH)D concentration, calcium, phosphate, and 1.25-$(OH)_2$D were not significantly different between HF and LF group (Table 3). On the opposite, obese animals displayed decreased plasma cholecalciferol, and increased PTH. These modifications were corrected by weight loss in HF/LF animals, except for plasma cholecalciferol concentration, which remained lower than in LF group (1.43±0.12 ng/mL vs. 2.58±0.13 ng/mL) and total plasma 25(OH)D became significantly lower in HF/LF group compared with HF group (34.78±1.63 ng/mL vs. 42.70±1.42 ng/mL) but not different from control animals. A significantly higher plasma free 25(OH)D was observed in HF/LF compared with HF group (7.05±0.42 pg/mL vs. 5.94±0.26 pg/mL). Furthermore, plasma free form of 25(OH)D was inversely correlated with body weight of mice (Fig. 2A), adiposity index (Fig. 2B) and serum PTH (Fig. 2C).

Since adipose tissue (AT) is a major storage site of lipophilic molecules, the concentration of cholecalciferol, 25(OH)D and $1.25(OH)_2D$ was investigated in epididymal white adipose tissue (eWAT; Supplemental Table 4) by LC-MS/MS, and quantities were calculated (concentration x mass of epididymal adipose tissue; Table 3). After 11 weeks of diet, a significant increase of quantity of cholecalciferol and 25(OH)D amount was observed in AT of HF fed mice whereas $1.25(OH)_2D$ levels remained unchanged in LF group (Table 3). After weight loss, eWAT tissue quantity of content for all metabolites returned to LF control values, even if a borderline significant decrease in cholecalciferol amount was noticed between HF/LF (165.05±35.44 ng) and LF groups ($p=0.105$). This tendency was confirmed by looking at tissue concentration (Supplemental Table 4): cholecalciferol concentrations in the eWAT of HF/LF animals was similar to that of HF animals, whereas 25(OH)D and 1.25(OH)D were remained unchanged.

Interestingly, we found an inverse correlation between plasma free form of 25(OH)D and the amount of 25(OH)D in AT ($p<0.01$, Fig. 2D).

Impact of weight loss on expression of genes involved in vitamin D metabolism in liver, kidney and white adipose tissue of mice

Systemic 25(OH)D and 1.25(OH)2D levels are believed to under the control of liver 25- and kidney 1-hydroxylases, respectively. Therefore, we investigated the impact of dietary intervention on expression of genes coding for these enzymes. HF diet led to increased expression of genes involved in hepatic 25-hydroxylation (Cyp2r1, Cyp27a1 and Cyp2j6, Fig 3A). Similarly, the expression of the gene encoding the 1-hydroxylase, Cyp27b1, was upregulated in the kidney of the HF animals (Fig 3B). In eWAT, the expression of Cyp2r1 was increased in obese animals, whereas

Cyp27b1 was decreased (Fig 3C). The changes induced in gene expression were abolished by weight loss.

To further investigate the consequences of weight loss, a wider screening on the expression of genes related to the metabolism of vitamin D (i.e. Cyp3a11, Cyp24a1, Vdr, Gc, Megalin, Cubn, AMN and Dab2) in eWAT, liver and kidney (supplemental tables 4-6). Again, weight loss brought expression levels of most investigated genes to that of the control group, except hepatic Cyp3a11, renal Lrp2 (Megalin) and eWAT Gc and Cubn (-38%, -16%, +682% and -31%, respectively vs. LF group).

Correlations were established between the expression of eWAT Cyp2r1 gene and the body weight of mice ($r = 0.7963$, $p < 0.001$), eWAT mass ($r = 0.8024$, $p < 0.001$) and eWAT cholecalciferol concentration ($r = 0.7950$, $p < 0.001$) (Fig. 4A-C). On the other hand, a negative correlation was found between the eWAT expression level of Cyp2r1 and the concentration of plasma free 25(OH)D ($r = -0.7063$, $p < 0.001$, Fig. 4D).

Discussion

The prevalence of vitamin D deficiency, assessed by measuring circulating 25(OH)D levels, has been consistently reported to be higher in obese populations [17-19] compared to normal weight individuals. This observation raised the question of a causal relationship between vitamin D status and obesity, and whether obesity was the cause of vitamin D deficiency or vice versa [20]. Furthermore, several hypotheses have been elaborated, (dilution, sequestration in adipose tissue, modulation of vitamin D metabolism, [9, 11, 21, 22]) to explain this inverse relationship.

However, it seems that, at least in mice, vitamin D status as assessed by plasma 25(OH)D levels are not influenced by adiposity but rather by cholecalciferol intake. Indeed, we and others have observed that high fat diet feeding did not decrease plasma 25(OH)D in obese compared to age-matched lean mice fed with the same amount of vitamin D [10, 11]. Furthermore, in a recent paper, Seldeen and colleagues have tested whether obese animals submitted to a diet poor in cholecalciferol were more prone to plasma 25(OH)D deficiency than their lean counterparts [23]. Low cholecalciferol intake led to a similar drop in plasma 25(OH)D values (around 10 ng/mL) but the decline was slower in obese animals compared to normal weight animals, suggesting that obese mice possess mobilizable stores of vitamin D that might help maintain a normal 25(OH)D level during low intake periods.

This suggests that vitamin D status is actually dependent on the regular and sufficient cholecalciferol consumption and does not differ between obese and lean subjects. This condition is easy to set up in an animal study but extremely difficult to verify in humans, whose cholecalciferol intake during their life is erratic.

We had hypothesized that weight loss would result in increased plasma 25(OH)D in formerly obese animals, since meta-analyses of human trials indicated that weight loss marginally improves vitamin D status [24, 25]. As expected, morphological parameters linked to obesity were reversed

simply by switching obese mice from a HF to LF diet, and after 5 weeks under these dietary conditions, mice had recovered similar liver weight, body weight and adiposity. Obesity-induced increase of PTH was also corrected in the HF/LF group at the end of the experiment. Surprisingly, we found that plasma concentrations of cholecalciferol is lower in HF/LF compared to LF group and 25(OH)D total were actually lower in animals that undergone weight loss than in obese control, despite unchanged cholecalciferol intake. HF/LF animals displayed a significant decrease of adipose tissue and examination of eWAT cholecalciferol content showed that tissue concentration was kept as low as that of obese animals. These data suggest that cholecaciferol released from adipose tissue during weight loss is not reabsorbed in the adipose tissue and might be stored in another tissue or excreted in urine. Unfortunately, we did not measured the content in vitamin D metabolites in other major tissues involved in total vitamin D stores such as liver or skeletal muscle. In their metaregression analysis, Pannu and colleagues have observed that reduction in fat free mass should lead to a decreased plasma 25(OH)D, albeit their model was not significant [25]. Since fat free mass was not measured, we were not able to test this hypothesis in our study. According to this group, a decrease in 25(OH)D levels could also result from increased 25(OH)D conversion to inactive metabolites such as 24,25$(OH)_2$D. However, this possibility is not supported by our data, since renal Cyp24a1 expression was not regulated by our experimental conditions.

Previous studies have shown that high fat diet induced obesity produces metabolic dysfunctions (dyslipemia, insulin resistance) that are only partially corrected by weight loss, and that abnormal regulations persist in formerly obese mice [26-31]. We therefore examined the impact of weight loss on transcriptional regulation of genes involved in vitamin D metabolism in formerly obese mice to understand the observed phenotype. We have previously shown that high fat diet induced obesity interferes with the transcriptional regulation of expression of genes involved in vitamin D metabolism [10]. Whereas the majority of the genes tested were expressed identically in HF/LF and LF groups, two genes involved in vitamin D tissue uptake were downregulated. The cubilin/megalin

(Lrp2) complex is a major component of vitamin D protein-bound endocytosis in the kidney [4] but also in adipocytes [32]. We observed a decreased expression levels of Lrp2 in the kidney as well as the downregulation of cubilin in the eWAT in formerly obese compared to LF animals, suggesting an impairment in renal reabsorption as well as adipose tissue uptake of protein-bound vitamin D. The mechanisms responsible for such perturbation of vitamin D endocytosis capacity are unclear and deserve further investigation, but would explain the low levels of plasma cholecalciferol and 25(OH)D in HF/LF mice and the lower levels of WAT cholecalciferol. Another possibility would be that cholesterol transporters (Cd36, Scarb1, Npc1l1), which have been involved in intestinal absorption of cholecalciferol, are downregulated in HF/LF, hence reducing tissue uptake.

Despite similar cholecalciferol intake, HFD leads to diminished plasma free 25(OH)D levels, in line with what was reported recently in humans [33, 34]. Free 25(OH)D has been proposed as a better indicator of vitamin D status than total plasma 25(OH)D [35], especially in populations displaying low levels of 25(OH)D that are not associated with clinical signs of vitamin D deficiency such as cirrhotic patients and pregnant women [35]. Interestingly, whereas total 25(OH)D were lower, free 25(OH)D levels were higher in HF/LF group than in HF group. Combined with the fact that they presented unaltered plasma concentrations of calcium, phosphate and PTH compared to LF mice, this suggests that formerly obese animals were not vitamin D deficient.

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Legends for Figures

Figure 1: Effect of high fat diet on body weight of mice.

Body weight of control mice (low fat, LF), obese mice (high fat, HF) or thinner mice (HF/LF) for 11 weeks (n=10 for each group). Values are presented as means ± SEM. Bars not sharing the same letter were significantly different in LSD post hoc test ($p < 0,05$)..

Figure 2: Relationship between biochemical and morphological parameters and free 25(OH)D in high fat diet fed mice and thinner mice.

Correlation between plasma free form 25(OH)D concentration and body weight at the end (A), adiposity index (B), plasma PTH concentration (C) and the quantity of 25(OH)D in eWAT (D). Coefficient of correlation is R and test p is calculated with one-tailed probability.

Figure 3: Effect of high fat diet on vitamin D metabolism of mice in liver, kidney and adipose tissue.

Expression of genes coding proteins of vitamin D metabolism relative to 18S ribosomal RNA in liver (A), kidney (B) and epididymal adipose tissue (AT) (C) of control mice (low fat, LF), obese mice (high fat, HF) or thinner mice (HF/LF) for 11 weeks (n=10). Values are presented as means ± SEM. Bars not sharing the same letter were significantly different in LSD post hoc test ($p < 0,05$).

Figure 4: Correlation between Cyp2r1 expression in adipose tissue and biochemical and morphological parameters in mice.

Correlation between adipose tissue Cyp2r1 mRNA levels and mice body weight (A), epididymal adipose tissue weight (B), plasma free form of 25(OH)D (C) and quantity of cholecalciferol in adipose tissue (D). Coefficient of correlation is R and test p is calculated with one-tailed probability.

Table 1: Mice morphologic parameters

	LF	HF/LF	HF
Body weight at start (g)	22,4 ± 0,22 [a]	22,2 ± 0,13 [a]	22,2 ± 0,13 [a]
Body weight at the end (g)	31,5 ± 0,49 [a]	32.3 ± 0.55 [a]	42.2 ± 1.31 [b]
Liver weight (g)	0.96 ± 0.02 [a]	0.99 ± 0.03 [a]	1.21 ± 0.09 [b]
Adiposity index	5.30 ± 0.47 [a]	4.56 ± 0.75 [a]	10.61 ± 0.28 [b]

Values are presented as means ± SEM, n = 10 for each group. Bars not sharing the same letter were significantly different in LSD post hoc test ($p < 0,05$) between control group (low fat, LF), high fat group (HF) and weight loss (HF/LF) for 11 weeks.

Table 2: Food intake parameters

	Energy intake (kcal/g)	Vitamin D intake (UI/g/day)
LF	15.51 ± 0.97 [a]	3.71 ± 0.23 [a]
HF/LF	13.02 ± 0.25 [a]	3.12 ± 0.06 [a]
HF	14.13 ± 0.45 [a]	3.6 ± 0.11 [a]

Values are presented as means ± SEM, n = 10 for each group. Bars not sharing the same letter were significantly different in LSD post hoc test ($p < 0,05$) between control group (low fat, LF), high fat group (HF) and weight loss group (HF/LF) for 11 weeks.

Table 3: Plasma and adiposse tissue vitamin D related parameters

		LF	HF/LF	HF
Plasma concentration	**Cholecalciferol (ng/mL)**	2.58 ± 0.13 [a]	1.43 ± 0.12 [b]	1.27 ± 0.12 [b]
	Total 25(OH)D (ng/mL)	38.40 ± 0.89 [a.b]	34.78 ± 1.63 [a]	42.70 ± 1.42 [b]
	Free form 25(OH)D (pg/mL)	6.77 ± 0.21 [a.b]	7.05 ± 0.42 [a]	5.94 ± 0.26 [b]
	1,25(OH)$_2$D (pg/mL)	240 ± 21 [a]	180 ± 6 [a]	239 ± 9 [a]
	PTH (pg/mL)	122.76 ± 11.74 [a]	139.63 ± 10.69 [a]	196.766 ± 19.20 [b]
	Calcium (mM)	0.099 ± 0.003 [a]	0.104 ± 0.008 [a]	0.1 ± 0.002 [a]
	Phosphate (mM)	0.045 ± 0.003 [a]	0.042 ± 0.002 [a]	0.046 ± 0.002 [a]
Epididymal AT quantity	**Cholecalciferol (ng)**	283.9 ± 28.5 [a]	165.04 ± 29.65 [a]	547.5 ± 60.7 [b]
	25(OH)D (ng)	43.9 ± 5.2 [a]	45.5 ± 5.3 [a]	140.4 ± 11.9 [b]
	1,25(OH)$_2$D (ng)	40.2 ± 6.1 [a]	33.8 ± 8.9 [a]	60.6 ± 13.6 [a]

Values are presented as means ± SEM, n = 10 for each group. Bars not sharing the same letter were significantly different in LSD post hoc test ($p < 0,05$) between control group (low fat, LF), high fat group (HF) and weight loss group (HF/LF) for 11 weeks.

Figure 1

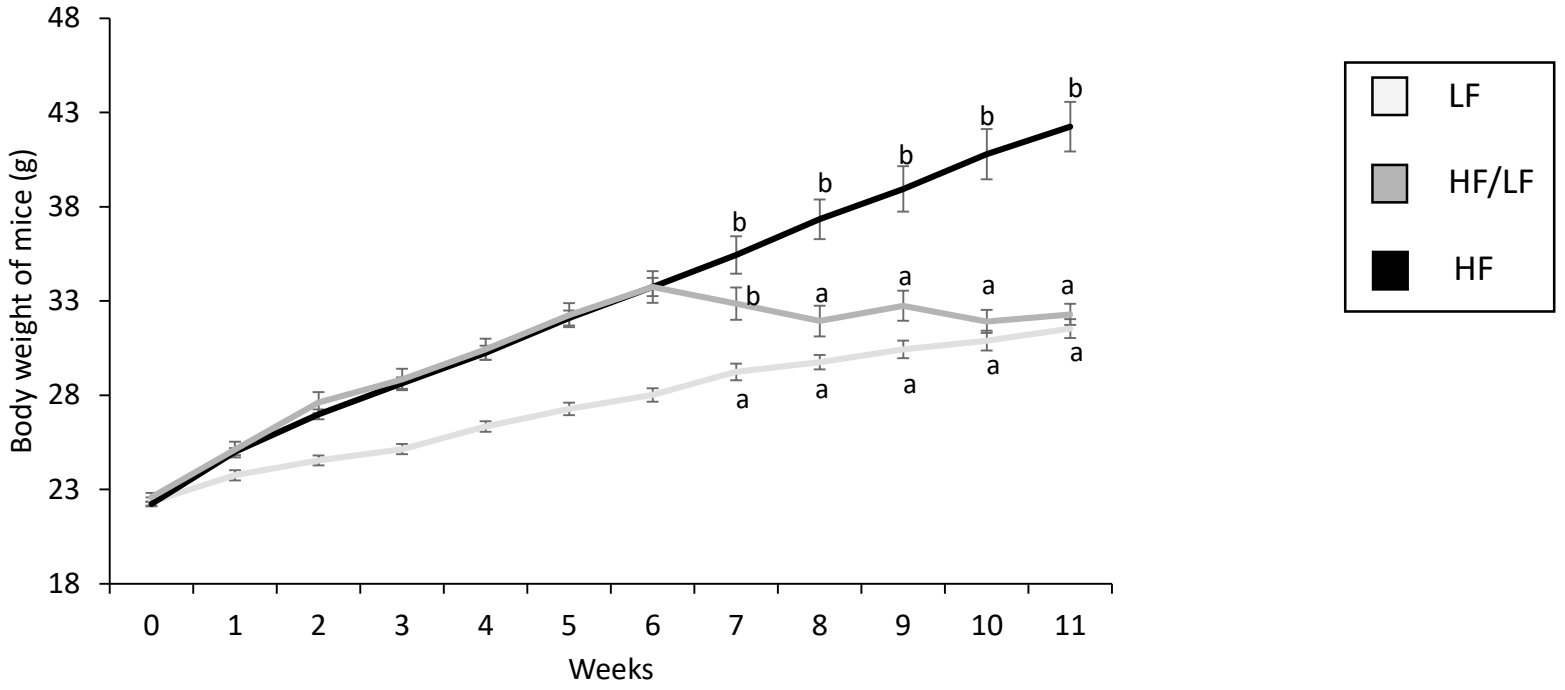

Body weight of mice (g)
48
43
38
33
28
23
18
0
1
2
3
4
5
6
7
8
9
10
11
Weeks
LF
HF/LF
HF

Figure 2

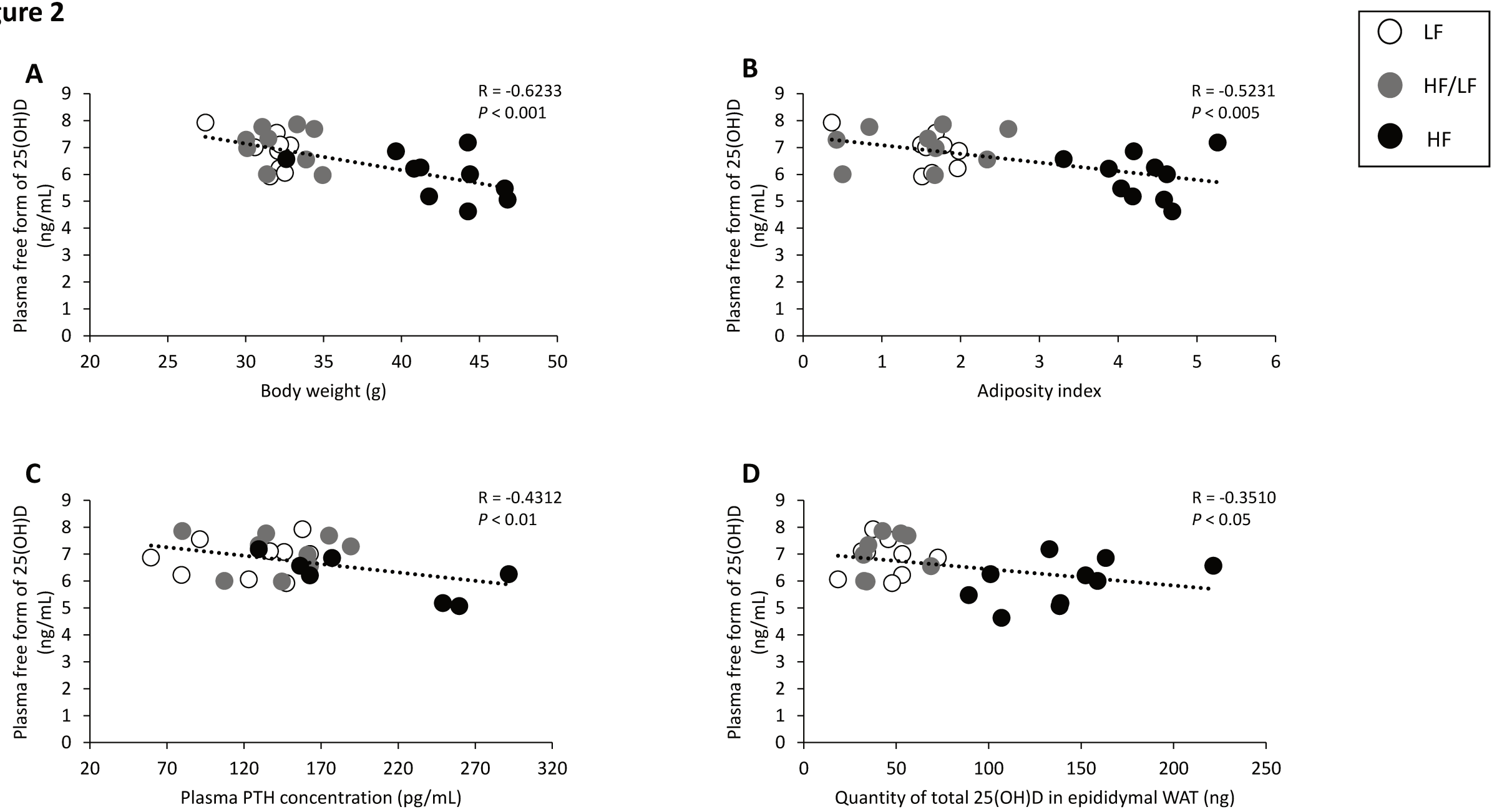

A
R = -0.6233
P < 0.001
Plasma free form of 25(OH)D (ng/mL)
Body weight (g)
B
R = -0.5231
P < 0.005
Plasma free form of 25(OH)D (ng/mL)
Adiposity index
C
R = -0.4312
P < 0.01
Plasma free form of 25(OH)D (ng/mL)
Plasma PTH concentration (pg/mL)
D
R = -0.3510
P < 0.05
Plasma free form of 25(OH)D (ng/mL)
Quantity of total 25(OH)D in epididymal WAT (ng)
LF
HF/LF
HF

Figure 3

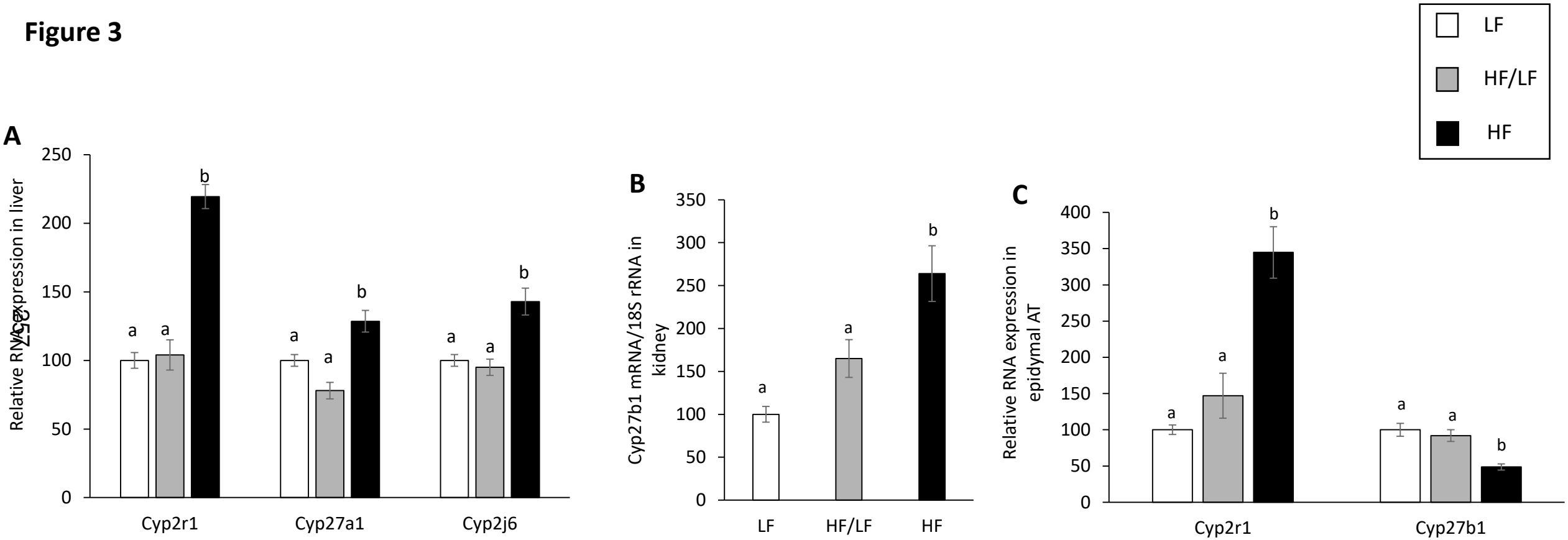
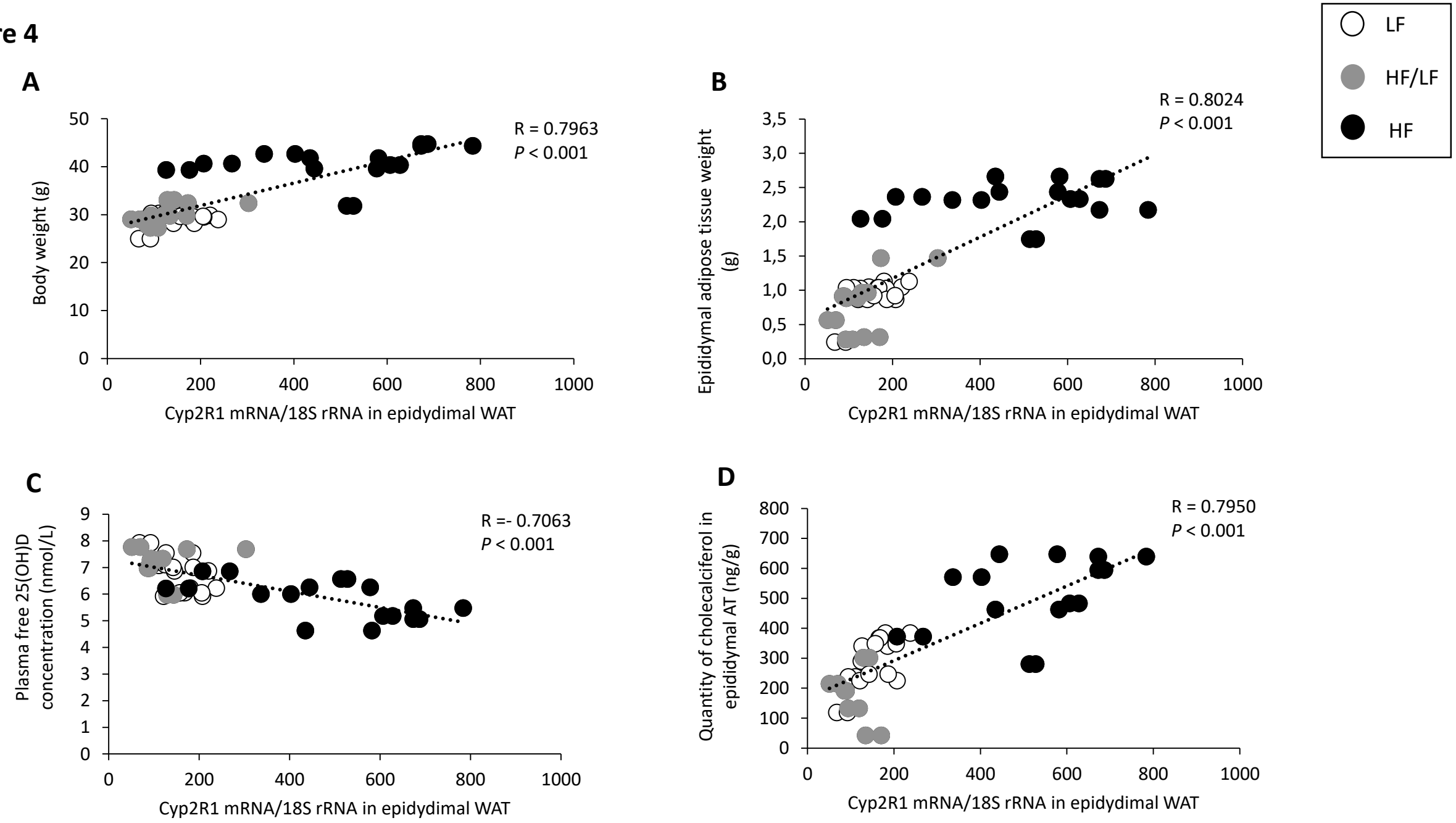

LF
HF/LF
HF
A
Relative RNA expression in liver
250
200
150
100
50
0
a
a
b
a
a
b
a
a
b
Cyp2r1
Cyp27a1
Cyp2j6
B
Cyp27b1 mRNA/18S rRNA in kidney
350
300
250
200
150
100
50
0
a
a
b
LF
HF/LF
HF
C
Relative RNA expression in epidymal AT
400
350
300
250
200
150
100
50
0
a
a
b
a
a
b
Cyp2r1
Cyp27b1

Figure 4


A
Body weight (g)
Cyp2R1 mRNA/18S rRNA in epidydimal WAT
R = 0.7963
P < 0.001
B
Epididymal adipose tissue weight (g)
Cyp2R1 mRNA/18S rRNA in epidydimal WAT
R = 0.8024
P < 0.001
C
Plasma free 25(OH)D concentration (nmol/L)
Cyp2R1 mRNA/18S rRNA in epidydimal WAT
R =- 0.7063
P < 0.001
D
Quantity of cholecalciferol in epididymal AT (ng/g)
Cyp2R1 mRNA/18S rRNA in epidydimal WAT
R = 0.7950
P < 0.001
LF
HF/LF
HF

Supplemented table 1: Experimental diets composition

Item (g)	Control diet	High fat diet
Sucrose	33.1290	8.8470
Dextrin	29.8560	0
Casein –Vitamin, Tested	18.9560	25.8450
Powdered Cellulose	4.7390	6.40610
Maltodextrin	3.3170	16.1530
Soybean oil	2.3700	3.2310
Lard	1.8960	31.6600
Potassium Citrate, Tribasic Monohydrate	1.5640	2.1320
Calcium Phosphate	1.2320	1.6800
DIO Mineral Mix	0.9480	1.2920
AIN-76A Vitamin Mix	0.9480	1.2920
Calcium Carbonate	0.5210	0.7110
L-Cystine	0.2840	0.3880
Choline Bitartrate	0.1900	0.2580
FD&C Yellow 5 Lake	0.0500	0.0500
Total	100	100

Supplemental table 2: Primers sequences

Gene	Forward sequence	Reverse sequence
Cyp2r1	TTTGTCGGCAACATCTGCT	TGCCTCCAAGATCTAAACTGAAA
Cyp3a11	TGAATATGAAACTTGCTCTCACTAAAA	CCTTGTCTGCTTAATTTCAGAGCT
Cyp27a1	CCTCACCTATGGGATCTTCATC	TTTAAGGCATCCGTGTAGAGC
Cyp2j6	CCCTCTACCCAGAAGTCCAA	TTCTGGCCAATCACCCTATC
Cyp24a1	AAGCCTACGCGCTGATGAT	CACGGGCTTCATGAGTTTC
Cyp27b1	AGTGGGGAATGTGACAGAGC	GGAGAGCGTATTGGATACCG
Megalin	GATGGATTAGCCGTGGACTG	TCCGTTGACTCTTAGCATCTGA
Cubilin	GCCATCCAGATGCAACCT	GGTGCAGACAGGCAACAAG
Vdr	AACCCCTCATAAAGTTCCAGGT	CTGTACCCCAGGTCGGTCT
Gc	CTACCTCAGAGGATTGCATGG	CTTTTTGGATAAGTTTTGACAGATTTT
Dab2	GCAGTCGAACTTTCTGCATCTC	GGTGTTACTGGGACCGTACCT
Amn	AGACAGTCACGCCATCTCG	GAGGCCAGGACCAACTCC.
18S rRNA	CGCCGCTAGAGGTGAAATTCT	CATTCTTGGCAAATGCTTTCG

Supplemental table 3: Method validation for LC-MS/MS analysis of vitamin D metabolites after Amplifex derivatization.

Analytes	Linear range ng/ml	LOD ng/ml	LOQ ng/ml	Inter-assay CV%	Intra-assay CV%
Vitamin D3	0.78-75	0.049	0.78	14.4	6.0
25OH D3	0.2-75	0.049	0.2	12.5	11.0
1,25(OH)$_2$D3	0.2-75	0.049	0.2	5.5	11.6

Supplemental table 4: Concentration of vitamin D metabolites in epididymal adipose tissue quantified by liquid chromatography-tandem mass spectrometry (LC-MS/MS)

	Cholecalciferol (ng/g of tissue)	25(OH)D (ng/g of tissue)	1,25(OH)$_2$D (ng/g of tissue)
LF	331.6 ± 25.4 [a]	50.9 ± 4.4 [a]	45.4 ± 5.9 [a]
HF/LF	223.9 ± 28,8 [b]	57.2 ± 8.1 [a]	43 ± 7.9 [a]
HF	236 ± 31.3 [b]	61.9 ± 8.3 [a]	26 ± 5.9 [a]

Values are presented as means ± SEM, n = 10 for each group. Bars not sharing the same letter were significantly different in LSD post hoc test ($p < 0,05$) between control group (low fat, LF), high fat group (HF) and weight loss group (HF/LF) after 11 weeks.

Supplemental table 5: Effect of high fat diet on gene expression of vitamin D metabolism in liver for 11 weeks.

	Cyp27a1	Cyp2r1	Cyp2j6	Cyp3a11	Cyp24a1	Cyp27b1	Vdr	Gc	Megalin	Cubilin	AMN	Dab2
LF	100 ± 4 [a]	100 ± 6 [a]	100 ± 4 [a]	100 ± 8 [a]			100 ± 14 [a]	100 ± 4 [a]	100 ± 6 [a]			
HF/LF	78 ± 6 [a]	104 ± 11 [a]	95 ± 6 [a]	62 ± 5 [b]	Nd	Nm	127 ± 17 [a]	81 ± 4 [a]	88 ± 9 [a,b]	Nm	Nd	Nd
HF	128 ± 8 [b]	219 ± 9 [b]	143 ± 10 [b]	39 ± 4 [c]			171 ± 23 [a]	94 ± 7 [a]	61 ± 7 [b]			

Expression of genes coding proteins of vitamin D metabolism relative to 18S ribosomal RNA in liver of mice fed with control diet (LF), high fat diet (HF) or weight loss group (HF/LF) after 11 weeks (n=10). Values are presented as means ± SEM. Bars not sharing the same letter were significantly different in LSD post hoc test ($p < 0,05$).

Supplemental table 6: Effect of high fat diet on gene expression of vitamin D metabolism in kidney for 11 weeks.

	Cyp27a1	Cyp2r1	Cyp2j6	Cyp3a11	Cyp24a1	Cyp27b1	Vdr	Gc	Megalin	Cubilin	AMN	Dab2
LF					100 ± 9 [a]	100 ± 9 [a]	100 ± 3 [a]	100 ± 8 [a,b]	100 ± 3 [a]	100 ± 4 [a]	100 ± 3 [a]	100 ± 3 [a]
HF/LF	Nm	Nm	Nm	Nm	86 ± 10 [a]	165 ± 22 [a]	94 ± 4 [a]	77 ± 5 [a]	84 ± 4 [b]	91 ± 3 [a,b]	99 ± 3 [a]	101 ± 3 [a]
HF					72 ± 8 [a]	264 ± 32 [b]	93 ± 6 [a]	113 ± 11 [b]	93 ± 4 [a,b]	78 ± 4 [b]	106 ± 5 [a]	96 ± 5 [a]

Expression of genes coding proteins of vitamin D metabolism relative to 18S ribosomal RNA in kidney of mice fed with control diet (low fat, LF) , high fat diet (HF) or weight loss group (HF/LF) after 11 weeks (n=10). Values are presented as means ± SEM. Bars not sharing the same letter were significantly different in LSD post hoc test ($p < 0,05$).

Supplemental table 7: Effect of high fat diet on gene expression of vitamin D metabolism in adipose tissue for 11 weeks.

	Cyp27a1	Cyp2r1	Cyp2j6	Cyp3a11	Cyp24a1	Cyp27b1	Vdr	Gc	Megalin	Cubilin	AMN	Dab2
LF	100 ± 8 [a]	100 ± 7 [a]	100 ± 8 [a]	Nd	Nd	100 ± 9 [a]	100 ± 12 [a]	100 ± 11 [a]	100 ± 8 [a]	100 ± 19 [a]	Nm	Nm
HF/LF	115 ± 24 [a,b]	147 ± 31 [a]	100 ± 18 [a]			92 ± 8 [a]	64 ± 5 [a]	782 ± 177 [b]	73 ± 8 [a,b]	69 ± 26 [b]		
HF	66 ± 6 [b]	344 ± 36 [b]	75 ± 6 [a]			48 ± 4 [b]	138 ± 11 [b]	340 ± 85 [a]	58 ± 5 [b]	140 ± 13 [a]		

Expression of genes coding proteins of vitamin D metabolism relative to 18S ribosomal RNA in epididymal white adipose tissue (WAT), of mice fed with control diet (low fat, LF), high fat diet (HF) or weight loss group (HF/LF) after 11 weeks (n=10). Values are presented as means ± SEM. FBars not sharing the same letter were significantly different in LSD post hoc test ($p < 0,05$).

ARTICLE 7 : Simultaneous quantification of cholecalciferol, 25-hydroxyvitamin D and 1.25-dihydroxyvitaminD in plasma, adipose and brain tissue.

Article soumis à Journal of Chromatography A.

Une partie de ma thèse a été consacrée à l'élaboration d'une méthode de quantification de la vitamine D et de ses métabolites au niveau du plasma et tissu adipeux par chromatographie en phase liquide couplée à une spectrométrie de masse en tandem (LC-MS/MS), la méthode de référence pour la quantification de ces molécules.

Le nombre d'articles publiés relatifs à la quantification du cholécalciférol et de la 25(OH)D dans le plasma par LC-MS/MS est assez élevé mais très peu parviennent à détecter la $1,25(OH)_2D$ simultanément. En ce qui concerne les tissus, les articles sont encore plus rares. En effet, seulement 4 articles sont répertoriés pour la quantification du cholécalciférol et de la 25(OH)D dans le tissu adipeux et un seul rapporte la quantification de la $1,25(OH)_2D$.

L'objectif dans cet article était de développer et de valider une méthode LC-MS/MS pour déterminer simultanément et précisément la quantité de cholécalciférol, de 25(OH)D et de $1,25(OH)_2D$ dans le plasma et le tissu adipeux de souris. Le défi est multiple et concerne surtout la faible concentration de ces métabolites, en particulier pour la $1,25(OH)_2D$ (picomolaires), mais aussi les faibles quantités d'échantillons disponibles qu'il s'agisse des tissus ou du plasma, compte tenu du faible poids des souris. Pour amplifier le signal, deux types de réactifs de dérivatisation ont été utilisés : PTAD et Amplifex©.

Simultaneous quantification of cholecalciferol, 25-hydroxyvitamin D and 1.25-dihydroxyvitaminD in plasma, adipose and brain tissue.

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Abbreviated title: Quantification of vitamin D metabolites in plasma and tissues by LC-MS/MS.

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Disclosure statement: The authors have nothing to disclose

Abstract

In the last years, the investigations of the role of vitamin D and its metabolites with a view to characterize its metabolic pathways have made necessary the development of accurate and sensitive method to measure these metabolites in biological fluid but also in tissues. To that end we aimed to develop a specific LC-MS/MS method allowing simultaneous quantification of 3 vitamin D metabolites of interest cholecalciferol, 25-hydroxyvitamin D (25(OH)D) and 1.25-dihydroxyvitamin D ($1.25(OH)_2D$) in 3 different matrix that were plasma, adipose tissue and brain tissue. A pretreatment with liquid-liquid extraction for plasma samples or solid phase extraction for tissue was followed by derivatization using Amplifex or PTAD reagents to improve the stability and ionization efficiency of the metabolites. The method was optimized using co-eluting stable isotope labelled internal standards (IS) for the calibration of each analogues and in the same manner biological samples were spiked with the IS. The intra- and inter-day relative standard deviations were respectively 0.78-5.95% and 2.04-14.4% for the 3 metabolites derivatized with Amplifex reagent and we obtained similar values for the metabolites derivatized with PTAD reagent. Limits of quantification (LOQ) obtained with Amplifex derivatization were respectively 0.02 ng/mL; 0.19ng/mL and 0.78 ng/mL for $1.25(OH)_2D$, 25(OH)D and cholecalciferol. LOQ obtained with PTAD derivatization were 0.78 ng/mL for the 3 vitamin D metabolites.

Simultaneous quantification of 3 relevant vitamin D metabolites was successfully developed and validated here.

Introduction

Vitamin D (VD) or cholecalciferol is a hormone involved in calcium and phosphate homeostasis. Well-known bone diseases such as rickets, osteoporosis and osteomalacia are directly linked to VD insufficiency or deficiency [1]. The involvement of VD has also been suggested in many other physiopathological disorders including cardiovascular disease or type 2 diabetes. After endogenous synthesis or intestinal absorption, cholecalciferol is metabolized in the liver by 25-hydroxylases and produced 25-hydroxyvitamin D (25(OH)D) [2]. In plasma, 25(OH)D is the major form of VD and classically considered as the biomarker of VD status [3]. 25(OH)D can be hydroxylated in the kidney by CYP27B1 into 1α,25-dihydroxyvitamin D ($1.25(OH)_2D$) which is the active form of VD.

Adipose tissue is considered as one of the main storage site for cholecalciferol and 25(OH)D as highlighted in several studies and recently reviewed [4, 5].

In brain, VD signalling plays an essential role in its function and development [6] and VD deficiency plays an important role in diseases of central nervous system (Parkinson's disease, epilepsy etc…) [3, 7].

To go further in the elucidation of VD impact in term of health effect and in the kwnoledge of its metabolism, it is of particular interest to simultaneous measure VD metabolites in key organs.

Consequently, the frequency of routine analytical measurement of VD status has increased greatly [8], necessitating the development of reliable and accurate analytical methods to measure concentration levels of VD metabolites not only in plasma but also in relevant body tissues [9]. Numerous LC-MS/MS methods have been described for the measurement of either a single or a combination of VD metabolites. In human serum, very few methods include $1.25(OH)_2D$ achieving the required sensitivity for all [10, 11].

In adipose and brain tissues, some papers reported the quantification of cholecalciferol and 25(OH)D by high performance liquid chromatography tandem-mass spectrometry (HPLC-MS/MS)[12-15] but none of them even the most recent paper [16] include the detection of $1.25(OH)_2D$. Our objective in this paper was to develop and validate a LC-MS/MS method for simultaneous and accurate determination of cholecalciferol, 25(OH)D and $1.25(OH)_2D$ in mice plasma, adipose and brain tissue. The challenge is multiple and concerns the low concentration of these metabolites especially for $1.25(OH)_2D$ which is biologically active at picomolar concentrations but also the small available amount of tissue sample or plasma volume in mice study. The necessity to increase the ionization efficiency and sensitivity implies a sample pretreatment and two type of derivatizing reagent applicable to diene analytes have been used.

Materials and methods

Materials

Cholecalciferol, 25OHD and 1.25$(OH)_2$D, 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD), dibasic potassium phosphate (K_2HPO_4), methyl tert-butyl ether (MTBE), phosphate buffer saline (PBS) were purchased from Sigma –Aldrich. Amplifex Diene Reagent was purchased from Sciex (Chemistry and consumables R&D, Framingham, MA). Isotopically labelled d3-cholecalciferol, d3-25OHD and d3-1.25$(OH)_2$D were purchased from Cambridge Isotope Laboratories (MA, USA). All vitamin D metabolites were stored at -80°C in the dark. LC-MS grade acetonitrile (AcN) and formic acid (FA), HPLC grade methanol (MeOH) and ethyl acetate (EtOAc) were obtained from CarloErba. Water used was generated from Direct-Q Ultrapure Water System from Millipore (Bedford, MA) with a specific resistance of 18.2 MΩ cm. Oasis HLB cartridges used for solid-phase extraction (3 CC / 60 mg) were supplied by Waters (Guyancourt, France).

Instrumentation

The chromatographic separation was carried out on a Dionex UltiMate 3000 (Thermo Fisher Scientific) consisted of a rapid separation pump (RS) (LPG-3400 RS), an autosampler (WPS-3000 TRS) and a column compartment (TCC-3000 RS) all operated by Chromeleon 6.8 software. Samples were injected on a Hypersil GOLD C18 column (2.1 x 100 mm; Thermo Scientific, Les Ulis, France). PRM and accurate mass measurements were performed on Q-Exactive Plus mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) with a Heated Electrospray Ionization (H-ESI II) probe. Thermo Xcalibur 3.0.63 software was used as well for the instrument setup and control of the LC-MS system during acquisition as for the data

99 treatment. Tune Q Exactive Plus 2.5 application was used for the direct control of the mass
100 spectrometer.
101
102 Chromatographic conditions
103 Autosampler tray was kept at 4 °C during the experimentation and the column oven temperature
104 regulated at 40 °C. 5 µL of sample were injected onto the column. Mobile phase A consisted of
105 0.1% formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile. Solvent
106 flow rate was set to 400 µL/min. Two different solvent gradients for the compounds derivatized
107 by Amplifex or PTAD reagent were used for the separation of compounds according to their
108 polarities. For the Amplifex assay, the starting mobile phase consisted of 30% of solvent B and
109 were held that way for 4min. A linear gradient was applied with B% increasing to 65% 6
110 minutes, held isocratic during 2 min and increasing to 100% of B during 4 minutes. Start
111 conditions were reinstated in 2 minutes, making the total run of 18 minutes. For the PTAD
112 derivatization assay, gradient started with 50% of B during one minute, then ramped to 100%
113 of B for 12 minutes and stayed isocratic for 2 minutes. During one minute, phase B descended
114 to 50% and then stayed for the equilibration at 50% for 2 minutes. The total run time was 18
115 minutes.
116
117 Mass spectrometric conditions
118 LC-HRMS/MS analyses were performed with external calibration in positive mode providing
119 a mass precision lower than 5 ppm. The H-ESI probe and the transfer capillary temperature
120 were kept at 310°C and 320°C, respectively. Spray voltage was set at 3500 V and the S-lens
121 RF level at 55 V. Sheath and auxiliary gas were maintained at 30 and 8 (arbitrary units).
122 PRM (Parallel Reaction Monitoring) method was set up as MS/MS acquisition mode and full
123 higher-energy collision dissociation spectra (HCD) spectra were acquired. For each compound,

the most abundant ion was selected as precursor ion and isolated in a 2 uma window in a specified time segment and fragmented under previously optimized collision energies (Table 1). The resolving power was set to 35000 full width at half maximum (FWMH) at m/z 200 and the AGC was set to 2*10e5. The injection time was set at 100 ms maximum in order to optimize the analytical cycle time.

Table 1: Precursor and product ions m/z, retention times, and normalized collision energies for cholecalciferol, 25(OH)D, 1,25$(OH)_2$D and the corresponding isotopically labeled molecule in the two LC-MS/MS methods for the absolute quantification.

Compound Name	Amplifex derivatization					PTAD derivatization				
	Precursor adduct	Precursor m/z	Product m/z	Retention Time (min)	Collision Energy (%)	Precursor adduct	Precursor m/z	Product m/z	Retention Time (min)	Collision Energy (%)
Cholecalciferol	[M]+	716.5	657.4368	13.2	21	[M+H]+	560.37	298.1185	11.1	20
25(OH)D	[M]+	732.51	673.4311	9.7	21	[M-H2O+H]+	558.37	298.1178	5.0	20
1,25$(OH)_2$D	[M]+	748.51	689.4297	8.6	21	[M-H2O+H]+	574.37	314.1123	2.8	20
d3-Cholecalciferol	[M]+	719.5	660.4552	13.2	21	[M+H]+	563.37	301.1371	11.1	20
d3-25(OH)D	[M]+	735.5	676.4534	9.7	21	[M-H2O+H]+	561.37	301.1371	5.0	20
d3-1,25$(OH)_2$D	[M]+	751.5	692.4458	8.6	21	[M-H2O+H]+	577.37	317.1318	2.8	20

Preparation of stock and working solutions of deuterated standards (IS) and analytical standards

A working solution of deuterated analytes was prepared at 0,02 ng/mL of each internal standards (IS) (d3-cholecalciferol, d3-25(OH)D and d3-1,25$(OH)_2$D).

A primary stock solution of cholecalciferol, 25(OH)D and 1.25$(OH)_2$D standards were prepared at concentrations respectively of 100, 50 and 10 ng/mL in ethanol and stored at -80°C in the dark. Calibration curves were prepared by serial dilution of the 3 analytes stock solution to obtain calibration standards from 0 to 50 ng/mL. After addition of 1.5 µL of the working solution of deuterated analytes to each dilution, derivatization was proceeded using Amplifex® or PTAD. A batch of QC samples was constituted for method validation.

145

146 Plasma sample preparation

147 The preparation was inspirited to the Wang paper [17]. The extraction procedure was conducted
148 under low light because vitamin D and its metabolites are light sensible. After thawing in ice,
149 mice plasma was centrifuged at 4°C, for 15 min, at 13 600 g. 100 µL of each sample was
150 transferred to hemolysis tube with 10 µL of deuterated working solution. Proteins were
151 precipitated by adding 500 µL of acetonitrile, vortex-mixed, and centrifuged for 10 min at 3 000
152 g. The supernatant was transferred to a glass tube, and the volume was reduced to a half under
153 a nitrogen stream. 500 µL of ethyl acetate was added to the solution for liquid-liquid extraction.
154 After shaking vigorously for 10 min, samples were centrifuged for 20 min at 590 g and the
155 upper organic layer was transferred to a glass tube and reduced under nitrogen stream. The
156 plasma samples were derivatized with amplifex diene reagent.

157

158 Adipose tissue preparation

159 Epididymal white adipose tissue were collected and stored at -80°C. The preparation of adipose
160 tissue was inspirited to the Lipkie paper [14]. After being thawed on ice, 50 mg of tissue were
161 grinded with 1 mL of PBS 30 mvt/sec for 15 min (Retsch, Eragny sur Oise, France). Tissue
162 homogenate was transferred in hemolysis tube and spiked with 25 µL of deuterated stock
163 solution. AcN (1 mL) was added and the mixture vortexed for 5 min and centrifuged at 4°C at
164 6 000 g for 5 min. After addition of 1 mL of MTBE the mixture was vortexed for 5 min,
165 centrifuged (the condition as previously) and the upper organic layer was collected in other
166 tube. The same extraction was repeated twice more with addition of 300 µL of PBS, 1 mL of
167 AcN (vortex+centrifugation) and 1 mL of MTBE (vortex+centrifugation). The supernatant
168 were pooled then dried under nitrogen. Oasis HLB cartridges were prepared with 3 mL each of
169 EtOAc, MetOH and H2O. The samples were reconstituted with 1 mL each of MetOH and 1 mL

of K2HPO4 (0.4 M) and loaded on the cartridge. The cartridge was washed with 3 mL of H2O and 2 mL of 70% MetOH before being dried for 2 min under vacuum. Tips were washed with AcN and analytes were eluted with 1.5 mL of AcN and dried under nitrogen. AT tissue samples were derivatized with Amplifex reagent.

Brain tissue preparation

Brains were collected and stored at 80°C. The preparation of adipose tissue was inspirited to the Xue paper [12]. After being thawed on ice, 90 mg of brain tissue were grinded at 30 mvt/sec for 15 min and homogenized after addition of 1 ml of AcN. 25µL of working solution of deuterated analytes were added and the mixture was vortexed for 10 min then centrifuged at 4°C for 10 min at 15 000 g. The supernatant was transferred into hemolysis tube tube and dried under nitrogen. The samples were derivatized with Amplifex or PTAD reagent.

Amplifex derivatization

A one-step derivatization was employed to improve the ionization efficiency of the metabolites using Amplifex diene as reagent [18]. 30 µL of the Amplifex were added to the dried sample above, vortexed for 15 s and incubated for 30 min at room temperature. Next, 30 µL of deionized water were added, vortexed for 15 s and transferred for LC injection.

PTAD derivatization

Working solution of PTAD was prepared at a concentration of 2mg/mL in AcN. For derivatization 100 µL of PTAD working solution were added to each sample, vortex for 10 min then these 2 steps were repeated. The samples were dried under nitrogen and finally 100 µL of AcN were added before LC injection.

195 Method validation

196 Linearity and LOQ

197 In order to assess linearity of the quantification methods, we analyzed increasing amount of
198 analytical standards solution spiked with the deuterated pool of the three corresponding
199 analytes. For each derivatization reagent and each analyte cholecalciferol, 25(OH)D and
200 $1,25(OH)_2D$, calibration curves were plotted with peak area ratio of vitamin D metabolite to
201 the respective internal standard versus a range of concentrations of the analyte. Five
202 concentrations of standards were chosen considering the concentration range expected in the
203 study. The series of concentrations were respectively for cholecalciferol, 25(OH)D and
204 $1,25(OH)_2D$ 0.20 to 50 ng/mL, 0.10 to 12 ng/mL and 0.020 to 2.5 ng/mL.
205 The LOQ was set as the concentration at which the precision is less than 20% of the relative
206 standard deviation with the accuracy between 80 and 120 % of the theoretical value.
207

208 Accuracy and precision

209 For the two types of derivatization, replicate samples at three levels were analyzed in separate
210 runs to determine the intra and inter day precision and accuracy.
211 The intraday accuracy and precision were calculated by processing five replicates at three
212 concentration levels. The inter-day accuracy and precision were determined by analyzing five
213 replicates samples in 3 batches (n=15) at three concentration levels.
214 The precision of the assay was estimated by the coefficient of variation for each concentration
215 level. Accuracy was represented as the relative error to the nominal concentrations (RE%).
216

217 Recovery and matrix effects

218 Percent recovery was determined as 100 x (concentration in spiked sample - concentration in
219 unspiked matrix) / concentration added. For each analyte (cholecalciferol, 25OHD and

220 1,25$(OH)_2$D) all samples were analyzed by the ratio of the analyte to the corresponding
221 isotopically labeled IS (d3-cholecalciferol, d3-25OHD and d3-1,25$(OH)_2$D). The recovery was
222 calculated for plasma, adipose tissue and brain samples spiked with a standard solution
223 containing the 3 analytes. Native and spiked samples were prepared and analyzed at the same
224 time.

Results and discussion

Method validation

Chromatographic separation

The optimal separation was achieved with linear gradient conditions using acetonitrile/water (with 0.1% formic acid) as mobile phase on a C18 column as shown in Figure 1, 2 and 3. The 3 vitamin D-Amplifex as well as the 3 vitaminD-PTAD were chromatographically resolved from each other (Rs>1.5). The use of tandem mass detection and satisfactory chromatographic separation ensured the high specificity of the method.

Linearity, LOQ and calibration curves

The data were fitted using linear regression. Table 1 showed the linear range and LOQ for the metabolites using Amplifex or PTAD reagent for derivatization. They were similar with both derivatization type for cholecalciferol. For 25OHD and $1,25(OH)_2D$ derivatization with Amplifex reagent showed a lower LOQ compared to the LOQ values obtained with PTAD reagent. Regression analysis showed good linearity with correlation coefficients (r^2) of > 0.993 for the three analytes whatever the signal amplification method was.

Table 2: Linear range, LOQ and RE[a] for the 3 metabolites derivatized with Amplifex and PTAD reagent

	Amplifex derivatization			PTAD derivatization		
Analytes	Linear range ng/ml	LOQ ng/ml	RE%	Linear range ng/ml	LOQ ng/ml	RE%
Cholecalciferol	0.20-50	0.78	-5.3	0.20-50	0.78	17.4
25OHD	0.10-12.5	0.19	4.0	0.10-12.5	0.78	-9.3
$1,25(OH)_2D$	0.02-2.5	0.02	17.3	0.02-2.5	0.08	-18.5

[a] RE was determined for LOQ concentration

246

247 Precision and accuracy

248 Table 3 summarizes the precision and accuracy of the 2 methods according the derivatization
249 procedure for simultaneous quantification of the 3 vitamin D metabolites. Using amplifex
250 derivatization the intra-assay variation was between 0.8 and 6% according vitamin D
251 metabolites and the concentration. The inter-assay variation was between 2 and 14.4%. The
252 relative error of the three tested concentrations in 3 independents assays were within ±3%, ±9
253 and ±13% for respectively cholecalciferol, 25(OH)D and $1.25(OH)_2D$. With PTAD procedure
254 derivatization the intra-assay variation was between 0.7 and 11% according the vitamin D3
255 metabolite and the concentration. The inter-assay variation was between 4 and 14.5%. The
256 relative error of the three tested concentrations in 3 independent assays were within ±14%, ±13
257 and ±8% for respectively cholecalciferol, 25(OH)D and $1.25(OH)_2D$. These results indicate
258 good reproducibility and accuracy of the methods whatever the derivatization procedure was.
259

Table 3: Precision and accuracy of cholecalciferol, 25(OH)D and 1.25(OH)$_2$D. measurements.

Compound and concentration	Intra-assay (n=5)[a]			Inter-assay (n=3)[b]		
	Measured[c]	%RE[d]	%CV[e]	Measured[c]	%RE[d]	%CV[e]
			Cholecalciferol-amplifex			
1.56 ng/ml	1.53±0.009	-1.66	0.96	1.57±0.09	0.86	12.7
3.13 ng/ml	3.31±0.059	6.18	2.61	3.09±0.28	-1.22	9.03
12.5 ng/ml	12.9±0.13	3.2	1.34	12.1±0.25	-2.85	2.04
			25(OH)D-amplifex			
0.78 ng/ml	0.81±0.03	4.11	5.95	0.74±0.09	9.26	11.1
1.56 ng/ml	1.70±0.04	8.99	3.74	1.49±0.10	-4.17	7.05
6.25 ng/ml	6.36±0.12	1.84	2.72	5.83±0.18	-6.79	3.10
			1.25(OH)$_2$D-amplifex			
0.16 ng/ml	0.15±0.001	-6.26	1.51	0.18±0.01	14.4	13.0
0.31 ng/ml	0.32±0.002	5.13	0.78	0.31±0.04	0.10	14.4
1.25 ng/ml	1.32±0.02	5.84	1.86	1.23±0.03	-1.63	2.78
			Cholecalciferol -PTAD			
1.56 ng/ml	1.64±0.15	5.63	9.23	1.58±0.18	1.15	11.3
3.13 ng/ml	3.28±0.38	5.09	11.5	2.97±0.43	-4.87	14.5
25 ng/ml	25.9±1.62	3.68	6.25	24.2±3.48	-3.1	14.4
			25(OH)D-PTAD			
0.78 ng/ml	0.71±0.07	-9.3	9.45	0.67±0.09	-14.6	13.2
6.25 ng/ml	6.03±0.04	-3.44	0.67	6.22±0.29	-0.39	4.67
12.5 ng/ml	13.0±0.61	3.82	4.68	12.7±1.43	1.64	11.3
			1.25(OH)$_2$D -PTAD			
0.312 ng/ml	0.31±0.02	-1.05	6.94	0.31±0.01	0.34	3.93
1.25 ng/ml	1.29±0.02	3.6	1.74	1.24±0.08	-0.59	6.51
2.5 ng/ml	2.57±0.09	2.74	3.63	2.37±0.19	-5.13	7.93

a. Intra-assay precision was calculated for 5 replicates measured in a single run
b. Inter- assay precision was calculated from 3 independent assays
c. Means+/- SD of measured concentrations
d. Percent of relative error (%RE) is defined as the ratio of the deviation to the theoretical value x 100
e. Coefficient of variance is defined as the ratio of the standard deviation to the mean value x 100

271

272 Recovery and matrix effect

273 Plasma, adipose tissue and brain tissue samples were spiked with increased concentrations of
274 metabolite standards (2 levels) and percent recovery were calculated for cholecalciferol,
275 25(OH)D and 1.25$(OH)_2$D. The recovery results were consistently greater than 72% for the 3
276 vitamin D metabolites in plasma and tissue matrix (Table 4). The deuterated internal standards
277 which demonstrated the same mass spectrometric behavior as the analytes were able to correct
278 for variations caused by matrix (Fig. 4 and 5).

279

280 **Table 4: Recovery results of cholecalciferol, 25(OH)D and 1.25$(OH)_2$D in plasma, adipose**
281 **and brain tissue.**

	Concentration	Recovery %		
	ng/ml	Plasma	Adipose tissue	Brain tissue
Cholecalciferol	25	73.2	100.5	97.5
	6.25	82.1	91.2	99.6
25(OH)D	12.5	97.5	99.6	98.3
	3.25	87.4	88.4	71.8
1.25$(OH)_2$D	2.5	93.9	73.1	-
	0.625	107.6	71.8	-

282 Plasma and adipose tissue were derivatized with Amplifex and brain tissue were derivatized
283 with PTAD for 25(OH)D and with Amplifex for cholecalciferol

284

285 Stability

286 Stability of derivatized vitamin D metabolites was tested. After 48h the PTAD 3 derivatized
287 metabolites were stable in the cooled autosampler (4 °C) with no loss of signal between the
288 first and third day. These results are in agreement with litérature [19]. The Amplifex
289 derivatized metabolites have shown a signal decrease from 24h in the autosampler
290 respectively from -15.3±0.5%, -19.7±2.0%, -42.6±2.0% for cholecalciferol, 25(OH)D and

1,25(OH)2D. These results were as expected according to the AB Sciex recommendation notice.

Conclusion

One more time LC-MS/MS appears as a powerful analytical technique to quantify simultaneously several analytes of interest. As expected compared to PTAD, Amplifex reagent has allowed to get a lower LOQ and could appear as the favorite method for derivatization and that is why we have preferably applied Amplifex derivatization to plasma or tissues samples. Nevertheless PTAD derivatization presents the advantages to be more stable at 48h and also for a reason that we did not demonstrate yet, for brain tissue samples only PTAD derivatization could allow quantification of 25(OH)D. We can suppose that the tissue composition play a role in the derivatization reaction and even if both tissue, brain and adipose tissues present lipidic environment they differ in their composition. The scope of LC-MS/MS methods published to date are limited to the quantification of 1 or 2 vitamin D metabolites in a single run. It is now accepted that derivatization techniques could maximized selectivity and sensitivity and we show here the interest of these derivatization methods to quantify simultaneously and with accuracy cholecalciferol, 25(OH)D and $1.25(OH)_2D$ in plasma, adipose tissue and brain tissue by LC-MS/MS.

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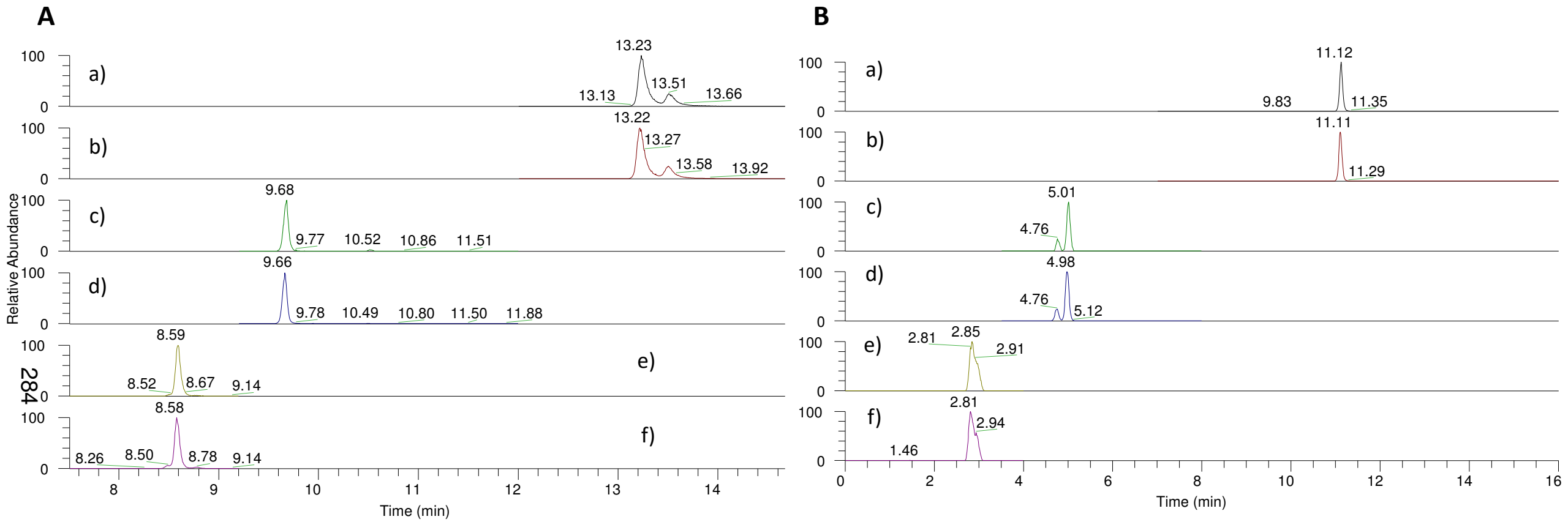

Figure 1: XIC of standards mixed solution derivatized either with Amplifex reagent (A) a) m/z 657.4368 extracted from the MS/MS spectrum of m/z 716.50 ion of cholecalciferol, b) m/z 660.4552 extracted from the MS/MS spectrum of m/z 719.50 ion of d3-cholecalciferol, c) m/z 673.4311 extracted from MS/MS spectrum of m/z 732.51 ion of 25(OH)D, d) m/z 676.4534 extracted from MS/MS spectrum of m/z 735.50 ion of d3-25(OH)D, e) m/z 689.4297 extracted from MS/MS spectrum of m/z 748.51 ion of $1,25(OH)_2D$ and m/z 692.4458 extracted from MS/MS spectrum of m/z 751.50 ion of d3-$1,25(OH)_2D$, or **with PTAD reagent (B)** a) m/z 314.11234 extracted from the MS/MS spectrum of m/z 574.37 ion of cholecalciferol, b) m/z 317.13177 extracted from the MS/MS spectrum of m/z 577.37 ion of d3-cholecalciferol, c) m/z 298.11780 extracted from MS/MS spectrum of m/z 558.37 ion of 25(OH) D, d) m/z 301.13708 extracted from MS/MS spectrum of m/z 561.37 ion of d3-25(OH)D, e) m/z 298.11850 extracted from MS/MS spectrum of m/z 560.37 ion of $1,25(OH)_2D$ and m/z 301.13708 extracted from MS/MS spectrum of m/z 563.37 ion of d3-$1,25(OH)_2D$

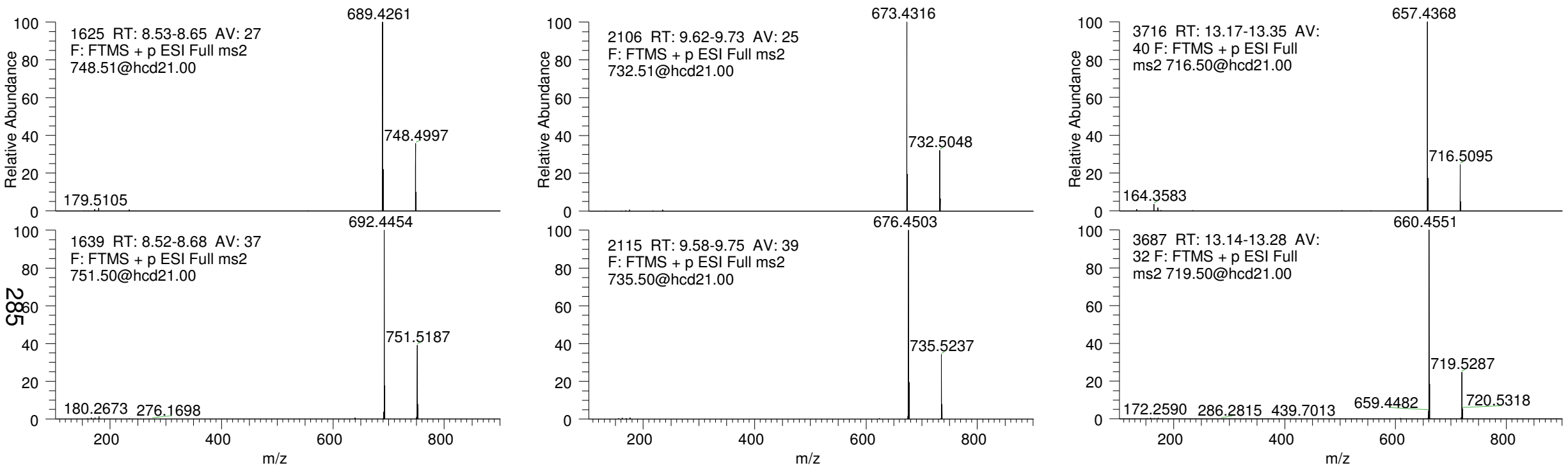

Figure 2: MS/MS spectrum of 1,25(OH)$_2$D, 25(OH)D and cholecalciferol derivatized with Amplifex reagent and their respective deuterated forms.

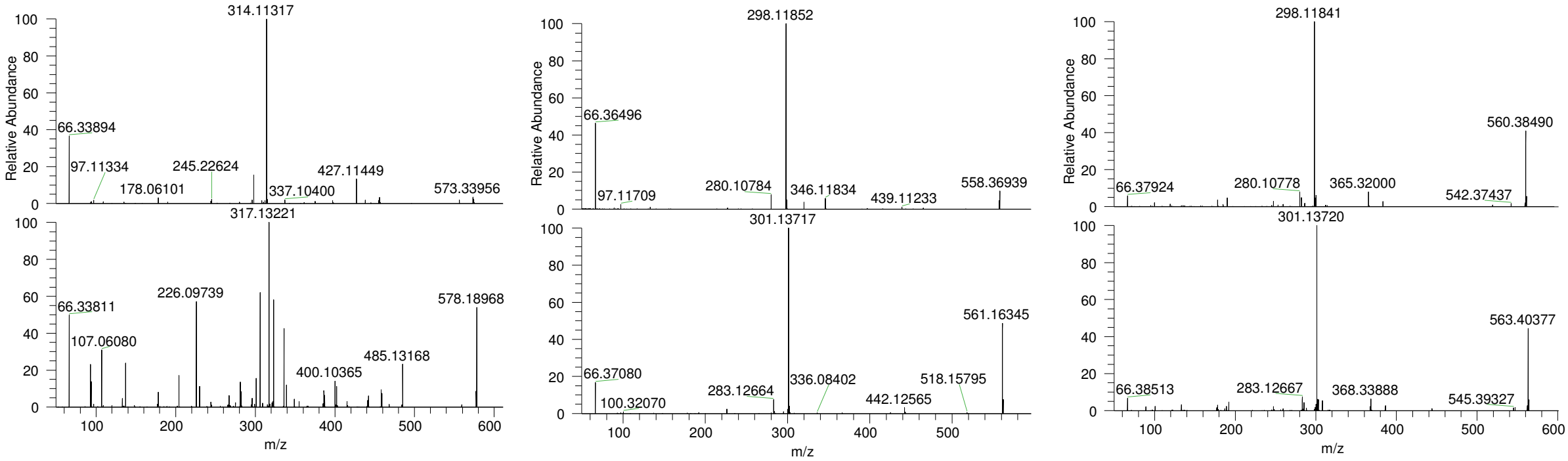

Figure 3: MS/MS spectrum of 1,25(OH)$_2$D, 25(OH)D and cholecalciferol derivatized with PTAD reagent and their respective deuterated forms.

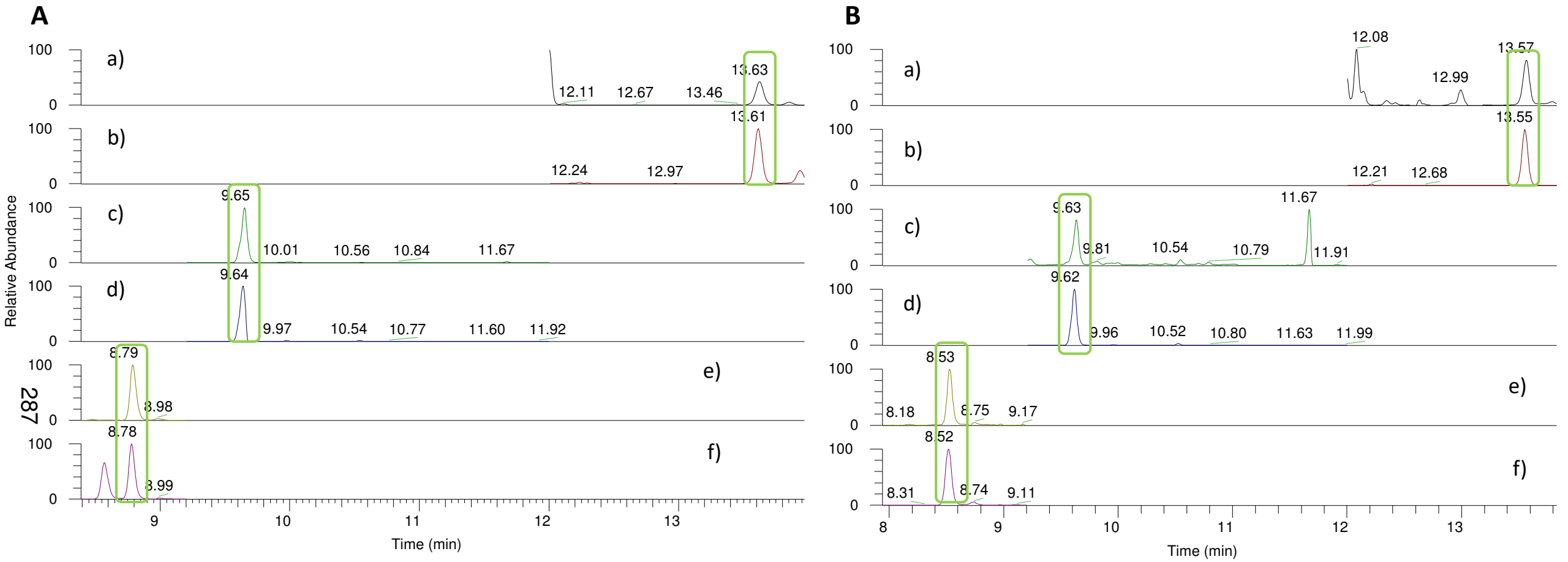

Figure 4: XIC of plasma (A) and adipose tissue (B) sample derivatized with Amplifex reagent a) m/z 657.4368 extracted from the MS/MS spectrum of m/z 716.50 ion of cholecalciferol, b) m/z 660.4552 extracted from the MS/MS spectrum of m/z 719.50 ion of d3-cholecalciferol, c) m/z 673.4311 extracted from MS/MS spectrum of m/z 732.51 ion of 25(OH)D, d) m/z 676.4534 extracted from MS/MS spectrum of m/z 735.50 ion of d3-25(OH)D, e) m/z 689.4297 extracted from MS/MS spectrum of m/z 748.51 ion of $1,25(OH)_2D$ and m/z 692.4458 extracted from MS/MS spectrum of m/z 751.50 ion of d3-$1,25(OH)_2D$.

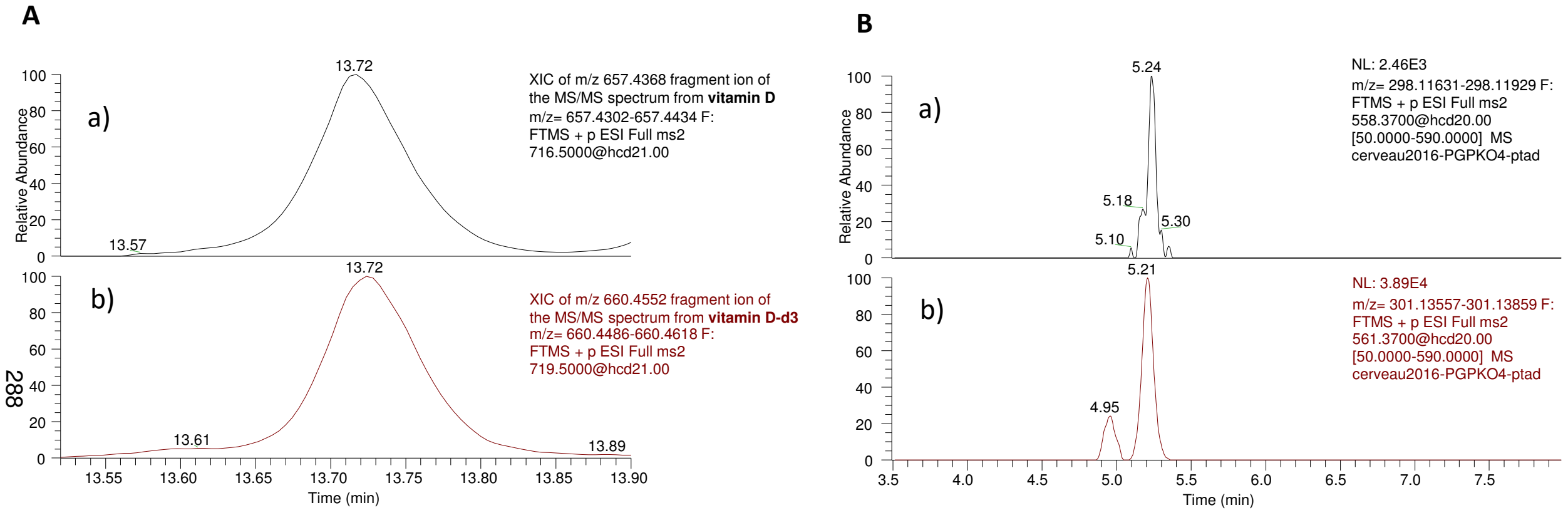

Figure 5: XIC of brain tissue sample derivatized either with Amplifex reagent (A) a) m/z 657.4368 extracted from the MS/MS spectrum of m/z 716.50 ion of cholecalciferol and b) m/z 660.4552 extracted from the MS/MS spectrum àof m/z 719.50 ion of d3-cholecalciferol, or **with PTAD reagent (B)** a) m/z 298.11780 extracted from MS/MS spectrum of m/z 558.37 ion of 25(OH)D and b) m/z 301.13708 extracted from MS/MS spectrum of m/z 561.37 ion of d3-25(OH)D,

Autres résultats

VITADOSE : Mise en place d'une cohorte de patients obèses et non-obèses

L'étude est conduite dans le service de Nutrition, Maladies Métaboliques, Endocrinologie à l'Hôpital de la Conception, sous la direction des Pr René VALERO et Patrice DARMON.

Objectif principal de l'étude

L'objectif principal de cette étude est de quantifier les taux de cholécalciférol ainsi que de ses deux autres métabolites 25(OH)D et $1,25(OH)_2D$, par LC-MS/MS, dans le tissu adipeux sous-cutané et viscéral de sujets obèses et de les comparer à ceux de sujets non-obèses afin de mettre en évidence d'éventuelles différences de concentration entre ces deux groupes de sujets.

Objectifs secondaires

Les objectifs secondaires de ce travail sont de quantifier l'expression des gènes impliqués dans le métabolisme de la vitamine D, des gènes codants pour des marqueurs de l'inflammation associée à l'obésité ainsi que des gènes impliqués dans la β-oxydation dans le tissu adipeux sous-cutané et viscéral et d'établir des corrélations entre ces niveaux d'expressions géniques et les concentrations en métabolites actifs de la vitamine D dans les deux groupes de sujets obèses et non-obèses. Des corrélations entre la concentration plasmatique en cholécalciférol et les concentrations des différents métabolites de la vitamine D dans le tissu adipeux, seront également étudiées.

Schéma expérimental

Afin de comparer les concentrations en cholécalciférol et de ses métabolites dans le tissu adipeux sous-cutané et viscéral de sujets obèses et non-obèses, deux groupes de 30 sujets seront constitués en fonction de leur indice de masse corporelle (IMC).

- Groupe 1, IMC<30 kg/m² (groupe non-obèse : NO)
- Groupe 2, IMC≥ 35 kg/m² (groupe obèse : OB)

Les patients devront répondre aux critères de sélection suivants :

Critères d'inclusion

- Sujet de sexe masculin ou féminin âgé de 18 à 65 ans

- Sujet ayant un IMC supérieur à 35 kg/m^2 (groupe OB) ou inférieur à 30 kg/m² (groupe NO)

- Sujets devant subir une chirurgie bariatrique (groupe OB) ou abdominale (groupe NO)

- Sujet étant affilié ou bénéficiaire d'un régime de sécurité sociale

- Sujet acceptant de participer à l'étude et ayant signé un consentement éclairé

Critères de non inclusion

- Personne particulièrement protégée : incapable majeur et personne privée de liberté, personne hospitalisée pour une autre pathologie

- Personne ne maîtrisant pas la lecture de la langue française

- Consommation régulière de compléments alimentaires ou de suppléments vitaminiques au cours des trois derniers mois.

- Femme enceinte (la prise d'une contraception efficace sera nécessaire pendant la durée de l'étude). Ces critères font déjà partie des recommandations de prise en charge chirurgicale de l'obésité de l'adulte avec la recherche systématique d'une grossesse chez les femmes en période d'activité génitale avant le geste chirurgical et la recommandation d'une contraception dès que la chirurgie est programmée puis pendant 12 à 18 mois après l'intervention [256].

- Pour les sujets du groupe NO :

• Sujet dénutri (IMC < 18,5 kg/m^2 ; perte de poids supérieure à 5% en 1 mois ou 10% en 6 mois)

• Sujet présentant une infection ou un syndrome inflammatoire (CRP > 10 mg/L et/ou globules blancs > 12000/mm3)

- Sujet cancéreux

Déroulement de l'étude

J-*30 avant l'opérat*ion : Dans le cadre d'une hospitalisation de jour, les sujets participant à l'étude doivent remplir un recueil alimentaire sur 3 jours dans le mois précédant leur chirurgie. Ils réalisent aussi une mesure de leur composition corporelle par l'absorption bi-photonique à rayons X (DEXA), ainsi qu'une évaluation de leur adiposité viscérale et sous-cutanée par la réalisation d'une coupe de scanner abdominal à hauteur de la 4ème vertèbre lombaire L4. Un prélèvement sanguin est nécessaire pour mesurer la concentration en cholécalciférol, 25(OH)D et $1,25(OH)_2D$ plasmatique, ainsi que les paramètres biologiques d'intérêt (glycémie, insulinémie, peptide C, hémoglobine glyquée chez les sujets diabétiques, NFS-plaquettes, CRP, calcémie, albuminémie, phosphorémie, PTH). Un autre tube de plasma est utilisé afin de doser les cytokines pro et anti-inflammatoires ainsi que l'adiponectine.

Jour de l'opération : Lors des procédures de chirurgie bariatrique : anneaux gastriques, sleeve gastrectomies et bypass gastriques chez les sujets obèses (groupe OB) et lors des interventions chirurgicales abdominales réglées chez les sujets non-obèses (groupe NO), il est réalisé, en fin d'intervention, un prélèvement de tissu adipeux sous-cutané de 0.25 cm^3 au niveau de l'entrée du trocard (hypochondre gauche) et un prélèvement de TAv au niveau du grand épiploon (corne gauche) de 2 cm^3.

Résultats attendus :

Nous postulons qu'il existe une différence de concentrations du cholécalciférol et de ses métabolites dans le TAsc et TAv ainsi que dans le plasma mesuré par LC-MS/MS entre les patients obèses et les patients non-obèses. Nous postulons qu'il existe des différences d'expression des gènes impliqués dans le métabolisme de la vitamine D, des gènes codants pour les marqueurs de l'inflammation associée à l'obésité et de ceux impliqués dans la β-oxydation au niveau du TAsc et TAv entre les 2 groupes de patients. Nous postulons que ces niveaux d'expression génique sont corrélés avec les concentrations au niveau du TA des métabolites actifs de la vitamine D.

DISCUSSION GÉNÉRALE

La vitamine D est impliquée dans de nombreux processus physiologiques, notamment dans le contrôle de certains paramètres de la biologie du tissu adipeux blanc, son principal site de stockage. En effet, elle y exerce des effets anti-inflammatoires et métaboliques en régulant l'expression de nombreuses cytokines ou chimiokines, sécrétées par le tissu adipeux lui-même, et de certains facteurs impliqués dans l'adipogénèse. Bien que le tissu adipeux exprime de nombreuses enzymes et acteurs du métabolisme de la vitamine D, la régulation adipocytaire de ce métabolisme n'a jamais été étudiée. Celui-ci pourrait être régulé par la vitamine D elle-même et par l'obésité, cependant les mécanismes mis en jeu ne sont pas clairement établis. De plus, au cours de l'obésité, la physiologie du tissu adipeux est fortement perturbée. Cela se traduit par une hypertrophie et une hyperplasie des adipocytes qui sont associées à d'autres désordres physiologiques aboutissant à la dérégulation du sécrétome caractéristique de l'inflammation métabolique [257]. Par ailleurs de très nombreuses études ont montré que les taux sériques en 25(OH)D, la forme circulante de la vitamine D, d'individus obèses sont généralement plus bas que ceux d'individus normo-pondéraux [207], suggérant une relation forte entre métabolisme de la vitamine D et obésité.

Dans ce contexte, le but de ce travail de thèse a consisté à faire avancer les connaissances relatives à l'interrelation entre la vitamine D, le tissu adipeux et l'obésité. Pour ce faire, nous avons poursuivis les travaux du Laboratoire sur l'effet anti-inflammatoire de la vitamine D dans l'adipocytes, et nous avons également entrepris l'étude de la régulation du métabolisme adipocytaire de la vitamine D, sous l'effet d'une supplémentation en vitamine D elle-même, ou au cours de l'obésité.

Concernant nos travails relatifs à l'effet anti-inflammatoire de la vitamine D, il avait été récemment mis en évidence que la vitamine D, ou plus exactement sa forme active, était capable de limiter l'inflammation au niveau du tissu adipeux de souris et des adipocytes humains et murins, in vitro et in vivo, en diminuant l'expression de cytokines et de chimiokines participant ainsi à une diminution de la migration macrophagique [146]. De plus, certains miRs, dont le mir155, sont connus pour être impliqués dans l'amplification du signal inflammatoire via l'induction de l'expression des cytokines et des chimiokines [126]. Fort de ces données, il nous a paru pertinent d'étudier l'impact de la vitamine D sur l'expression des miRs dans les adipocytes soumis à un stress inflammatoire.

Nous avons pu mettre en évidence, de façon tout à fait originale, que la $1,25(OH)_2D$ est capable de limiter l'expression des 3 miRs (miR-146a, miR-150 et miR-155) induite par le TNFα dans des adipocytes humains et murins **(Article 2)**. De plus, nous avons été en mesure de confirmer cet effet in vivo dans un modèle murin d'inflammation chronique **(Article 2)**. Dans ce modèle, les souris ont été soumises à différents régimes : régime standard, régime riche en graisses ou régime riche en graisse supplémenté en vitamine D pendant 10 semaines. Il est important de souligner que nous avons supplémenté les animaux avec une dose 10 fois supérieure à la dose recommandée pour les animaux de laboratoire soit une dose de 15000 UI/kg de nourriture qui équivaut à 90 UI/souris. Enfin, nous avons montré que la régulation de l'expression des miRs (miR-146a et miR-155) est médiée par la voie de signalisation NF-κB **(Article 2)**. En effet, les souris sur-exprimant p65, une sous-unité activatrice du complexe NF-κB, présentent une augmentation de l'expression des miR-146a et miR155. A l'inverse, les souris F-p65-KO, invalidées pour cette même sous-unité p65 présentent une diminution de l'expression du miR-155. Nous avons montré que les effets anti-inflammatoires de la $1,25(OH)_2D$ sont liés à une inhibition de la voie de signalisation NF-κB qui se traduit par une réduction du niveau de phosphorylation de p65 et IκB dans les adipocytes 3T3-L1 (**Figure 24**). Ces résultats sont concordants avec les études précédentes sur la $1,25(OH)_2D$ montrant l'inhibition de l'expression de la protéine IκB dans les adipocytes 3T3-L1 [144], les adipocytes [147] et les préadipocytes humains [142], ainsi que l'inhibition de la translocation de p65 dans les adipocytes humains [148]. Ainsi nos travaux ont permis de contribuer à la mise en évidence d'un nouveau niveau de régulation de l'inflammation par la vitamine D dans l'adipocyte, impliquant les miRNA.

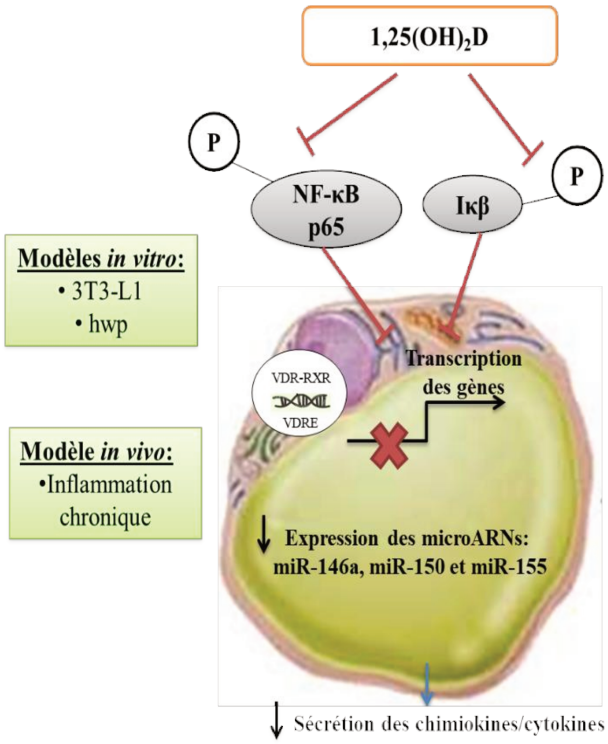

Figure 24 : Effet de la 1,25(OH)$_2$D sur la régulation des miRNAs au niveau des adipocytes et du tissu adipeux.

En préambule de l'étude de la régulation du métabolisme de la vitamine D, nous avons, avec l'aide du Groupe « Métabolomique » du Laboratoire, mis au point un dosage de la vitamine D (cholécalciférol) et de ses métabolites (25(OH)D et 1,25(OH)$_2$D par chromatographie en phase liquide couplée à une spectrométrie de masse en tandem (LC-MS/MS). En effet ce dosage constituait un véritable verrou technologique qu'il a fallu lever. Ainsi une partie de mon travail de thèse a été consacrée à la mise au point et à la validation d'une méthode de quantification du cholécalciférol, de la 25(OH)D et de la 1,25(OH)$_2$D au niveau du plasma et du tissu adipeux par LC-MS/MS (**Article 7**), qui est considérée à l'heure actuelle comme la méthode de référence pour la quantification de ces molécules. Les aspects techniques et méthodologiques ne seront pas développés d'avantage dans cette discussion. Il est toutefois important de souligner que l'utilisation d'un réactif de dérivation (Amplifex) de conception récente, nous a permis d'obtenir une plus forte sensibilité et de meilleurs résultats que les autres réactifs couramment utilisés. Par la suite, cette technique a été utilisée en routine dans les **Articles 3, 4, 5 et 6**.

S’il est établi de longue date que le tissu adipeux et l’adipocyte expriment un grand nombre d’enzymes impliquées dans le métabolisme de la vitamine D comme celles permettant l’hydroxylation en position 25 [127, 128], l’hydroxylation en position 1α [128, 129] ainsi que l’hydroxylation en position 24 [128, 129, 131], la régulation de ce métabolisme dans l’adipocyte n’avait jamais été étudié. Partant du fait que ce métabolisme de la vitamine D est fortement régulé au niveau du rein et du foie dans une moindre mesure [12], nous avons émis l’hypothèse que la vitamine D pourrait réguler transcriptionnellement son propre métabolisme dans le tissu adipeux et l’adipocyte. Dans l’**Article 3**, nous nous sommes donc intéressés à l’effet d’une supplémentation en vitamine D sur cette régulation transcriptionnelle. Pour ceci, des souris ont été supplémentées avec 15000 UI de cholécalciférol/kg de nourriture pendant 4 jours et nous avons étudié l’expression génique des différents acteurs impliqués dans le métabolisme de la vitamine D. Les taux sériques et les quantités de métabolites de la vitamine D retrouvés dans le tissu adipeux ont validé notre modèle expérimental. Nous avons pu mettre en évidence que le niveau d’expression des ARNm de Cyp24a1 et Cyp27a1 était régulé négativement au niveau du tissu adipeux épidydimal suggérant une diminution de la 25-hydroxylation et de l'inactivation des métabolites actifs. De plus, le niveau d’expression des ARNm codant pour la cubiline (Cubn) était lui aussi diminué. Des résultats similaires ont été obtenus lors du traitement d’adipocytes murins 3T3-L1 avec la forme active de la vitamine D durant 24h en ce qui concerne pour la régulation des gènes Cyp27a1 et de Cubn **(Article 3)**. Ainsi, nous avons pu utiliser ce modèle pour l’étude de mécanismes moléculaires impliqués dans la régulation. De façon intéressante, nous avons aussi observé une régulation positive de Vdr et Cyp24a1, qui sont des gènes cibles bien connus de VDR [129], validant ainsi notre modèle. De plus, nous avons pu constater que certains acteurs de la 25-hydroxylation hépatique [43] n'étaient pas exprimés dans les adipocytes. C'est notamment le cas de Cyp2r1 et Cyp3a11, qui n'ont pas été détectés dans nos conditions, comme précédemment décrit dans le cas de Cyp2r1 [127]. Grace à ce travail, nous avons donc montré pour la première fois que la vitamine D régule au niveau transcriptionnel son propre métabolisme dans le tissu adipeux et l’adipocyte.

Fort de ces résultats très innovants concernant la cubiline, nous avons poursuivis nos investigations. En effet, la cubiline est impliquée, avec la mégaline, dans l’endocytose de la

25(OH)D. Ceci a été particulièrement bien décrit au niveau du rein [61], mais n'a, à ce jour, jamais été démontré au niveau du tissu adipeux et de l'adipocyte. Les données obtenues concernant la régulation de l'expression du gène Cubn, au niveau du tissu adipeux et de l'adipocyte chez les souris, ont été reproduites également au niveau protéique ainsi que sur des adipocytes humains traités avec de la $1,25(OH)_2D$ pendant 24h **(Article 3)**. Ces régulations sont VDR-dépendantes comme nous avons pu le montrer grâce à l'utilisation d'un agoniste de VDR (EB1089) et de siRNA dirigés contre VDR au niveau des adipocytes murins. De plus, nous avons confirmé la présence d'un élément de réponse VDR dans les promoteurs murins et humains de la cubiline grâce à une analyse in silico (MatInspector of the Genomatix Suite) **(Article 3)**. Nous avons voulu ensuite confirmer, d'un point de vue fonctionnel, l'implication de la cubiline dans l'absorption de 25(OH)D au niveau des adipocytes par l'utilisation de la 25(OH)D radiomarquée dans des conditions physiologiques (87% de la 25(OH)D radioactive étaient liés à la VDBP, 12% à l'albumine et 1% était sous forme libre). Les données suggèrent que la 25(OH)D complexée avec l'albumine est plus facilement absorbée par les cellules ; cependant, la forme libre peut également y être internalisée **(Article 3)**. Il semblerait que la VDBP ne soit probablement pas le meilleur moyen d'absorption de la 25(OH)D au niveau des adipocytes mais correspondrait plus à un site de stockage dans le plasma, comme indiqué précédemment [42]. L'implication de la cubiline dans l'absorption de la 25(OH)D couplée à l'albumine et à la VDBP dans les adipocytes a été confirmée par la mise en place de deux stratégies : l'utilisation de RAP (inhibiteur du complexe de mégaline/cubiline [53]) et l'utilisation de siRNA dirigés contre Cubn. L'absorption de la 25(OH)D libre se produit indépendamment de la voie de mégaline/cubiline. Enfin, nous avons confirmé que la $1,25(OH)_2D$ qui régulait négativement l'expression de la cubiline était associée à une diminution de l'absorption 25(OH)D **(Article 3)**. L'ensemble de ces données originales décrit donc pour la première fois les mécanismes d'absorption de la 25(OH)D par les adipocytes et met en avant le rôle de la cubiline dans ce processus (**Figure 25**)

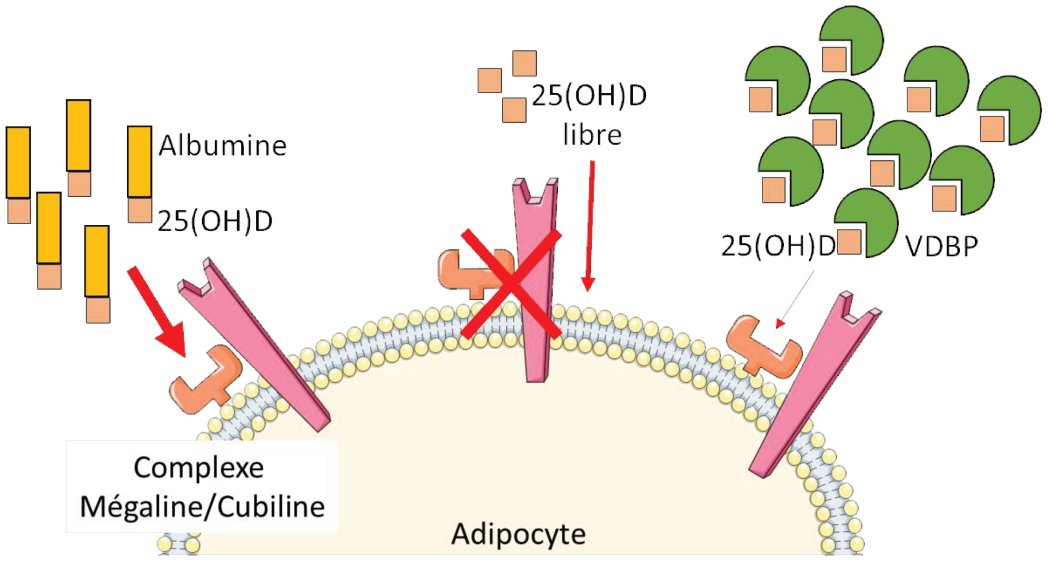

Figure 25 : Implication de la cubiline dans l'absorption de la 25(OH)D au niveau des adipocytes.

Depuis de nombreuses années, d'innombrables études montrent que l'obésité est associée à une diminution du taux plasmatique 25(OH)D totale [209], la forme circulante de la vitamine D et plus récemment de la 25(OH)D libre [208]. L'hypothèse la plus connue afin d'expliquer cette observation est la possible séquestration de la 25(OH)D dans le tissu adipeux, son site principal de stockage. Néanmoins, certains auteurs se sont intéressés à la modification transcriptionnelle du métabolisme adipocytaire de la vitamine D. Une première étude a montré que l'expression de l'enzyme Cyp2j2, impliquée dans la 25-hydroxylation, était diminuée chez les patients obèses comparés aux patients non-obèses dans le tissu adipeux sous-cutané [128]. Plus récemment, Park et al. ont montré qu'un régime riche en graisse pendant 18 semaines chez la souris modifie l'expression des ARNm codants pour les enzymes impliquées dans le métabolisme de la vitamine D au niveau du foie (augmentation de Cyp2r1, Cyp27a1 et Cyp2j3), du rein (augmentation de Cyp27b1 et diminution de Cyp24a1) et du tissu adipeux viscéral (augmentation de Cyp27a1, Cyp2j3 et Vdr) [220]. Ces deux études ne permettent cependant pas d'établir si ces modifications de profils d'expression ont un rôle causal dans la mise en place de la diminution plasmatique en 25(OH)D retrouvée au cours de l'obésité ou s'ils sont juste la résultante de phénomènes adaptatifs consécutifs à l'obésité. Afin d'identifier des éléments causaux et potentiellement initiateurs, nous avons étudié l'effet d'un régime riche en graisse, mimant une alimentation déséquilibrée et

obésogène, pendant 4 jours afin d'étudier des évènements précoces à la mise en place de l'obésité (**Article 4**) sur la régulation transcriptionnelle du métabolisme de la vitamine D, notamment au niveau du tissu adipeux, afin d'expliquer les taux circulants en vitamine D associés à l'obésité.

Nous avons eu recours à un modèle in vivo, de souris nourries soit avec de l'aliment standard (type A04 ; groupe contrôle) soit par une alimentation riche en graisse avec 60% d'énergie sous forme de lipides. Suite à 4 jours de régime, la masse corporelle des souris ne diffère pas entre les deux groupes, pourtant celles nourries avec un régime riche en graisse ont une augmentation de l'index d'adiposité. De plus, la consommation de cholécalciférol ne varie pas entre les deux groupes. De façon originale, nous avons mis en évidence une diminution des taux plasmatiques en cholécalciférol chez les souris nourries avec une alimentation riche en graisse (**Figure 26**). Cette diminution pourrait être due au piégeage de cette molécule dans le tissu adipeux qui est en pleine expansion. Les résultats liés à la 25(OH)D libre (diminution) et à la $1,25(OH)_2D$ (augmentation) (**Article 4**) (**Figure 26**) chez les souris nourries avec un régime riche en graisse sont conformes aux données retrouvées dans une étude récente illustrant des résultats similaires chez des sujets obèses [208]. Cependant, cette étude clinique a également rapporté une diminution de la 25(OH)D totale plasmatique ce qui n'était pas le cas dans notre étude. La diminution de la 25(OH)D libre pourrait être amplifiée par la diminution de l'expression de l'enzyme Cyp3a11 impliquée dans le 25-hydroxylation au niveau hépatique. De plus, ces résultats ne sont pas en accord avec l'hypothèse de dilution volumétrique telle que rapportée par Drincic et al. [255] qui suggère que la teneur plasmatique en 25(OH)D serait mieux corrélée au volume total des individus qu'à la masse grasse. En effet, dans notre étude, les souris nourries avec une alimentation riche en graisse ont une augmentation de la quantité de masse grasse sans modification du poids corporel (**Article 4**). L'augmentation de la $1,25(OH)_2D$ pourrait quant à elle être expliquée par la diminution de l'expression des ARNm de l'enzyme Cyp24a1, impliquée dans la dégradation des métabolites de la vitamine D, au niveau rénal. Cette $1,25(OH)_2D$ pourrait à son tour réguler négativement l'expression génique de la Cyp27b1, impliquée dans la 1α-hydroxylation au niveau du rein (**Article 4**).

Au niveau du tissu adipeux, nous avons pu mettre en évidence une augmentation de l'expression de 2 enzymes impliquées dans la 25-hydroxylation, Cyp27a1 et Cyp2r1 ainsi qu'une diminution de l'expression de Cyp27b1 qui code pour l'enzyme impliquée dans l'hydroxylation 1,25 à 4 jours

de régime riche en graisse (**Article 4**). Une diminution de l'expression de Cyp27b1 dans le tissu adipeux sous-cutané des sujets obèses par rapport aux sujets normaux pondéraux a déjà été rapportée par Wamberg et al. [128] et chez la souris [220]. Ces résultats suggèrent un possible stockage de la vitamine D sous forme de 25(OH)D.

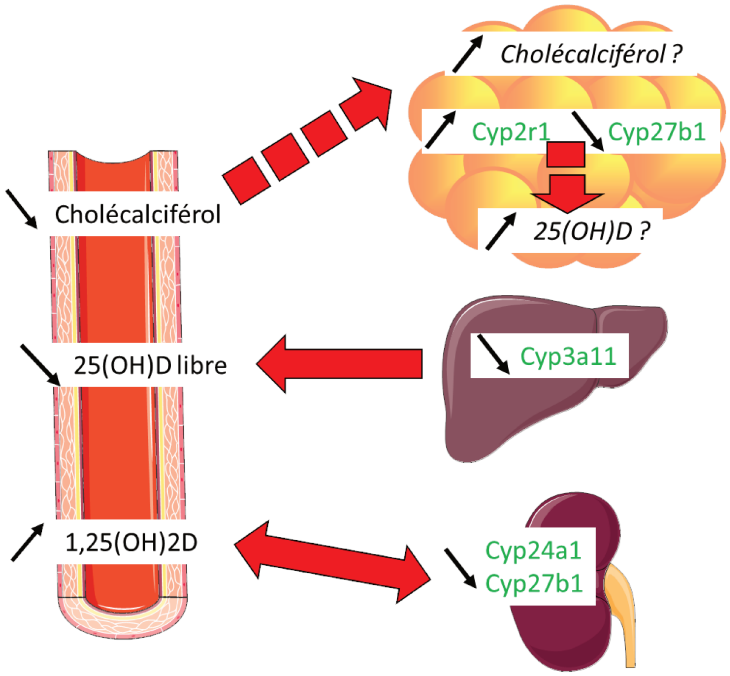

Figure 26 : Modulation du métabolisme de la vitamine D au niveau plasmatique et adipeux lors d'une alimentation riche en graisse pendant 4 jours chez des souris (Article 4).

Comme nous venons de le voir (**Article 4**), un régime riche en graisse apporté à des souris pendant 4 jours, mimant des évènements précoces de la survenue de l'obésité, modifie les concentrations plasmatiques de certains métabolites de la vitamine D ce qui peut s'expliquer en partie par la régulation des gènes impliqués dans ce métabolisme au niveau du foie, du rein et du tissu adipeux. Au vu de ces résultats, nous avons voulu étudier l'effet d'un régime riche en graisse pendant 7 et 11 semaines, chez des souris, sur la modification des paramètres plasmatiques liés au métabolisme de la vitamine D, sur la teneur en cholécalciférol et métabolites dans le tissu adipeux ainsi que l'expression génique des acteurs impliqués dans le métabolisme de la vitamine D, principalement au niveau du tissu adipeux (**Article 5**).

Nous avons eu recours à un modèle murin expérimental constitué de deux groupes de souris, l'un nourri avec une alimentation riche en graisse contenant 10% d'énergie sous forme de lipides (groupe contrôle) et l'autre contenant 60% d'énergie sous forme de lipides (groupe HF,), pendant 7 et 11 semaines. Les souris du groupe HF présentaient une augmentation de leur masse corporelle ainsi que de leur index d'adiposité. Ces changements morphologiques n'étaient pas accompagnés de variation de l'apport énergétique et de la consommation de cholécalciférol entre les groupes. Ce dernier paramètre était particulièrement important à contrôler dans notre cas, car une modification de l'apport de cholécalciférol induite par un mauvais équilibrage du régime aurait pu conduire à elle seule une déficience en vitamine D.

Suite à 7 semaines de régime nous avons mis en évidence que les souris obèses présentaient une diminution de la concentration plasmatique en cholécalciférol bien que l'apport en cholécalciférol alimentaire soit équivalent tout au long de l'expérience (**Article 5**). Cette diminution pourrait être due au piégeage de cette molécule dans le tissu adipeux qui est en pleine expansion. De plus, nous avons mis en évidence pour la première fois une augmentation plasmatique en 25(OH)D totale exclusivement à 7 semaines d'un régime riche en graisses (**Article 5**) qui pourrait participer à la diminution de la teneur en cholécalciférol, ce dernier étant convertit en 25(OH)D. L'augmentation de l'expression génique des enzymes hépatiques impliquées dans la 25-hydroxylation (Cyp27a1, Cyp2r1 et Cyp2j6) à 7 semaines participe très probablement à l'augmentation de la 25(OH)D totale. Une telle augmentation n'avait à notre connaissance jamais été observée. A l'inverse, il avait été rapporté une régulation négative de l'expression de ces enzymes [220]. Ces différences pourraient provenir des protocoles expérimentaux mis en place, notamment de la durée de régime. De façon intéressante, malgré l'augmentation de l'index d'adiposité mis en évidence chez nos souris, aucune diminution de la teneur plasmatique en 25(OH)D totale n'a été constatée. Cette observation n'est clairement pas en accord avec l'hypothèse de Wortsman et a1. [250] qui ont suggéré que la diminution plasmatique de 25(OH)D observée pendant l'obésité est le résultat direct de l'expansion du tissu adipeux.

De façon originale, nous avons mis en évidence qu'à 11 semaines de régime, la concentration plasmatique en 25(OH)D libre était diminuée lors d'un régime riche en graisse sans modification de celle-ci à 7 semaines (**Article 5**), ce qui suggère qu'un temps relativement long de régime obésogène est nécessaire pour observer une modification de ce paramètre. Cette 25(OH)D libre

était par ailleurs bien corrélée au poids corporel plutôt qu'à la masse grasse. Ces observations sont donc globalement conformes à l'hypothèse de dilution volumétrique de Drincic et al. [255]. Cependant, cette hypothèse a été émise en ce qui concerne la 25(OH)D totale et non pas la 25(OH)D libre. L'extrapolabilité à la 25(OH)D libre reste donc discutable et nécessitera d'autres recherches. De plus, nous avons pu mettre en évidence de fortes corrélations entre la concentration de 25(OH)D libre et les différents paramètres morphologiques testés (poids corporel total, niveau de PTH, index d'adiposité), alors que ces relations étaient inexistantes à 7 semaines (**Article 5**). De cette façon, nous pourrions supposer que la 25(OH)D libre dans le plasma pourrait être un meilleur marqueur du statut en vitamine D que la 25(OH)D totale durant l'obésité conformément avec la « free hormone hypothetisis » [258]. Curieusement, nous avons également remarqué que le taux plasmatique en 25(OH)D libre était inversement corrélé à la quantité de 25(OH)D dans le tissu adipeux à 11 semaines, ce qui suggère que plus le tissu adipeux s'expand au cours de l'obésité, plus les quantités de 25(OH)D stockées dans le tissu adipeux sont importantes, réduisant ainsi une partie de la 25(OH)D libre dans le plasma. Cependant, l'évolution cinétique du taux plasmatique en 25(OH)D libre pendant l'obésité entre 7 semaines et 11 semaines dans notre étude supporte clairement un rôle causal de l'obésité dans cette diminution [216, 259]. Néanmoins, cette diminution plasmatique en 25(OH)D libre pourrait à son tour contribuer à amplifier l'obésité, puisque nous avons déjà montré que la supplémentation en cholécalciférol limite l'apparition de l'obésité induite par un régime riche en graisse en diminuant l'oxydation lipidique [167, 260].

Au niveau du tissu adipeux, nous avons pu mettre en évidence une augmentation de l'expression de Cyp2r1 à 7 et 11 semaines, ainsi qu'une diminution de l'expression de Cyp27b1 à 11 semaines chez des souris nourries avec un régime riche en graisse (**Article 5**). Les données sur l'expression de Cyp2r1 codant pour une enzyme majeure de 25-hydroxylation [44] sont novatrices et suggèrent une capacité accrue du tissu adipeux à stocker la vitamine D sous forme de 25(OH)D. Pour valider cette hypothèse, nous avons quantifié le cholécalciférol et la 25(OH)D dans le tissu adipeux. A 11 semaines, nous avons observé que le régime riche en graisse augmentait la quantité de cholécalciférol (**Article 5**) en accord avec les résultats de Carrelli et al. [85], démontrant que les sujets obèses possédaient des stocks de cholécalciférol adipeux plus importants que les sujets normo-pondéraux. En ce qui concerne la 25(OH)D, nous observons une augmentation de sa quantité dans le tissu adipeux à 11 semaines, compatible avec l'induction de Cyp2r1. Nous pouvons donc en conclure qu'un régime riche en graisse induisant l'obésité entraîne une modification

transcriptionnelle du métabolisme de la vitamine D allant dans le sens d'un stockage des métabolites de la vitamine D au niveau du tissu adipeux, pouvant ainsi expliquer la diminution des concentrations plasmatiques en cholécalciférol et 25(OH)D libre observée chez les souris obèses.

Au vu de ces résultats, il nous a semblé pertinent d'étudier la régulation du métabolisme de la vitamine D lors d'une perte de poids médiée par l'alimentation chez la souris, afin d'étudier la réversibilité des phénotypes mis en évidence (**Article 6**). En effet, aucune donnée n'est disponible à ce jour à ce sujet.

Les régimes utilisés sont identiques à ceux de l'**Article 5** et la perte de poids est engendrée par un changement de régime à partir de 7 semaines et ceci pendant 4 semaines. Lors de cette expérience, nous observons une perte de masse corporelle progressive au cours du temps pour le groupe soumis au changement de régime (groupe HF/LF) ce qui entraîne aussi une diminution de la masse du foie et du tissu adipeux (index d'adiposité) jusqu'à un retour à l'état basal (valeurs identiques à celles du groupe contrôle). Comme nous l'avons vu précédemment dans l'**Article 6**, un régime riche en graisse pendant 11 semaines chez les souris (groupe HF) entraîne des modifications des concentrations plasmatiques (augmentation de la PTH et diminution du cholécalciférol) et adipocytaires (augmentation des quantités de cholécalciférol et de 25(OH)D), ainsi que l'expression de certains acteurs du métabolisme de la vitamine D au niveau du foie (augmentation de l'expression génique de Cyp2r1, Cyp27a1 et Cyp2j6), du rein (augmentation de l'expression génique de Cyp27b1) et du tissu adipeux (augmentation de l'expression génique de Cyp2r1 et diminution de celle de Cyp27b1) comparé aux souris contrôles (groupe LF). Tous ces paramètres reviennent à l'état basal lors d'une perte de poids de 4 semaines à l'exception de la concentration plasmatique en cholécalciférol (**Article 6**).

Alors que la majorité des gènes testés ont été exprimés de manière identique dans les groupes HF/LF et LF, deux gènes impliqués dans l'absorption des tissus de la vitamine D ont vus leur expression diminuer. En effet, le complexe mégaline/cubiline est un composant majeur de l'endocytose liée à la VDBP dans le rein [61], mais aussi dans les adipocytes comme nous avons pu le voir dans l'**Article 3**. Nous avons observé une diminution des niveaux d'expression des ARNm codants pour la cubiline dans le tissu adipeux épididymal ainsi qu'une régulation négative du gène codant pour la mégaline au niveau rénal chez les souris HF/LF comparées aux animaux LF (**Article 6**), ce qui suggère une altération de la réabsorption rénale ainsi que l'absorption dans

tissu adipeux de la vitamine D liée à ses protéines de transport. Les mécanismes responsables d'une telle perturbation de la capacité d'endocytose de la vitamine D ne sont pas clairs et méritent d'être approfondis, mais pourraient expliquer les faibles taux de cholécalciférol plasmatiques et 25(OH)D ainsi que les taux inférieurs de cholécalciférol adipocytaire chez les souris HF/LF.

L'ensemble de ces travaux ont permis de montrer la réversibilité des modifications induites par un régime obésogène sur le métabolisme de la vitamine D au niveau plasmatique et transcriptionnel au niveau du foie, du rein et du tissu adipeux.

En conclusion, les résultats obtenus au cours de ce travail de thèse ont permis de générer de nouvelles connaissances relatives à l'impact de la vitamine D sur la biologie du tissu adipeux et de l'adipocyte. En effet, nous avons mis en évidence que la vitamine D modulait l'expression de plusieurs miRs au niveau du tissu et de l'adipocyte via une désactivation de la voie de signalisation NF-κB. Nous avons également montré que le métabolisme de la vitamine D était régulé transcriptionnellement au niveau adipocytaire et ces régulations nous ont permis d'identifier la cubiline comme un acteur majeur de l'internalisation du cholécalciférol et le la 25(OH)D dans l'adipocyte. Enfin, nous avons montré que l'obésité modulait le métabolisme de la vitamine D notamment au niveau de l'expression génique suggérant pour la première fois un rôle actif du tissu adipeux dans les variations de teneurs plasmatiques en 25(OH)D libres observées au cours de l'obésité. L'ensemble de ces données confirment donc le lien très fort qu'il existe entre vitamine D, obésité et tissu adipeux. .

PERSPECTIVES

Si de nombreux résultats innovants ont été obtenus quant à une meilleure compréhension de la relation entre la vitamine D, l'obésité et le tissu adipeux, certains devront faire l'objet d'approfondissements.

Lors de notre travail de thèse, nous avons mis en évidence la régulation transcriptionnelle des enzymes Cyp2r1 et Cyp27a1 au niveau du tissu adipeux lors d'un régime riche en graisse chez les souris. Il serait intéressant d'étudier plus précisément le rôle de ces enzymes dans le développement de l'obésité en utilisant par exemple des souris invalidées de façon tissu spécifique pour Cyp2r1 et/ou Cyp27a1 nourries avec un régime riche en graisse pendant plusieurs semaines. L'impact de cette invalidation sera notamment évalué sur les capacités de stockage de vitamine D et de 25(OH)D dans le tissu adipeux.

Afin de parfaire l'étude de la relation entre l'obésité et la vitamine D, une étude in vivo sur les souris ob/ob va être entreprise. Elle aura pour but d'évaluer le métabolisme de la vitamine D au cours de la prise de poids en s'affranchissant de l'impact du facteur nutritionnel (le régime riche en graisse) et en regardant donc l'expression des différents acteurs du métabolisme de la vitamine D au niveau des différents tissus et plus spécifiquement du tissu adipeux, l'activité des enzymes clés et la quantité des métabolites de la vitamine D stockée dans le tissu adipeux.

Afin de valider chez l'Homme un certain nombre de résultats obtenus lors de cette thèse, une étude clinique est en cours afin d'obtenir des biopsies de tissus adipeux de patients normo-pondéraux et obèses pour comparer les quantités de vitamine D et de ses métabolites dans ces prélèvements ainsi que l'expression de gènes en lien avec le métabolisme de la vitamine D.

Enfin, nous avons montré que les souris nourries avec une alimentation riche en graisse dont les apports en cholécalciférol sont similaires aux souris soumises à une alimentation standard, n'expérimentaient pas de diminution de leur teneur plasmatique en 25(OH)D totale. Ceci suggère que les apports en vitamine D dans la population obèse sont peut-être insuffisants. Dans le cadre d'une collaboration avec des épidémiologistes, il sera pertinent d'aller estimer l'apport en vitamine D dans une population obèse et de le comparer à celui d'une population non obèse (à l'aide des

données de la cohorte INCA2) afin de mettre en évidence une différence des apports exogènes en vitamine D. La diminution de la 25(OH)D totale plasmatique associée à l'obésité pourrait être en effet partiellement expliquée par une simple insuffisance en vitamine D dans l'alimentation des personnes obèses.

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ANNEXES

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Research Article

GPR40 mediates potential positive effects of a saturated fatty acid enriched diet on bone

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Scope: The stimulation of the free fatty acid receptor G-protein coupled receptor (GPR) 40 by GW9508 prevents bone loss by inhibiting osteoclast activity, both in vitro and in vivo. Here, we questioned whether the stimulation of the GPR40 receptor by dietary fatty acids may lead to the same beneficial effect on bone.

Methods and results: We investigated (i) the impact of a fatty acid enriched diet (high-fat diet [HFD]) on bone health in C57/BL6 female mice depending on (ii) the estrogen status (ovariectomy) and (iii) the genotype (GPR40$^{+/+}$ or GPR40$^{-/-}$). Bone mineral density (BMD), body composition, weight, inflammation and bone remodeling parameters were monitored. HFD decreased BMD in HFD-SH-GPR40$^{+/+}$ mice but OVX failed to further impact BMD in HFD-OVX-GPR40$^{+/+}$mice, while additional bone loss was observed in HFD-OVX-GPR40$^{-/-}$ animals. These data suggest that when stimulated by fatty acid enriched diets GPR40 contributes to counteract ovariectomy-induced bone alteration. The sparing effect is supported by the modulation of both the osteoprotegerin/receptor activator of nuclear factor kappa-B ligand (OPG/RANKL) ratio in blood stream and the expression level of inflammatory markers in adipose tissues. Bone preservation by GPR40 stimulation is dependent on the presence of long-chain saturated fatty acids.

Conclusion: GPR40 contributes to counter ovariectomy-induced bone loss in a context of saturated fatty acid enrichment.

Keywords:
Free fatty acid receptor / GPR40 / High-fat diet / Nutrition / Osteoporosis

Additional supporting information may be found in the online version of this article at the publisher's web-site

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Abbreviations: **ANOVA**, analysis of variance; **BMD**, bone mineral density; **GPR**, G-protein coupled receptor; **HFD**, high-fat diet; **MCP-1**, monocyte chemoattractant protein 1; **OPG**, osteoprotegerin; **RANKL**, receptor activator of nuclear factor kappa-B ligand

Colour Online: See the article online to view Fig. 1 in colour.

1 Introduction

A growing body of data has revealed complex relationships between dietary lipids and the skeleton [1]. Depending on the type of fatty acids, the biological activity on bone may differ. Literature has mainly evidenced that high-fat diets (HFDs) negatively impact bone health. However, most of the studies have focused their investigations on hyper caloric diets enriched with saturated fatty acids in healthy groups without asking whether a beneficial effect could exist under certain circumstances. Regarding the unsaturated fatty acid family, the ω-6 family has been shown to enhance osteoporosis establishment while ω-3 fatty acids seem to preserve bone tissues [2–5]. These seemingly conflicting results have raised the idea that in some ways the modulation of the lipid intakes may represent a preventive opportunity for the management of bone complications. However, mechanisms of action are poorly understood and further investigations are required to develop innovative strategies regarding fat involvement in osteoporosis management.

About a decade ago, several groups demonstrated that the G-protein coupled receptor (GPR) 40 was able to interact with medium- to long-chain fatty acids and to transduce a specific signal across the cell membrane [6–11]. The role of GPR40 (also known as Free Fatty Acid Receptor-1) was mainly examined in beta-cells for its involvement in insulin secretion [12–14]. Later, GPR40 roles were discovered in taste buds [15], enteroendocrine cells [16], and in the brain [17, 18].

Recently, we demonstrated in vivo and in vitro the implication of this receptor in bone homeostasis [19–22]. GPR40$^{-/-}$ mice were characterized by an osteopenic/osteoporotic phenotype when compared to wild-type (WT) littermates. In addition, phenotype onset was prevented in WT ovariectomized mice when a GPR40 synthetic agonist, GW9508, was given orally. We further demonstrated that prevention by GW9508 was driven by an inhibition of osteoclastogenesis in a GPR40 dependent way. This synthetic agonist was used to decipher the role of GPR40 and to avoid confounding parameters. However, since free fatty acids are natural ligands for GPR40, we further questioned the relevance of this receptor in an integrative approach investigating whether this fatty acid receptor may represent an innovative opportunity in the design of nutritional and therapeutic strategies to prevent bone loss in an ageing population.

2 Materials and methods

2.1 Ethics

All animal procedures were approved by the institution's animal welfare committee (Comité d'Ethique en Matière d'Expérimentation Animale Auvergne: CEMEAA) and were conducted in accordance with the European guidelines for the care and use of laboratory animals (CE 80-12). Animals were housed in the animal facility of the INRA Research for Human Nutrition (Agreement N°: C6334514). Throughout the study, animals were housed in a controlled environment characterized by a 12:12 h light-dark cycle, 20–22°C, 50–60% relative humidity. Mice were kept one per plastic cage with free access to water. At the end of the protocol, blood was withdrawn from anesthetized animals. Then, animals were sacrificed by cervical dislocation and tissues were harvested, frozen, and stored prior to investigation.

2.2 Genotype

GPR40$^{-/-}$ mice were obtained from AMGEN Inc. As described by Latour et al. [19], GPR40$^{-/-}$ mice on a mixed C57BL/6/129 background were generated by homologous recombination in embryonic stem cells at Lexicon Genetics (The Woodlands, TX, USA). Exon 2 of the GPR40 gene was replaced with a LacZ gene. The mice were backcrossed onto the C57BL/6 strain over nine generations. Pups were screened by PCR performed on genomic DNA. WT littermates were used as controls.

2.3 Experimental design

In order to accustom the mice to the texture, animals were provided with free access to a standard growth powdered diet during an acclimatization period of 3 weeks from weaning (weeks 4–7). On week 7, mice were randomly divided into 16 groups ($n = 12$, individual cage), depending on the diet (six different diets), the type of surgery (SH or OVX) and the genotype (GPR40$^{+/+}$ or GPR40$^{-/-}$; Tables 1 and 2). From this point, mice were provided with their specific diet. On week 9, mice were sham-operated or ovariectomized to induce bone loss. Diets were delivered ad libitum for a total of 7 weeks including 5 weeks postsurgery (Fig. 1A and B). Briefly, every 48 h, each mouse received exactly 10 g of the diet in a small bowl (Fig. 1) to cover their needs (about 3–4 g per day; Fig. 4B). Food was changed every 48 h to minimize fatty acid oxidation. Once a week, the leftover food was weighted to evaluate the consumption of each mouse.

2.4 Ovariectomized mice

Mice were either sham-operated to mimic the side effects of the surgery or ovariectomized to induce an estrogen deprivation and a subsequent bone loss.

2.5 Diets

Diets were purchased from our experimental facility dedicated to the conception of animal diets (INRA UE0300, UPAE Unité de Préparation des Aliments Expérimentaux, Jouy-en-Josas, France). Compositions are summarized in Table 2. Diets were provided to the mice as powders and not as croquettes to avoid fatty acid heating and alteration during the croquettes' production process. The fatty acid enrichment in

Table 1. Distribution of the groups

Diets	Control				HFD				HFD saturated		HFD ω-6		HFD ω-9		HFD ω-3	
Surgery	SH		OVX		SH		OVX		OVX		OVX		OVX		OVX	
Genotype	WT	KO	WT	KO	WT	KO	WT	KO	WT	KO	WT	KO	WT	KO	WT	KO

HFDs was obtained by a modulation of the corn starch content and in these cases, 42.18% of the calories were related to the fat content and 46.54% from the carbohydrate content. The total amount of calories was higher in the fatty acid enriched groups than in the control ones (464 kcal/100 g of diet versus 373 kcal/100 g of diet, respectively). The groups with fatty acid enrichment were isocaloric. This parameter was taken into account to allow group comparisons and to avoid any bias in the conclusions. The fatty acid profile of the HFD was designed to meet French dietary guidelines in terms of fatty acid ratios and set a reference point for the fatty acid content (Table 3). To test whether one fatty acid class may better activate GPR40 than another, selective enrichments of the diet were designed to produce saturated, omega-3, -6, and -9 HFDs (Table 2).

2.6 Bone mineral density analysis

After removing soft tissues, left femurs were placed in PBS with 10% formaldehyde at 4°C for 1 week. Bone mineral density (BMD) was measured using an eXplore CT 120 scanner (GE Healthcare, Canada). Acquisitions were performed with X-ray tube settings at 100 kV and 50 mA. We limited our investigation to the trabecular region of the distal metaphysis of the left femurs. ROI was set at 2000 μm height. Measurements started 300 μm in the proximal direction from the growth plate.

2.7 Body mass and composition

Mice were weighed every week throughout the experimental period and subjected to body composition analysis at the beginning and at the end of the protocol using QMR EchoMRI- 900 system. Whole body fat and lean (excluding bone mass) mass were measured in living animals with no need of sedation.

2.8 Tissue weight analysis

Mice total body weight was measured weekly throughout the study. Spleen and uterus were collected from mice and weighed immediately after sacrifice.

Table 2. Composition of HFDs

Diets	Control	HFD	Saturated HFD	Omega-6 HFD	Omega-3 HFD	Omega-9 HFD
Ingredients	%	%	%	%	%	%
Casein	14.00	14.00	14.00	14.00	14.00	14.00
Sucrose	10.00	10.00	10.00	10.00	10.00	10.00
Dextrin (maltodextrin)	15.50	15.50	15.50	15.50	15.50	15.50
Cellulose	5.00	5.00	5.00	5.00	5.00	5.00
Corn oil	7.00	—	4.00	4.00	4.00	4.00
Rapeseed oil	—	6.72	—	—	—	—
Sunflower oil	—	—	—	20.00	—	—
Fish oil concentrated 22/60 TG (EPA/DHA)	—	0.48	—	—	—	—
Fish oil Omegavie DHA 70 TG QS ICE	—	—	—	—	20.00	—
Olive oil	—	—	—	—	—	20.00
Lard	—	16.80	20.00	—	—	—
l-cystine	0.18	0.18	0.18	0.18	0.18	0.18
Choline bitartrate	0.25	0.25	0.25	0.25	0.25	0.25
Mineral mix (AIN-93M) Ca normal (0.5% per kg of diet)	3.50	3.50	3.50	3.50	3.50	3.50
Vitamins (AIN-93M) normal vitD (1000 UI diet)	1.00	1.00	1.00	1.00	1.00	1.00
***t*-Butyl-hydroxyquinon**	0.008	0.008	0.008	0.008	0.008	0.008
Corn starch (by difference)	43.56	26.56	26.56	26.56	26.56	26.56
Verification	100	100	100	100	100	100

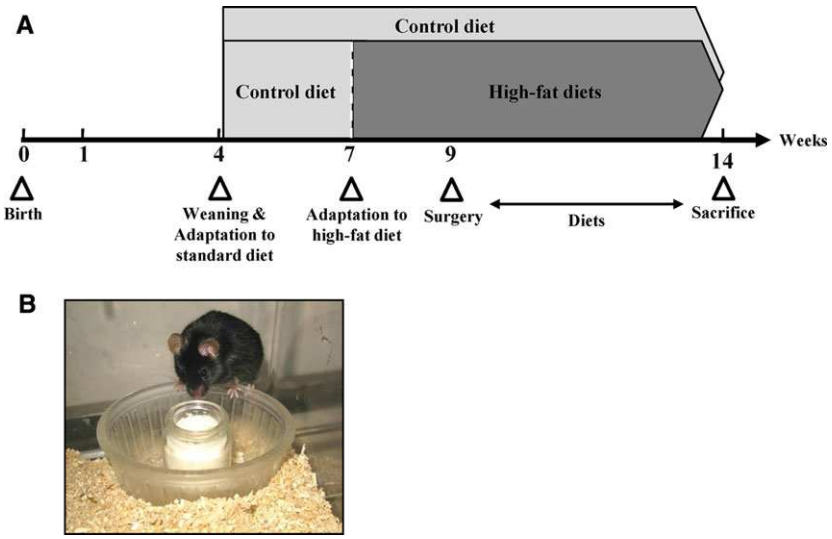

Figure 1. Scheme of experimental protocol design (A) and presentation of the food distribution mode (B). Acclimatization period (weeks 4–7); On week 7, mice were randomized into 16 groups ($n = 12$, individual cage) and provided with their specific diet (six different diets; two different genotypes; see Tables 1 and 2); On week 9, mice were ovariectmized or sham-operated to induce bone loss. Diets were delivered ad libitum for a total of 7 weeks including 5 weeks postsurgery.

Table 3. Validation of fatty acid composition of diets

Fatty acid	Control (%)	HFD (%)
C12:0	—	0.18
C14:0	0.24	1.95
C16:0	15.29	25.71
C16:1 *n*-9	—	0.32
C16:1 *n*-7	—	2.41
C17:0	—	0.23
C17:1	—	0.2
C18:0	1.54	9.12
C18:1 *n*-9	27.07	36.51
C18:1 *n*-7	—	5.98
C18:2 *n*-6 cis	55.04	12.91
C20:1 *n*-9	—	0.34
C18:3 *n*-3	0.82	3.07
C20:5 *n*-3	—	0.6
C22:6 *n*-3	—	0.48
Verification	100%	99.53%

2.9 Biochemical parameters

Blood samples from 14-week-old mice were collected in tubes for serum chemistry, and centrifuged. Serum was isolated, aliquoted, and stored at −80°C. Osteoprotegerin (OPG), receptor activator of nuclear factor kappa-B ligand (RANKL), and monocyte chemoattractant protein 1 (MCP-1) were measured by Quantikine ELISA for mouse (R&D Systems Europe).

2.10 Statistics

The experimental procedure was conducted with a total of 12 mice per group. The 16 groups were processed at the same time but Ctrl and HFD groups (for a total of eight groups; see Table 1) were investigated first and were the only groups fully investigated for all parameters. Statistics were performed to either investigate the role of the genotype ($GPR40^{+/+}$ versus $GPR40^{-/-}$), the role of the ovariectomy (Ctrl-SH versus Ctrl-OVX), the role of the diet (Ctrl-SH versus Ctrl-HFD), or the role of the diet when mice were ovariectomized (HFD-SH versus HFD-OVX). Then, the obtained data were analyzed via a multivariate analysis of variance (ANOVA) followed by a Tukey's test. Data are presented as mean ± SD. (ExcelStat Pro software—Microsoft Office 2007.) Groups with significant differences ($p < 0.05$) are indicated with different letters or (*).

3 Results

3.1 Validation of the bone loss model

To validate ovariectomy-induced oestrogen deficiency, mice uteri were weighed and checked for atrophy. As expected, uterus mass of the ovariectomized animals was significantly lower than those isolated from sham-operated females (Fig. 2). Consistently, BMD was significantly decreased in Ctrl-**OVX**-$GPR40^{+/+}$ mice compared to Ctrl-**SH**-$GPR40^{+/+}$ (Fig. 3A). In addition, as previously described, BMD in **GPR40**$^{-/-}$ mice was significantly lower than in **GPR40**$^{+/+}$ WT littermates (Fig. 3A), thus validating the consistency of both the OVX and the $GPR40^{-/-}$ mouse models, and allowing further investigation.

3.2 GPR40 limits bone loss induced by ovariectomy upon HFD

As shown in Fig. 3B, HFD-induced bone loss in **HFD-SH**-$GPR40^{+/+}$ mice but OVX failed to further impact BMD in **HFD-OVX**-$GPR40^{+/+}$mice. In contrast, the absence of GPR40 revealed an additional BMD decrease in HFD-OVX-**GPR40**$^{-/-}$ mice (Fig. 3C). These data suggest that, when stimulated by fatty acid enriched diets, GPR40 contributes to counteract ovariectomy-induced bone alteration.

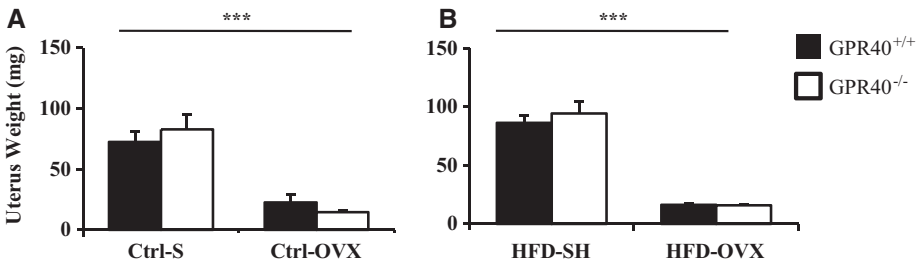

Figure 2. Ovariectomy validation. (A and B) Weight analysis for uterine horns. Significantly different groups are represented by $***p < 0.0001$ as analyzed via a multivariate ANOVA followed by a Tukey's test. (Ctrl, control diet; SH, sham-operated mice; OVX, ovariectomized mice.) Data are presented as mean ± SD.

3.3 The protective effect of GPR40 is not related to mechanical protection

To ensure that GPR40 protective effects were not related to weight gain and subsequent mechanical loading due to an HFD, mice weight was monitored weekly throughout the experiment. Only ovariectomy significantly increased animals' weight, whereas diet or genotype had no, or little effect on weight gain, suggesting that the absence of further bone loss in HFD-OVX-**GPR40$^{+/+}$** is not related to a modulation of the mechanical loading (Fig. 4A).

3.4 The absence of GPR40 leads to higher fat/lean mass ratio

EchoMRI data reveal that ovariectomy exerted a significant influence on body composition pattern with a decreased lean mass and an increased fat mass (Fig. 4C, D, and E). HFD had no particular effect on body composition, except in the absence of GPR40. Indeed, HFD-SH-**GPR40$^{-/-}$** mice showed a greater percentage of fat, while HFD failed to induce such a gain in HFD-SH-**GPR40$^{+/+}$** mice (Fig. 4D and E). This difference reveals that GPR40 may contribute to limit diet-induced fat gain and adipose tissue hypertrophy.

3.5 HFD causes increased systemic inflammation

To elucidate the role of HFD on bone behavior we questioned its impact on inflammatory status. Mice fed with the HFD exhibited both higher spleen weight and MCP-1 level in the serum ($p < 0.0264$ and $p < 0.0001$, respectively) independently of the oestrogen status or genotype (Fig. 5A and B, second panels). These results support a global negative impact of the HFD on inflammatory parameters that may contribute to explain, at least in part, the subsequent loss of BMD observed in HFD-SH mice. Ovariectomy or genotype had no significant impact on systemic inflammatory status.

3.6 Inflammation in adipose tissue is increased by ovariectomy and enhanced in the absence of GPR40

As aforementioned, ovariectomy did not significantly impact the systemic inflammatory status. However, locally, it increased the production of the proinflammatory cytokine MCP-1 by the inguinal adipose tissue (Fig. 5C, left and right panels). In contrast, the diet was ineffective. Regarding genotype influence, the absence of GPR40 mostly increased the production of TNF-α. Interestingly, when mice were ovariectomized and fed an HFD, the absence of GPR40 led to a massive increase of both TNF-α and MCP-1 cytokines, supporting an anti-inflammatory role for GPR40 at the adipose tissue level.

3.7 The absence of GPR40 leads to a greater production of RANKL

The analysis of OPG and RANKL expression, two major cytokines involved in bone tissue homeostasis, reveals that BMD level parallels OPG/RANKL ratio. These data suggest a key role for this molecular cross-talk between osteoblasts and osteoclasts in mediating ovariectomy, diet, and genotype influences on BMD (Fig. 6C, all panels). Interestingly, while no or little impact on OPG was observed in the HFD-OVX-GPR40$^{-/-}$ group, the absence of GPR40 led to a remarkably increased level of RANKL (Fig. 6A and C, right panels). This

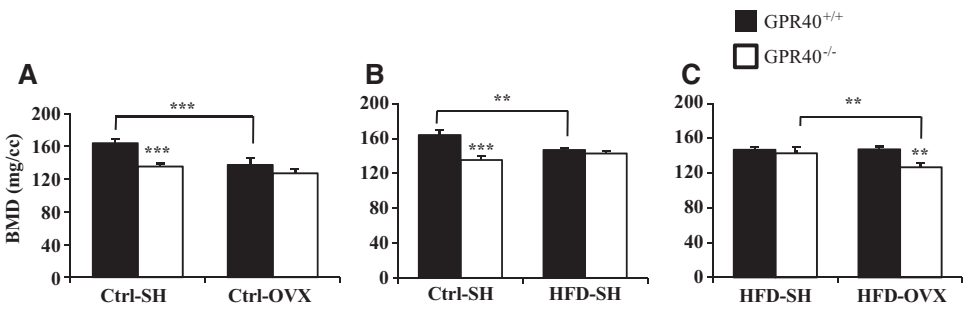

Figure 3. Bone parameters. (A and B) BMD. Significantly different groups are represented by $***p < 0.0001$ or $**p < 0.01$ as analyzed via a multivariate ANOVA followed by a Tukey's test. (Ctrl, control diet; SH, sham-operated mice; OVX, ovariectomized mice.) Data are presented as mean ± SD.

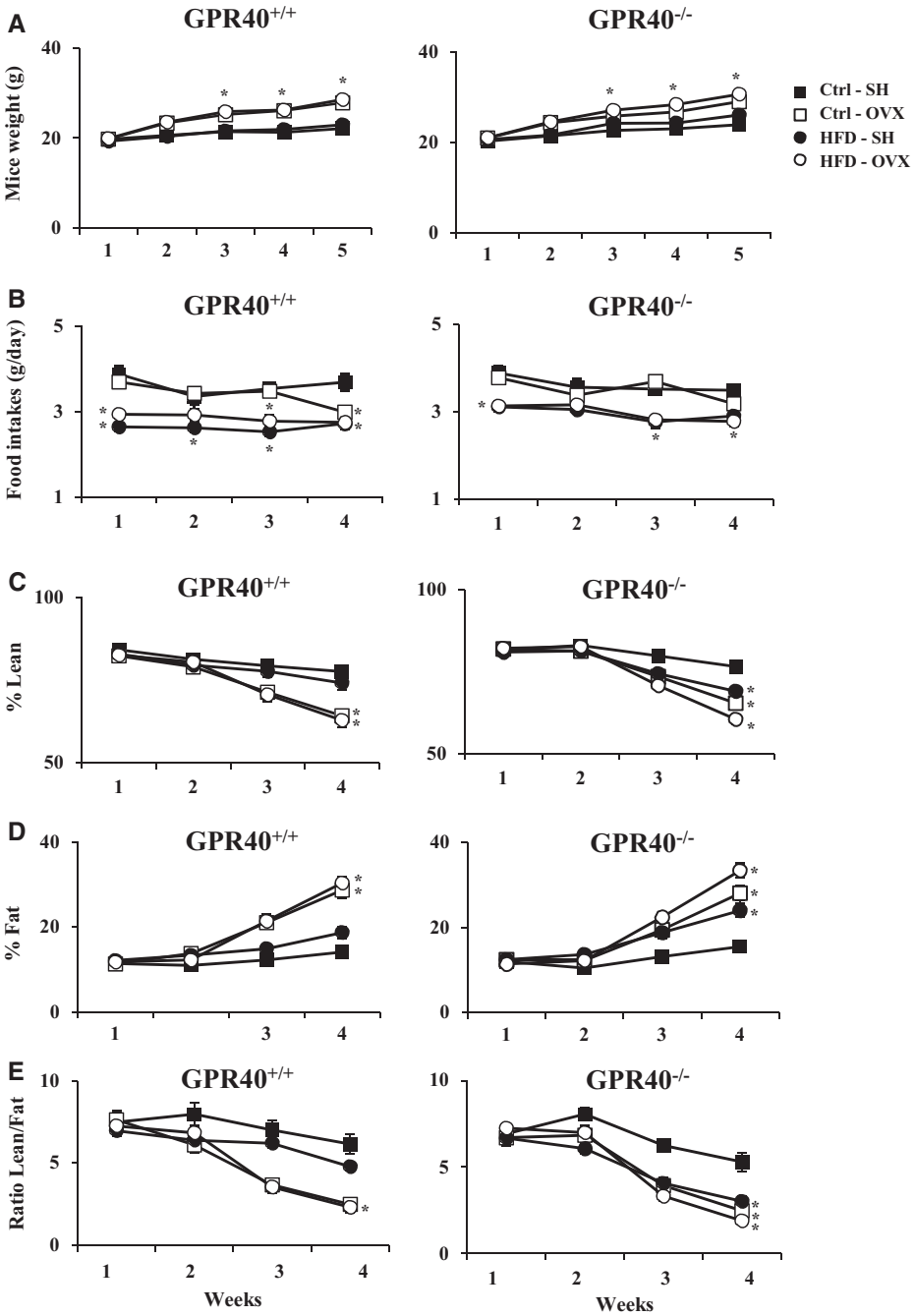

Figure 4. Weight, food intake, and body composition parameters. Total body weight gain over the complete study of *GPR40*$^{+/+}$ and *GPR40*$^{-/-}$ mice (A). Food intake over the complete study of *GPR40*$^{+/+}$ and *GPR40*$^{-/-}$ mice (B). Percentage of *GPR40*$^{+/+}$ and *GPR40*$^{-/-}$ lean gain over the complete study (C), percentage of *GPR40*$^{+/+}$ and *GPR40*$^{-/-}$ fat gain over the complete study (D), and ratio of *GPR40*$^{+/+}$ and *GPR40*$^{-/-}$ lean/fat over the complete study (E). Significantly different groups are represented by $*p < 0.05$ compared to control as analyzed via a multivariate ANOVA followed by a Tukey's test. (Ctrl, control diet; SH, sham-operated mice; OVX, ovariectomized mice.) Data are presented as mean ± SD.

increase correlates with the observed alteration of bone and strongly supports that HFD no longer limits a greater bone resorbing cell differentiation in the absence of GPR40.

3.8 GPR40 bone sparing effect may rely on stimulation by saturated fatty acids

Since we demonstrated the role of GPR40 in mediating HFD positive influence in OVX-induced bone loss, we questioned the specificity on the receptor. The HFD combines different fatty acid groups. In order to decipher which one of these could be responsible for GPR40-related protection, fatty acid mixture composition was revised to allow diet enrichment either with saturated, ω-3, ω-6, or ω-9 fatty acids. Only the diet rich in saturated fatty acid showed similar effects to the so-called HFD diet while diets rich in ω-3, ω-6, or ω-9 failed to promote GPR40-related protection of bone tissue (Fig. 7). These data support a specificity for GPR40 toward saturated fatty acids that has never been reported before.

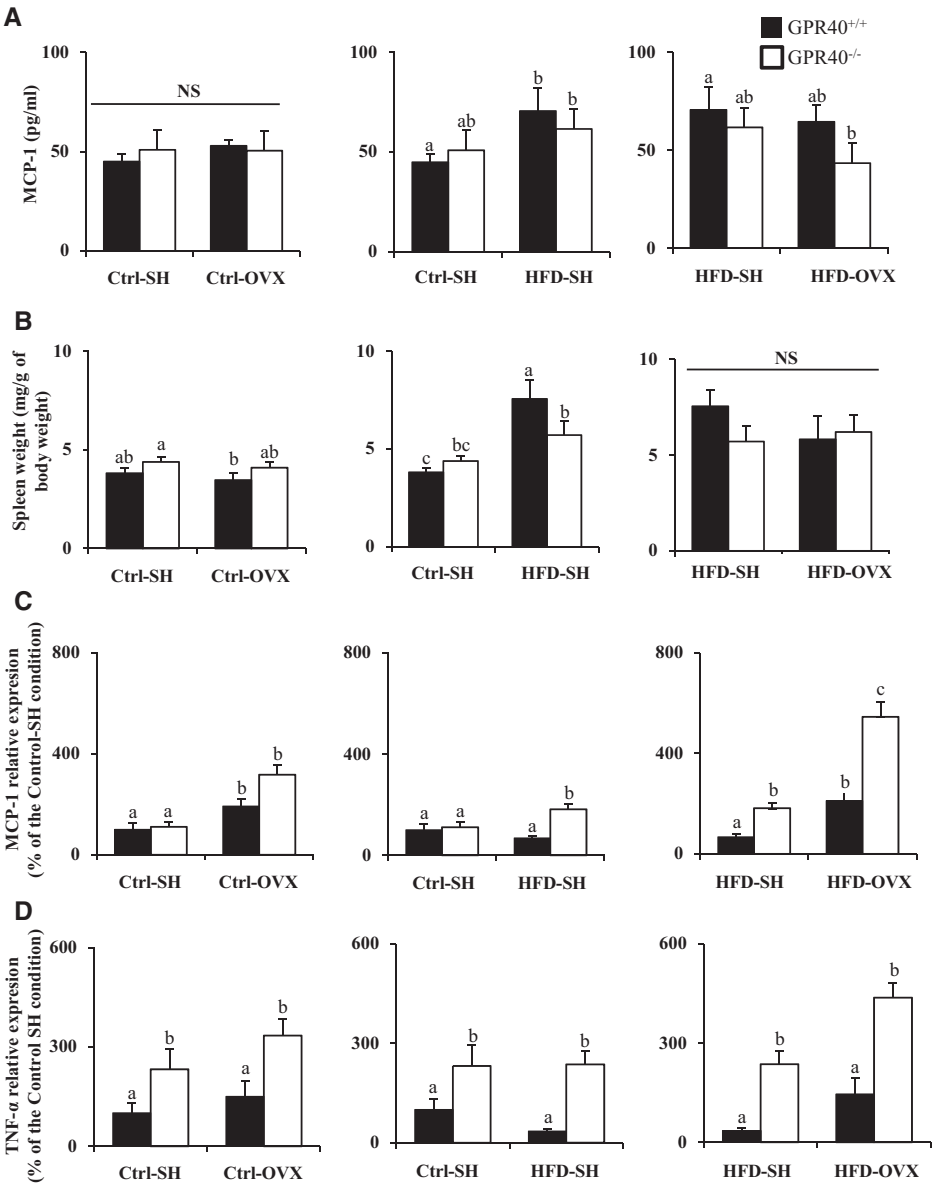

Figure 5. Inflammation parameters. (A) Monocyte chemotactic protein 1 (MCP-1) level analysis in blood samples. (B) Spleen weight measurement. (C) MCP-1 mRNA relative expression in white adipose tissue. (D) TNF-α mRNA relative expression in white adipose tissue. Significantly different groups are represented by different letters as analyzed via a multivariate ANOVA followed by a Tukey's test, $p < 0.05$. (Ctrl, control diet; SH, sham-operated mice; OVX, ovariectomized mice.) Data are presented as mean ± SD.

4 Discussion/conclusion

In this study, we demonstrated for the first time that GPR40 contributes to limit ovariectomy-induced bone loss when mice were fed an HFD. Limitation of bone loss in this model was associated with both a restrained production of proinflammatory cytokines by the white adipose tissue and a controlled level of RANKL. GPR40-related protection of bone tissue was dependent on the presence of saturated fatty acids. These results are consistent with previous published data obtained with the synthetic agonist GW9508.

We also confirmed that mice lacking GPR40 exhibited osteoporotic features [19]. As expected, oestrogen deficiency following ovariectomy was confirmed by uterine horns atrophy and led to bone loss independently of the diet or the genotype [23, 24]. Then, we provided evidence that HFD decreased BMD in HFD-SH-GPR40$^{+/+}$ mice. This deleterious impact of the diet on bone parallels previous studies from Le's and Faria's groups showing that bone loss upon HFD was associated with an unbalanced osteoblast/osteoclast coupling and an increased of inflammatory status [25, 26]. In our hands, HFD increased MCP-1 level and spleen weight. Alteration of the OPG/RANKL ratio by the diet was also observed. Besides, bone loss observed in HFD-SH-GPR40$^{+/+}$ mice occurred in the absence of weight gain supporting a metabolic deleterious effect of the diet uncovered by the absence of mechanical loading [27].

Interestingly, ovariectomy failed to further impact BMD in HFD-OVX-GPR40$^{+/+}$mice, while additional bone loss was observed in HFD-OVX-GPR40$^{-/-}$ animals. These data

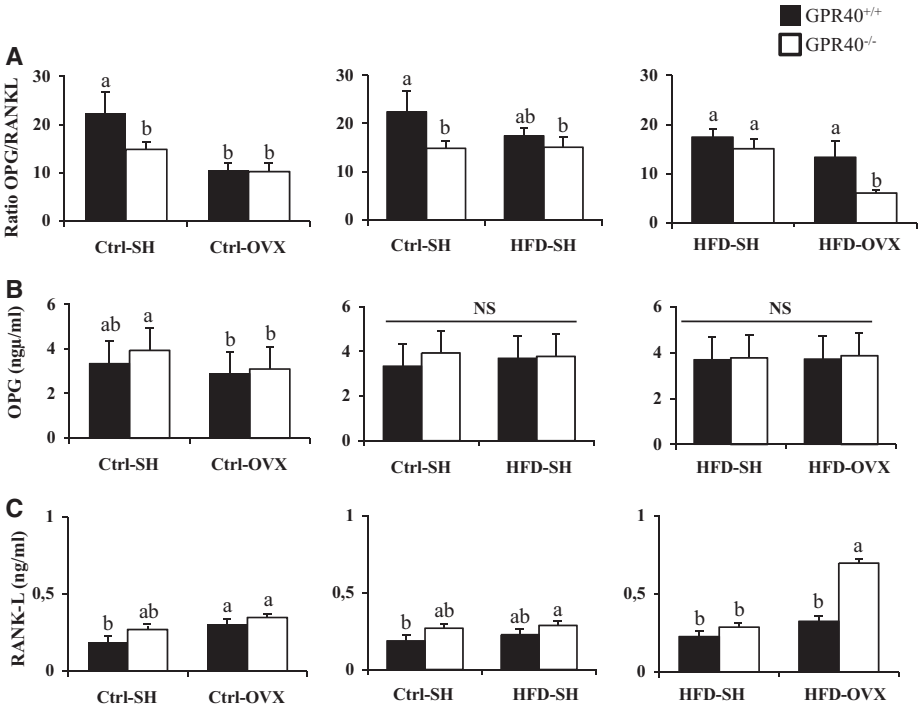

Figure 6. OPG and RANKL serum levels. (A and B) OPG and RANKL levels in blood samples. © OPG/RANKL levels ratio. Significant different groups are represented by different letters as analyzed via a multivariate ANOVA followed by a Tukey's test, $p < 0.05$. (Ctrl, control diet; SH, sham-operated mice; OVX, ovariectomized mice.) Data are presented as mean ± SD.

support a positive role for GPR40 in bone sparing effect and strongly suggest that, when stimulated by fatty acid enriched diets, GPR40 contributes to counteract ovariectomy-induced bone alteration.

Ovariectomy-induced bone loss mainly relies on a rapid increase of osteoclast activity [28]. Besides, both osteoclast activity and differentiation are stimulated by proinflammatory conditions [29]. The presence or the absence of GPR40 did not significantly impact circulating level of MCP-1 or spleen weight. Therefore, while inflammation induced by HFD may account for the bone loss observed, the limitation of the OVX-induced bone loss by GPR40 cannot be explained by a modulation of the systemic inflammatory status. At the tissue level, ovariectomy increased TNF-α and MCP-1 expression by the inguinal white adipose tissue. This increase was enhanced by the absence of GPR40 and further strengthened when combined with HFD in the HFD-OVX-GPR40$^{-/-}$ group. MCP-1 released by adipocytes contributes to recruit macrophages that enhance a local inflammatory vicious cycle. Interestingly, this local macrophage recruitment may involve specific populations such as Ly6C monocytes that have the ability to migrate from bone marrow to inflammation sites, get activated and then return to bone marrow where they may contribute to initiate or enhance an inflammatory context with deleterious effect on bone health [30]. This concept is of particular interest and may explain, at least in part, the seemingly conflicting

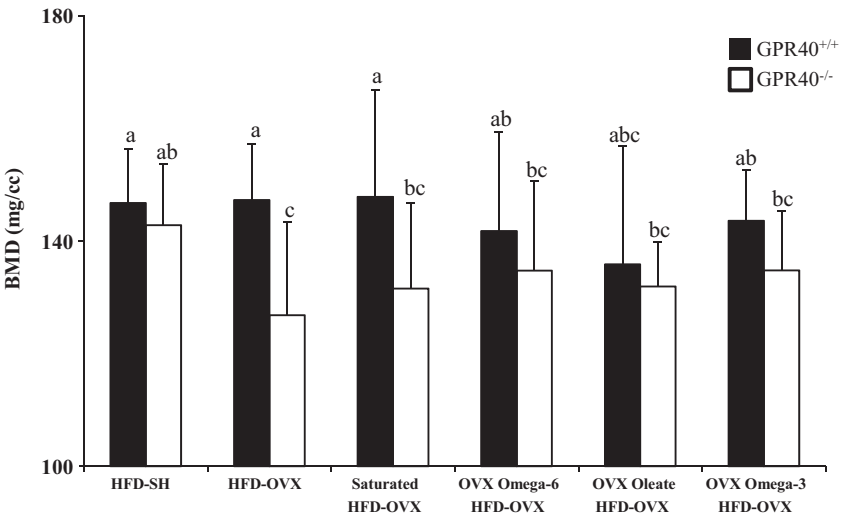

Figure 7. BMD of WT and knock-out GPR40 mice depending on fatty acid class-enriched diets. Significant different groups are represented by different letters as analyzed via a multivariate ANOVA followed by a Tukey's test. Data are presented as mean ± SD.

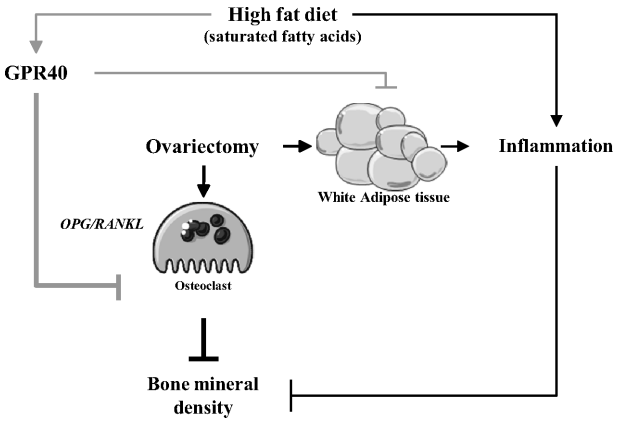

Figure 8. Conclusion GPR40 contributes to counteract ovariectomy-induced bone alteration in the presence of fatty acid enriched diets. The sparing effect is supported by the modulation of the OPG/RANKL ratio in blood stream and the expression level of inflammatory markers in local adipose tissues. The importance of GPR40 in osteoclastogenesis has been previously described.

results between the systemic and local level of MCP-1 in line with the role of GPR40 in both adipose and bone tissues [30]. Furthermore, these results parallel the fat gain observed in the OVX mice and strongly support that OVX and diet act in concert to increase the local production of proinflammatory cytokines by the adipose tissue while the presence of GPR40 limits it.

To further decipher the mechanism of action, the modulation of the OPG/RANKL ratio was assessed. Consistently, OPG/RANKL ratio was significantly decreased in HFD-OVX-GPR40$^{-/-}$. This unbalanced ratio was driven by an increase in RANKL production while OPG was unaffected. According to both, the role of RANKL in osteoclast differentiation and our previous published data [19] regarding the bone phenotype of the GPR40$^{-/-}$ mice, the additional decrease of bone mass observed in HFD-OVX-GPR40$^{-/-}$ animals may originate from an uncovered inhibition of bone resorption mediated by fatty acids and GPR40 interaction. Although Gao et al. [31] recently demonstrated that GPR40 may promote osteogenic differentiation through Wnt/β-catenin pathway, in our hands osteoblasts were only weakly affected in vivo by GPR40 [20]. This further supports the paramount role of the osteoclast in this story [20].

To sum up the results, our data suggest that a diet enriched with saturated fatty acids leads to decreased BMD but in a limited way due to GPR40 stimulation by fatty acids. The positive role of GPR40 in both adipose and bone tissues contributes to counter ovariectomy-induced bone loss (Fig. 8). Regarding fatty acid specificity, GPR40 was first described as a fatty acid receptor binding medium- to long-chain fatty acids independently of their degree of saturation [6,7,13]. Here, we demonstrate that GPR40-related bone protection seems to be preferentially mediated by saturated fatty acids. Despite this contrast with the literature, it is worth noting that just like GPR40, GPR120 was first described as a receptor for medium- to long-chain fatty acids lacking specificity before Oh's group evidenced it as a DHA sensor [32]. Besides, our data parallel those from Cornish's group [33]. They demonstrated that saturated fatty acids (C14:0–C18:0) inhibited osteoclastogenesis in bone marrow cultures and RAW264.7 cells expressing GPR40. Still, consistent with our results regarding the influence of diets either enriched in ω-3, ω-6, or ω-9, they found that the introduction of double bonds abrogated the inhibition of osteoclastogenesis with comparable low activity between ω-3 and ω-6 fatty acids.

In a context of increasing prevalence of obesity in occidental countries, it appears difficult from a nutritional view point to encourage long-chain saturated fat intake. Therefore, even if stimulation of the GPR40 receptor by saturated fatty acids contributes to limit excessive bone loss, this study may remain of mechanistic interest and our results rather support the relevance of pharmacologic approaches. Nevertheless, it is worth noting that GPR40 also binds medium-chain saturated fatty acids, such as myristic acid, that have been shown to limit the prevalence of metabolic complication [34,35]. The relevance of such a class of lipids regarding bone health prevention remains to be investigated.

C.P., F.W., V.C., and Y.W. designed the study and wrote the manuscript. G.Y.R., J.S., and E.M.-N. were in charge of the bone investigation. J.-F.L. and L.B. were in charge of the adipose tissue investigation. M.A. and F.R.J. were in charge of the insulin sensitivity. A.B. was in charge of the microbiota investigation.

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The authors have declared no conflict of interest.

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All-*trans*-retinoic acid represses chemokine expression in adipocytes and adipose tissue by inhibiting NF-κB signaling☆

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Abstract

An effect of the Vitamin A metabolite all-*trans*-retinoic acid (ATRA) on body weight regulation and adiposity has been described, but little is known about its impact on obesity-associated inflammation. Our objective was to evaluate the overall impact of this metabolite on inflammatory response in human and mouse adipocytes, using high-throughput methods, and to confirm its effects in a mouse model. ATRA (2 μM for 24 h) down-regulated the mRNA expression of 17 chemokines in human adipocytes, and limited macrophage migration in a TNFα-conditioned 3 T3-L1 adipocyte medium (73.7%, $P<.05$). These effects were confirmed in mice ($n=6$–9 per group) subjected to oral gavage of ATRA (5 mg/kg of body weight) and subsequently injected intraperitoneally with lipopolysaccharide. In this model, both systemic and adipose levels of inflammatory markers were reduced. The antiinflammatory effect of ATRA was associated with a reduction in the phosphorylation levels of IκB and p65 (~50%, $P<.05$), two subunits of the NF-κB pathway, probably mediated by PGC1α, in 3 T3-L1 adipocytes. Taken together, these results show a significant overall antiinflammatory effect of ATRA on proinflammatory cytokine and chemokine production in adipocyte and adipose tissue and suggest that ATRA supplementation may represent a strategy of preventive nutrition to fight against obesity and its complications.

Keywords: Inflammatory response; ATRA; Adipocyte; Chemokines; Macrophage migration, NF-κB

1. Introduction

The inflammatory response triggered by obesity involves many components of the classical inflammatory response to pathogens and is thought to play a major role in the onset of insulin resistance [1]. The main source of proinflammatory cytokines in obesity is adipose tissue [2]. Various products of adipose tissue have been characterized. Among the soluble factors it produces are the adipokines: these include leptin, adiponectin, tumor necrosis factor-alpha (TNFα), interleukin-6 (IL6), interleukin-1β (IL1β) and chemokines [3]. These cytokines are mainly produced by infiltrating macrophages, although adipocytes play a role [4].

Adipose tissue is also considered as the second most important storage site for retinol after the liver [5,6]. Other retinoids including several isomers of retinoic acid, of which all-*trans*-retinoic acid (ATRA) and retinaldehyde have been detected in this tissue [7,8].

Many studies indicate that Vitamin A inadequacy is associated with obesity and that Vitamin A may play an important role in body weight regulation and adiposity in humans (for a review [9]). Many animal and *in vitro* studies have demonstrated the role of Vitamin A metabolites in the control of adiposity (for a review [10]). Notably, the action of ATRA has been linked to increased oxidative metabolism and energy expenditure in different tissues including white adipose tissue (WAT) [10,11] and could be related to the ability of ATRA to impact oxidative phosphorylation (OXPHOS) capacity and mitochondrial content in adipocytes [12].

The *per se* antiinflammatory effect of ATRA has not yet been thoroughly investigated in adipocytes. A few studies have reported that ATRA can regulate the production of several adipokines linked to inflammatory tone, including leptin, adipsin and resistin. An inhibitory effect of ATRA on leptin expression has been described [13,14]. ATRA also suppressed the adipose production of resistin [15] *in vivo* and in adipocyte cell models. Similarly, adipsin was down-regulated by ATRA in adipocyte cell culture [16]. Our group has shown that ATRA is able to limit the expression of proinflammatory markers (IL6 and IL1β) in murine adipocytes pretreated with ATRA and incubated with TNFα [17].

The aim of the present study was to extend current knowledge on the impact of the active form of Vitamin A (ATRA) on inflammatory responses in human and mouse adipocytes, using high-throughput methods, and to confirm its effects in the adipose tissue of an inflammatory animal model.

☆ Conflict of interest statement: The authors have nothing to disclose.

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2. Materials and methods

2.1. Reagents

Dulbecco's modified Eagle's medium (DMEM) was purchased from Life Technologies (Cergy-Pontoise, France). Fetal bovine serum (FBS) was obtained from PAA Laboratories (Les Mureaux, France). Isobutylmethylxanthine, dexamethasone and insulin were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). TRIzol reagent, random primers and Moloney murine leukemia virus reverse transcriptase were obtained from Life Technologies (Saint-Aubin, France). SYBR Green reaction buffer was purchased from Eurogentec (Angers, France).

2.2. Cell culture

Macrophage and adipocyte cells were grown at 37 °C in a 5% CO_2 humidified atmosphere. The 3 T3-L1 preadipocytes (ATCC, Manassas, VA, USA; passage 10) were seeded in 3.5-cm diameter dishes at a density of 15×10^4 cells/well and grown in DMEM supplemented with 10% FBS, at 37 °C, as previously described [18]. To induce differentiation, 2-day postconfluent 3 T3-L1 preadipocytes (day 0) were stimulated for 72 h with 0.5-mM isobutylmethylxanthine, 0.25-μmol/l dexamethasone and -μg/ml insulin in DMEM supplemented with 10% FBS. The cultures were then treated with DMEM supplemented with 10% FBS and 1-μg/ml insulin (complete medium). In most cases, adipocytes were preincubated with all-*trans*-retinoic acid (ATRA) (0.2 and 2 μM; dissolved in absolute ethanol) for 24 h and incubated with TNFα (15 ng/mL) for an additional 24 h. To identify the signaling pathway involved in chemokine regulation, 3 T3-L1 cells were treated with a specific inhibitor of NF-κB signaling (BAY 117082, 10 μM) for 1 h and then stimulated with TNFα (15 ng/mL) for 24 h. To examine ATRA effects on NF-κB signaling, adipocytes were incubated with TNFα (15 ng/ml) for 5 min. All treatments were performed on day 8. Each experiment was reproduced in triplicate, at least three independent times. To examine the impact of PGC1α and PGC1β on NF-κB signaling, adipocytes were transfected with pCMX-PGC1α or pCMX-PGC1β for 24 h and then incubated with TNFα (15 ng/ml) for 5 min.

Raw 264.7 macrophages (ECACC, Salisbury, UK; passage 8) were seeded in 3.5-cm diameter dishes and grown in DMEM supplemented with 10% FBS, 2% HEPES and 1% antibiotics.

The human preadipocytes (five independent cultures) were supplied by Promocell (Heidelberg, Germany) and cultured according to the company's instructions. The mature adipocytes (day 15) were incubated with ATRA (2 μM, 24 h) followed by a 24-h incubation with TNFα (15 ng/mL). Experiments were performed in triplicate, on three independent times.

2.3. Microarray hybridization and data analysis

RNA was extracted from human adipocyte cultures (three independent cultures), and RNA quality control was performed on an Agilent 2100 Bioanalyzer (Massy, France), according to the manufacturer's instructions. RNA was hybridized to the Agilent Whole Human Genome 8×60 K microarray (Massy, France). For each independent culture, treated conditions (ATRA + TNFα) were labeled with cyanine 5 and control conditions (TNFα alone) with cyanine 3. In addition, a treated pool and a control pool made from an equal amount of each independent control and treated condition were made up and labeled likewise (treated pool with cyanine 5 and control pool with cyanine 3). The resulting four pairs of samples (three independent pairs of samples plus one pair of pooled samples) were further hybridized. All labeling, hybridization, washing and scanning were performed as described in the manufacturer's protocol and as previously described [17,19]. The same procedure was applied for 3 T3-L1 adipocytes treated only with ATRA.

The arrays were scanned using an Agilent Scanner (Massy, France). The data were extracted using Agilent Feature Extraction v10.5.1.1 software and analyzed with Agilent GeneSpring GX v11.0.2 software (Massy, France). The analyses were performed using GSEA (http://www.broadinstitute.org/gsea) (Gene Set Enrichment Analysis) and DAVID (Database for Annotation, Visualization, and Integrated Discovery) software as previously described [20]. A false discovery rate $q<0.25$ for normalized enrichment score was considered significant.

2.4. RNA isolation and qPCR

Total cellular RNA was extracted using TRIzol reagent according to the manufacturer's instructions. cDNA was synthesized from 1 μg of total RNA using random primers and Moloney murine leukemia virus reverse transcriptase. Real-time quantitative RT-PCR analyses were performed using the Mx3005P Real-Time PCR System (Stratagene, La Jolla, CA, USA) as previously described [21]. For each condition, the expression was quantified in duplicate, and the ribosomal protein 18S mRNA was used as the endogenous control in the comparative cycle threshold (C_T) method. The sequences of the primers used for qPCR determination of gene expression are displayed in Table S1.

2.5. Chemokine quantification in cell culture supernatants

A Proteome Profiler™ Array Human Chemokine Array Kit, Catalog Number ARY017 [R&D Systems (Lille, France)] was used to quantify 31 chemokines in human adipocyte cell culture supernatants according to the manufacturer's instructions. Pixel density was quantified with Image J software. A Luminex screening assay [R&D Systems (Lille, France)] was also used to quantify CXCL10 with the Luminex 200 platform.

2.6. Chemokine/Cytokine quantification in plasma and epididymal adipose tissue samples

Ccl2 and Ccl5 were quantified with mouse Ccl2 and Ccl5 DuoSet ELISA from R&D Systems. Tnfα and Il6 were quantified with Ready-SET-Go! ELISA from eBioscience.

2.7. Macrophage migration assay

Migration assays were performed using cell culture inserts of 3-μm membrane pore size (Transwell Millipore, Molsheim, France). The 3 T3-L1 adipocytes were preincubated with or without various concentrations of ATRA (0.2 and 2 μM) for 24 h. The adipocytes were then incubated with TNFα (15 ng/mL) for 24 h. The 3 T3-L1 conditioned media were transferred to plates containing inserts. RAW 264.7 macrophages (ECACC, Salisbury, UK) were seeded on these inserts at a density of 900 cells/cm^2. After migration for 4 h at 37 °C, macrophages in the lower compartment were fixed with 2.5% glutaraldehyde for 15 min and counted as previously described [22–24].

2.8. NF-κB activation

To examine ATRA effects on NF-κB signaling, adipocytes were preincubated with ATRA (0.2 and 2 μM) for 24 h and incubated with TNFα (15 ng/mL) for an additional 5 min. Phosphorylation levels of p65 (Ser536) and IκBα (Ser32/36) were quantified with an ELISA Instant One kit according to the manufacturer's instructions (eBiosciences SAS, Paris, France).

2.9. Transfection experiments

For RNA interference experiments, 3 T3-L1 differentiated cells were transfected with siRNA targeted against Ppargc1α or Ppargc1β (encoding peroxisome proliferator-activated receptor gamma coactivator (PGC)1α or PGC1β, respectively) or a nontargeting siRNA according to the manufacturer's instructions (Polyplus). Briefly, the cells were transfected overnight using a mixture of 10-μM siRNA and 2.8 μL of interferin reagent per well. The media were then replaced with complete medium. Adipocytes were then treated with ATRA for 24 h and incubated for a further 24 h.

For overexpression experiments, 3 T3-L1 adipocytes were transfected using Lipofectamine LTX Plus Reagent (Life Technologies) with empty or PGC1α-containing pCMX plasmid (pCMX-PGC1α; Addgene plasmid # 6 [25]). After 24-h incubation with transfection mixes (1 μg of plasmid, 0.9 μL of Lipofectamine LTX and 1 μL of Plus Reagent per well, according to the manufacturer's instructions), the medium was replaced, and cells were incubated with TNFα for 24 h. RNA was isolated from treated cells, and residual plasmidic DNA contamination was removed by DNase I digestion [Sigma-Aldrich (Saint-Quentin-Fallavier, France)]. PGC1α expression was quantified by qPCR.

2.10. Animal experiment

The protocol was approved by the local ethics committee. Six-week-old male C57BL/6 J mice were obtained from Janvier (Le Genest-Saint-Isle, France). Mice were fed *ad libitum* (chow diet A04, Safe, Augy, France), with full access to drinking water. The animals were maintained at 22 °C under a 12 h light–12 h dark cycle at 50% humidity.

To assess the impact of Vitamin A on acute inflammation, the mice received by gavage ($n=6$–9 per group) the active form of Vitamin A (ATRA) (5 mg/kg of body weight; Sigma-Aldrich, Saint-Quentin-Fallavier, France) or vehicle alone (olive oil), once a day for 4 days. On the fifth day, the mice were injected intraperitoneally with saline or *Escherichia coli* lipopolysaccharide (LPS) (4 mg/kg; serotype O111:B4, Sigma-Aldrich). The mice were sacrificed 4 h after LPS injection, and epididymal adipose tissue was dissected and stored at −80 °C.

2.11. Statistical analysis

The data are expressed as means ± S.E.M. Significant differences between the control and treated group were determined using the ANOVA analysis followed by the Tukey–Kramer *post hoc* test using Statview software: $P<.05$ was considered statistically significant.

3. Results

3.1. ATRA modulates the transcriptome of human adipocytes in inflammatory conditions

To study the impact of ATRA on chemokines under inflammatory conditions, these cells were preincubated with ATRA, or untreated, and then incubated with TNFα. The transcriptomic approach was applied in cells treated with TNFα compared with cells pretreated with ATRA before incubation with TNFα. The impact of these

treatments on the transcriptome was evaluated with a fold change filter of 1.3. Examination of the gene list by DAVID (Database for Annotation, Visualization, and Integrated Discovery) software revealed that the "chemokine family" was strongly impacted by the ATRA/TNFα treatment compared with TNFα alone (P=0.018) (Table S1). These data were confirmed by other software, GSEA (Gene Set Enrichment Analysis), with which analysis according to gene ontology terms highlighted inflammation-related processes such as "G protein coupled receptor binding," "defense response," "locomotory behavior," "cytokine activity" and "chemokine activity." The chemokine activity was impacted by the ATRA/TNFα treatment compared with TNFα alone (NES=−1.757, P<.05 and FDR q<0.25; the ten most frequently represented gene sets are given in Table S2). It is noteworthy that all these gene sets presented a negative enrichment score, meaning that chemokines were down-regulated by ATRA pretreatment in comparison with TNFα (Table S2). As seen in Table S3, 17 chemokines identified by GSEA software were regulated by ATRA pretreatment in human adipocytes. Interestingly, we also observed that five chemokine receptors were down-regulated by ATRA pretreatment (Table S4). To confirm our observations, we evaluated the gene expression profiles of six chemokines (randomly chosen, CCL5, CCL19, CX3CL1, CXCL1, CXCL5 and CXCL10) in human adipocytes incubated with ATRA (2 μM) for 24 h followed or not by incubation with TNFα (15 ng/mL) for a further 24 h. A significant increase in expression was observed in all the genes investigated: CCL5, CCL19, CXCL1, CX3CL1, CXCL5 and CXCL10 were increased 1611%, 2194%, 1865%, 993%, 3221% and 493%, respectively, in TNFα condition *versus* control (Fig. 1A). Interestingly, the incubation with ATRA reduced the mRNA expression levels of these chemokines 57.7%, 92.6%, 816.5%, 67.8%, 55.5% and 54.4%, respectively, compared with TNFα alone.

Very similar results were obtained when murine 3 T3-L1 differentiated adipocytes were treated: the mRNA expression levels of Ccl5, Ccl19, Cxcl1, Cx3cl1 and Cxcl10 were significantly increased by TNFα 34,163%, 3489%, 729%, 211% and 1221%, respectively. By contrast, ATRA incubation significantly reduced chemokine expression in comparison with the TNFα condition 49%, 92.3%, 60.6%, 80.1% and 20%, respectively (Fig. 1B).

The preventive effect of ATRA on the TNFα-mediated proinflammatory cytokine expression was also evaluated in the human adipocyte culture supernatants. An increase in protein secretion was obtained for CXCL1, CXCL10 and midkine under TNFα treatment (168%, 173% and 118%, respectively). Their levels were significantly decreased in adipocytes pretreated with ATRA followed by TNFα incubation compared with TNFα alone (18%, 68% and 23.7%, respectively) (Fig. 1C).

3.2. ATRA supplementation limits cytokine/chemokine expression induced by LPS in mice adipose tissue.

The effects of ATRA on cytokine/chemokine expression were studied in a model of acute inflammation, consisting of an intraperitoneal injection of LPS for 4 h in mice. As shown in Fig. 2A, a significant increase in the mRNA expression levels of Il6 (8667%), Tnfα (1180%) and Il1β (360%) was observed in epididymal fat pads under the LPS

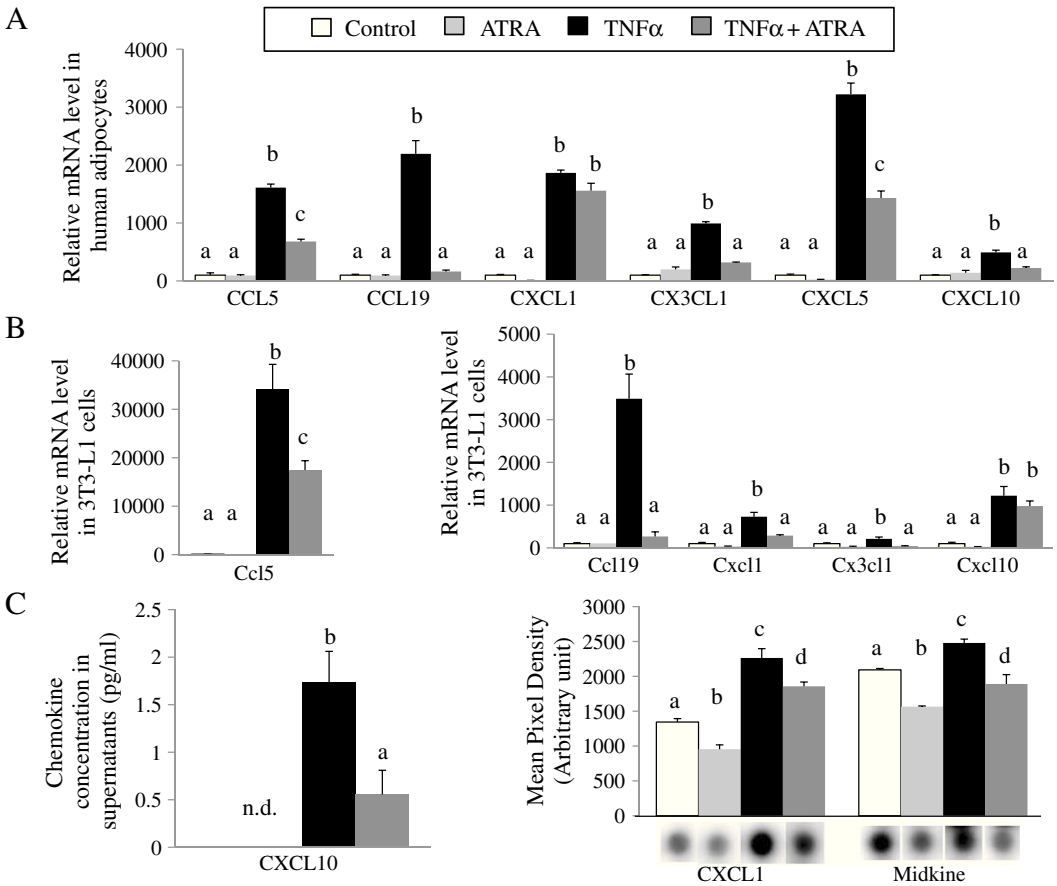

Fig. 1. ATRA decreases chemokine expression levels in adipocytes. Cells were preincubated with ATRA (2 μM) for 24 h and incubated with TNFα (15 ng/mL) for 24 h. (A) The mRNA levels of CCL5, CCL19, CXCL1, CX3CL1, CXCL5 and CXCL10 were quantified through qPCR in human adipocytes. The values are presented as means ± S.E.M. Experiments were performed in triplicate on three independent cultures. (B) The mRNA levels of Ccl5, Ccl19, Cxcl1, Cx3xl1 and Cxcl10 were quantified through qPCR in 3 T3-L1 adipocytes. The values are presented as means ± S.E.M. Experiments were performed in triplicate on three independent cultures. (C) CXCL10, CXCL1 and Midkine secretion in cell culture supernatants of human adipocytes was quantified by Luminex technology and Proteome Profiler Array. n.d.: not detected. Bars not sharing the same letters are significantly different, P<.05.

effect, whereas a 4-day ATRA supplementation prior to LPS stimulation significantly reduced inflammation in white adipose tissue 37.4%, 38.5% and 43.9%, respectively (Fig. 2A). Chemokine mRNA including Ccl2, Ccl5, Ccl11, Ccl19, Cxcl1, Cx3cl1 and Cxcl10 were also quantified. Their expression was significantly increased by LPS (4412%, 7097%, 547%, 956%, 3150%, 320%, 31,944%, respectively) and reduced by 4 days of ATRA supplementation (55.3%, 56.3%, 54%, 66%, 36.2%, 75.7% and 66.6%, respectively) (Fig. 2A).

We confirmed the preventive effect of ATRA on the LPS-mediated inflammation in the plasma mice. An increase in protein secretion was obtained for Il6, TNFα and Ccl5 after LPS intraperitoneal injection in mice (288.4%, 404% and 5597%, respectively) (Fig. 2B). Their levels were significantly decreased in the plasma of mice that received ATRA by gavage followed by LPS injection compared with LPS alone (46.2%, 14% and 13.5%, respectively) (Fig. 2B). We obtained similar results in the epididymal adipose tissue of these mice for Ccl2 and Ccl5, which increased in LPS-injected mice (275% and 30%, respectively) (Fig. 2C). Interestingly, their levels were significantly decreased in epididymal adipose tissue of mice that received ATRA by gavage followed by LPS injection compared with LPS alone (24.2% and 15.8%, respectively) (Fig. 2C).

3.3. ATRA limits RAW 264.7 macrophage migration in 3 T3-L1 conditioned medium.

To examine the potential of ATRA to limit the migration of macrophages, 3 T3-L1 cells were preincubated with ATRA (0.2 and 2 μM) prior to TNFα incubation and compared with cells incubated with TNFα alone. The resulting 3 T3-L1 conditioned medium was used to study the RAW 264.7 macrophage migration. As expected, 3 T3-L1 conditioned medium under TNFα proinflammatory stimulus induced a significant migration of macrophages (approximately 100%, Fig. 3). Interestingly, the preincubation of 3 T3-L1 with 2 μM of ATRA significantly reduced the migration of macrophages 26.3% (Fig. 3).

3.4. ATRA limits NF-κB activation in 3 T3-L1 adipocytes.

As previously demonstrated, the expression of several chemokines is up-regulated in inflammatory conditions, mainly *via* the NF-κB signaling pathway in human adipocytes [4], and in 3 T3-L1 adipocytes [23]. Since NF-κB activation is central in the induction of chemokines, we next examined whether the effect of ATRA on chemokine production could be mediated through limitation of NF-κB signaling in murine adipocytes. Cells were pretreated with ATRA for 24 h followed by a 5-min incubation with TNFα. As expected, phosphorylation levels of p65 and IκB, two subunits of the NF-κB pathway, were significantly increased in the inflammatory condition induced by TNFα (414% and 360%, respectively). On the other hand, preincubation of cells with ATRA strongly limited the phosphorylation of p65 and IκB, suggesting that ATRA was able to reduce NF-κB activation in 3 T3-L1 adipocytes (Fig. 4).

3.5. ATRA modulates NF-κB signaling and cytokine/chemokine expression through PGC1α in 3 T3-L1 adipocytes.

To identify the molecular mechanisms involved in ATRA-mediated NF-κB signaling deactivation, we hypothesized the involvement of PGC1α and/or β, since we had recently shown that ATRA induced the expression of PGC1α and PGC1β in 3 T3-L1 adipocytes [12], and these transcription factors are known to reduce NF-κB activity in muscle cells [26].

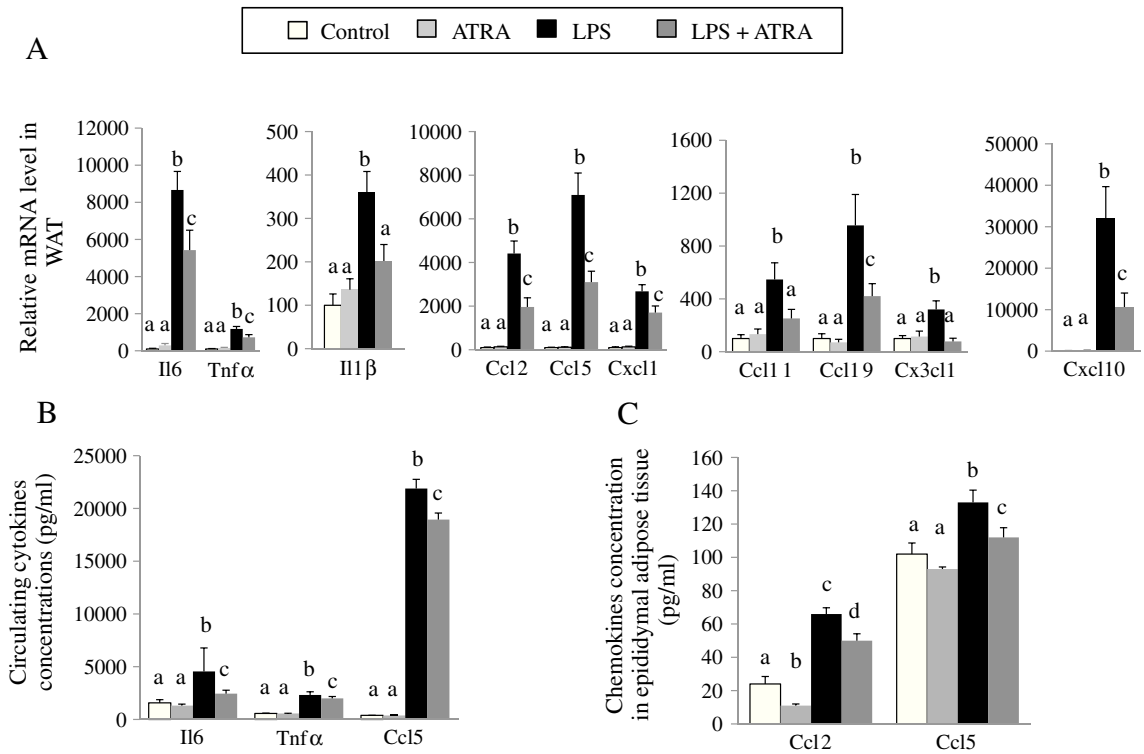

Fig. 2. ATRA decreases chemokine expression levels in LPS-mediated inflammation in mice epididymal adipose tissue. (A) In an acute inflammation model (LPS injection for 4 h), the mRNA levels of Il6, Tnfα, Il1β, Ccl2, Ccl5, Ccl11, Ccl19, Cxcl1, Cx3cl1 and Cxcl10 were quantified through qPCR in epididymal adipose tissue of mice (n=6–9 per group) and expressed relative to 18S ribosomal RNA. The data are expressed as relative expression ratios. The values are presented as means ± S.E.M. Bars not sharing the same letters are significantly different, P<.05. (B) In the LPS inflammation model, protein amounts of Il6, Tnfα and Ccl5 secreted were quantified by ELISA. The data are expressed as relative expression ratios. The values are presented as means ± S.E.M. Bars not sharing the same letters are significantly different, P<.05. (C) In the LPS inflammation model, protein amounts of Ccl2 and Ccl5 in epididymal adipose tissue of mice were quantified by ELISA. The data are expressed as relative expression ratios. The values are presented as means ± S.E.M. Bars not sharing the same letters are significantly different, P<.05.

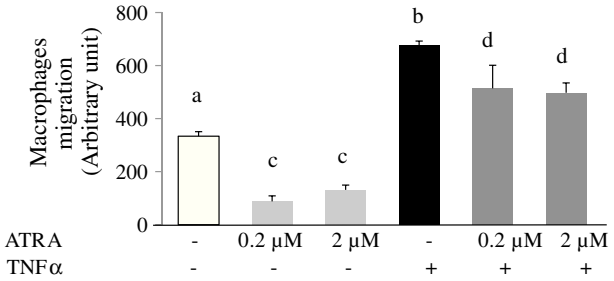

Fig. 3. ATRA decreases macrophage migration in 3 T3-L1 adipocytes. 3 T3-L1 cells were preincubated with ATRA (0.2 and 2 µM) for 24 h and incubated with TNFα (15 ng/mL) for 24 h. RAW 264.7 macrophage migration in 3 T3-L1 conditioned media (4 h, 37 °C) was then performed. Data are expressed as relative expression ratio. Values are presented as means ± S.E.M. Bars not sharing the same letter are significantly different, $P<.05$. Experiments were performed in triplicate on three independent cultures.

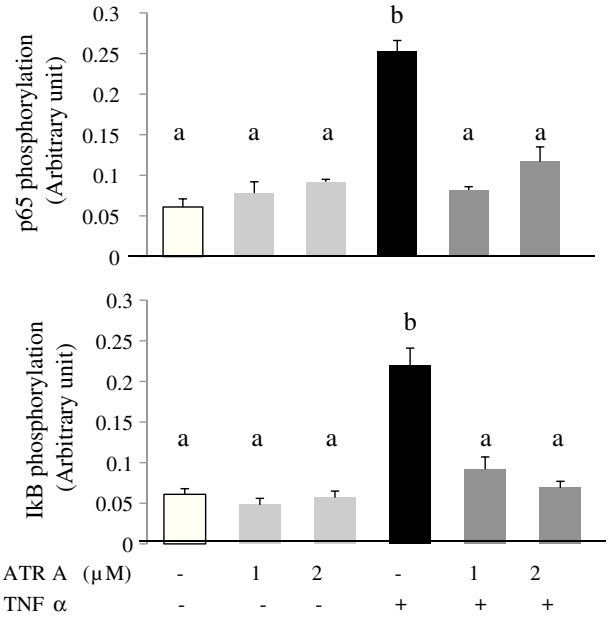

Fig. 4. ATRA limits NF-κB activation. Cells were preincubated with ATRA (1 and 2 µM) for 24 h dose dependently and incubated with TNFα (15 ng/mL) for 5 min. Phosphorylation levels of the NF-κB subunits (p65 and IκB) were evaluated by ELISA. Data are expressed as relative expression ratio. Values are presented as means ± S.E.M. Bars not sharing the same letter are significantly different, $P<.05$. Experiments were performed in triplicate on three independent cultures.

The involvement of PGC1α and/or β on cytokine/chemokine modulation by ATRA was investigated in 3 T3-L1 adipocytes transfected with siRNA designed against PGC1α or PGC1β. The cells were then preincubated with ATRA for 24 h followed by an incubation with TNFα for 24 h (Fig. 5A). As expected, TNFα treatment significantly increased the mRNA expression levels of Tnfα, Il6, Ccl2, Ccl5, Ccl11 and Cxcl10 (521%, 424%, 4420%, 3791%, 232% and 2787%, respectively), whereas ATRA incubation significantly decreased them (48.4%, 35%, 17%, 41.8%, 26.8% and 43%, respectively) compared with the TNFα condition (Fig. 5A). Interestingly, in the 3 T3-L1 adipocytes transfected with PGC1α siRNA, there was a modulation in the expression of these inflammatory markers (Tnfα, Il6, Ccl2, Ccl5, Ccl11 and Cxcl10, 211.9%, 160%, 154%, 189%, 160% and 196%, respectively compared with the ATRA + TNFα condition). However, in the 3 T3-L1 adipocytes transfected with PGC1β siRNA, no difference in Tnfα, Ccl2, Ccl5 and Cxcl10 expression was observed compared with the ATRA + TNFα condition, whereas the Il6 and Ccl11 decrease mediated by ATRA was slightly blunted (Fig. 5A). These results suggest that the cytokine/chemokine down-regulation mediated by ATRA in inflamed adipocytes is mainly PGC1α-dependent.

To further investigate the involvement of PGC1α in these regulations, we studied the effect of PGC1α overexpression on TNFα-mediated cytokine/chemokine expression (Fig. 5B). PGC1α overexpression induced a significant decrease in Tnfα, Ccl2, Ccl11 and Cxcl10 expression (46.3%, 14.6%, 33.3% and 13.3% compared with pCMX in the TNFα condition). However, in the case of Ccl5 and Il6 expression, no effect of PGC1α was observed. These data suggest that the up-regulation of some cytokine/chemokine expression mediated by TNFα can be partly blunted by PGC1α.

To confirm the effect of PGC1α on the NF-κB signaling pathway, cells transfected with pCMX-PGC1α were incubated with or without TNFα for 5 min, and IκB phosphorylation was evaluated. As expected, TNFα treatment significantly increased the IκB phosphorylation by 30% (Fig. 6). Interestingly, PGC1α overexpression in adipocytes significantly decreased IκB phosphorylation by 18.4% compared with TNFα-treated adipocytes, suggesting that PGC1α overexpression is able to deactivate NF-κB signaling in adipocytes (Fig. 6).

4. Discussion

Here we studied the putative role of ATRA on inflammatory marker expression in adipocytes and in adipose tissue, together with the molecular mechanisms involved.

To evaluate the impact of ATRA on the adipocyte inflammatory response, we used both human and murine mature adipocyte cultures subjected to TNFα incubation (to induce a low-grade inflammation). Analyses were conducted using high-throughput methods (microarrays and proteome profiler) combined with bioinformatic analysis of gene set enrichment. This approach demonstrated the ability of ATRA treatment to down-regulate the expression of TNFα-mediated chemokine expression (at mRNA level and at protein level in some cases). Numerous genes coding for chemokines were impacted, among which Ccl2 [27], Ccl5 [28,29], C-x-c motif chemokine ligand 5 (Cxcl5 [30]), Cxcl12 [31], Cxcl14 [32] and Ccl20, which are known to be involved in leukocyte recruitment in adipose tissue [33]. Several chemokine receptors were also down-regulated, which may add to the physiological consequences of ATRA-mediated chemokine down-regulation. The impact of these regulations was also evaluated in terms of leukocyte chemotaxis [34], since chemokines mediate this process, and we found that ATRA limited macrophage migration induced by adipocyte-conditioned medium, which gives our results a functional validation.

To confirm the physiological relevance of these regulations, mice underwent oral gavage of ATRA for 4 days and were injected with LPS to induce inflammation, both at the systemic level and in adipose tissue. In these conditions, not only were adipose tissue mRNA levels of inflammatory markers reduced but also was adipose tissue protein expression (Ccl2 and Ccl5). It is also of note that this improvement in adipose tissue inflammatory status was associated with a reduced systemic inflammatory tone, as shown by the reduction of several inflammatory markers, such as Il6, Tnfα and Ccl5. Surprisingly, our results diverge from the recent finding of a moderate increase in proinflammatory markers (Il18 and MIP-1γ) in male mice subjected to a moderate Vitamin A supplementation [35]. This discrepancy may derive from the major differences between the two experimental protocols (use of Vitamin A or ATRA, duration of treatment, concentrations of substances used, *etc.*). However, our results seem to be in line with the suspected role of Vitamin A deficiency in elevated proinflammatory cytokines and T-cell activation [36,37].

From a molecular point of view, it is well-established that NF-κB controls the transcription of proinflammatory cytokines and chemokines in many cell types, including preadipocytes and adipocytes [1,4,38]. Thus we evaluated the ability of ATRA to blunt NF-κB-

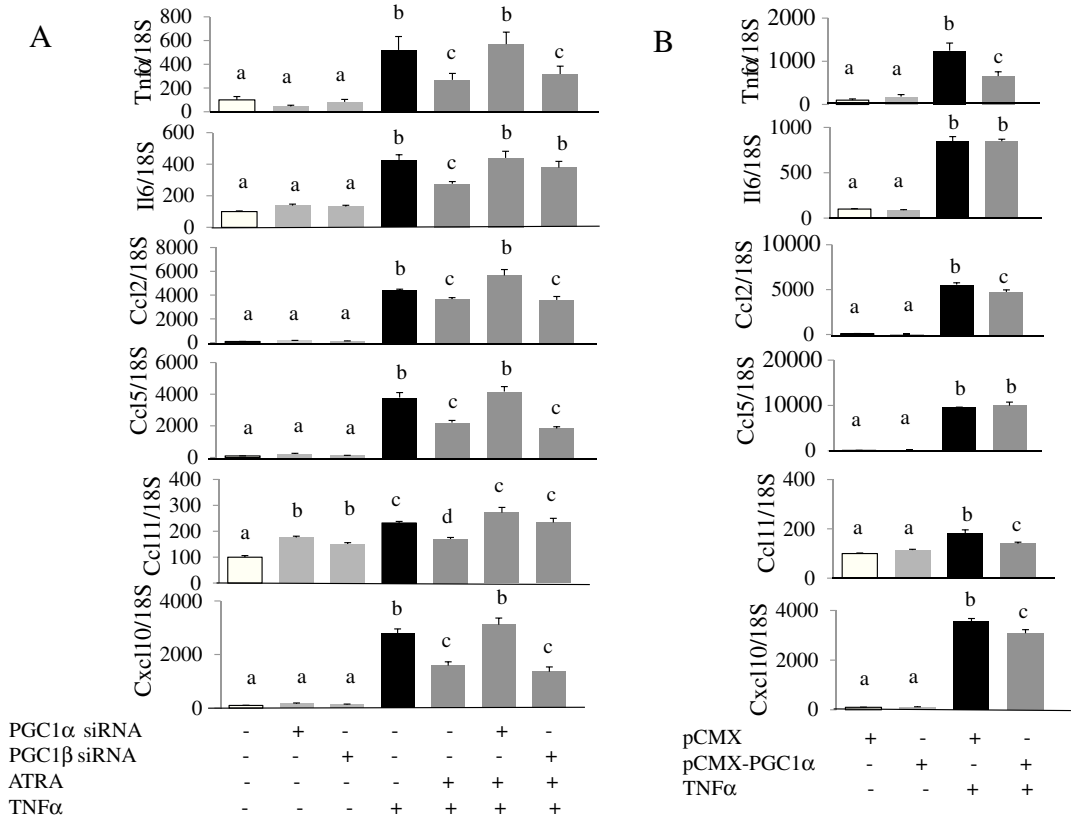

Fig. 5. ATRA modulates NF-κB signaling and cytokine/chemokine expression through PGC1α in 3 T3-L1 adipocytes. (A) Cells were transfected with siRNA designed against PGC1α or PGC1β or with nontargeted siRNA. The cells were then preincubated with ATRA for 24 h followed by an incubation with TNFα for 24 h. The mRNA levels of Tnfα, Il6, Ccl2, Ccl5, Ccl11 and Cxcl10 were quantified through qPCR. The values are presented as means ± S.E.M. (B) Cells were transfected with pCMX plasmids, empty or containing PGC1α (pCMX-PGC1α). After 24-h incubation, cells were incubated with TNFα for 24 h. The mRNA levels of Tnfα, Il6, Ccl2, Ccl5, Ccl11 and Cxcl10 were quantified through qPCR. Values are presented as means ± S.E.M. Bars not sharing the same letter are significantly different, $P<.05$. Each experiment was reproduced in triplicate at least three independent times.

mediated chemokine expression. First we demonstrated that ATRA displayed a strong inhibitory effect on NF-κB signaling in 3 T3-L1 adipocytes, characterized by reduced phosphorylation levels of IκB and p65 (two main intermediates in the NF-κB signaling pathway).

Similar inhibition of NF-κB signaling has already been reported in various cell models [39] and in transgenic NF-κB reporter mice [40]. To pursue the identification of the precise molecular mechanisms, we hypothesized the involvement of PGC1α and/or PGC1β. Peroxisome proliferator-activated receptor γ coactivator 1 α and β are members of the PGC1 family of transcriptional coactivators [41]. PGC1α is mainly involved in adaptive thermogenesis, glucose disposal and mitochondrial biogenesis [42], whereas PGC1β regulates saturated fatty acid-induced hepatic triglyceride synthesis and hyperlipidemia [43] and mitochondrial fatty acid β oxidation [44]. In addition, these two transcription factors are able to reduce phosphorylation of the NF-κB family member p65 and thereby its transcriptional activation potential in muscle cells [26], and we recently showed that ATRA up-regulated the two transcription factors in 3 T3-L1 adipocytes [12]. To confirm the involvement of PGC1α and/or β in cytokine/chemokine down-regulation, siRNA designed against PGC1α and PGC1β was used. Results clearly show that inflammatory marker limitation through ATRA was primarily mediated by PGC1α, the ATRA down-regulation of chemokines being abolished in the presence of siRNA directed against PGC1α. Furthermore, the ability of PGC1α to repress the TNFα-mediated up-regulation of chemokine mRNA levels together with NF-κB signaling, and notably IκB phosphorylation, was confirmed in overexpression experiments. Taken together, these findings strongly support the involvement of the ATRA-mediated up-regulation of PGC1α in the inhibition of NF-κB signaling and subsequent limitation of chemokine expression. However, other putative mechanisms cannot be excluded, notably the ATRA-mediated up-regulation of specific phosphatases leading to NF-κB signaling deactivation, similar to the antiinflammatory effect of the 1,25$(OH)_2$D, which is mediated in particular *via* an induction of Dusp10 [24], a phosphatase involved in stress-activated kinase dephosphorylation.

Data related to the impact of retinoic acid on inflammation in humans are particularly scant, but it is noteworthy that in a recent prospective study, an inverse association between retinoic acid (RA)

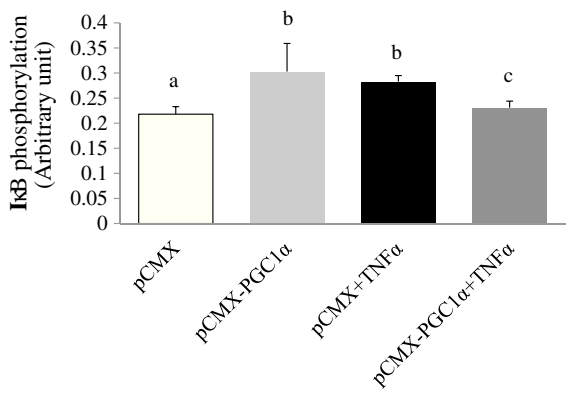

Fig. 6. PGC1α limits IκB phosphorylation in 3 T3-L1 adipocytes. Cells were transfected with pCMX plasmids, empty or containing PGC1α (pCMX-PGC1α). After 24-h incubation, cells were incubated with TNFα for 5 min. Phosphorylation levels of IκB were evaluated by ELISA. Data are expressed as relative expression ratio. Values are presented as means ± S.E.M. Bars not sharing the same letter are significantly different, $P<.05$. Experiments were performed in triplicate on three independent cultures.

with several inflammatory and oxidative stress biomarkers such as 8-iso-prostaglandin F2α, C Reactive Protein and IL6 was reported in a Chinese metabolic syndrome population [45]. Even if our results seem to be encouraging and in agreement with a clinical study, our data present several limitations due to the supraphysiological doses of ATRA used both *in vitro* and *in vivo* and to the fact that extrapolation from mice to human is sometimes hazardous.

In conclusion, the results of the present study show that ATRA reduces chemokine expression in adipocytes and macrophage migration *in vitro*. We confirmed these results in mice adipose tissue. These effects may be associated with the ability of ATRA to limit the activation of the NF-κB signaling pathway, probably through PGC1α.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jnutbio.2017.01.004.

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RESEARCH ARTICLE

Lycopene and tomato powder supplementation similarly inhibit high-fat diet induced obesity, inflammatory response, and associated metabolic disorders

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Scope: Several studies have linked the high intake of lycopene or tomatoes products with lower risk for metabolic diseases. The aim of the present study was to evaluate and to compare the effect of lycopene and tomato powder on obesity-associated disorders.
Methods and results: Male C57BL/J6 mice were assigned into four groups to receive: control diet (CD), high fat diet (HFD), high fat diet supplemented with lycopene or with tomato powder (TP) for 12 weeks. In HFD condition, lycopene and TP supplementation significantly reduced adiposity index, organ, and relative organ weights, serum triglycerides, free fatty acids, 8-iso-prostaglandin GF2α and improved glucose homeostasis, but did not affect total body weight. Lycopene and TP supplementation prevented HFD-induced hepatosteatosis and hypertrophy of adipocytes. Lycopene and TP decreased HFD-induced proinflammatory cytokine mRNA expression in the liver and in the epididymal adipose tissue. The anti-inflammatory effect of lycopene and TP was related to a reduction in the phosphorylation levels of IκB, and p65, and resulted in a decrease of inflammatory proteins in adipose tissue.
Conclusion: These results suggest that lycopene or TP supplementation display similar beneficial health effects that could be particularly relevant in the context of nutritional approaches to fight obesity-associated pathologies.

Keywords:
Adipose tissue carotenoids / Inflammation / Insulin sensitivity / Metabolic disease / Obesity / steatosis / Tomato product

1 Introduction

Obesity is characterized by a state of chronic low grade inflammation that is closely associated with the pathogenesis of obesity-related diseases, such as atherosclerosis, hypertension, and insulin resistance leading to type 2 diabetes [1]. This low-grade inflammatory state is notably related to the activation of inflammatory pathways (JNK and NF-$_{\kappa}$B) in adipose tissue [1] and increased production of cytokines such as IL-6, IL-1β, tumor necrosis factor alpha (TNF-α), or various chemokines [2], including monocyte chemotactic protein 1 (MCP-1) and other mediators of the immune response [1].

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Abbreviations: CD, control diet; **HFD**, high fat diet; **MCP-1**, monocyte chemotactic protein 1; **NEFA**, nonesterified fatty acids; **TNF-α**, tumor necrosis factor alpha; **TP**, tomato powder

Lycopene is a lipophilic nonprovitamin A carotenoid that is responsible for the red color in various fruits and vegetables such as tomato, water melon, guava, grapefruits [3]. This carotenoid is well known for its antioxidant properties [4]. In humans, lycopene is mainly stored in adipose tissue, where it represents more than half of total carotenoid concentration [5,6]. The uptake of lycopene by adipocytes and adipose tissue is mediated at least in part by CD36 [7] and is not related to its physicochemical properties [8]. Moreover, high intake of lycopene is associated with low waist circumferences, as well as low visceral and subcutaneous fat masses as reported by Sluijs et al., suggesting that lycopene could actively impact adipocyte physiology [9]. Several studies showed that lycopene has been reported to display anti-inflammatory effects in adipocytes and adipose tissue; [10–13] and liver [14, 15]. Among others, these effects could be responsible of the numerous health

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effect attributed to lycopene [16, 17] in the field of CVD [18], adiposity, and obesity [19].

Tomatoes are a valuable source of many micronutrients including lycopene, which is classically associated to the health benefits of tomato consumption [20]. However, in the field of obesity and associated disorders, despite the beneficial effect of lycopene has already been described [12], the effect of tomato powder (TP) consumption and the comparison between purified lycopene and TP has never been reported. Thus, the aim of present study was to investigate and to compare the effects of purified lycopene and TP to exhibit prevention/reduction of high fat diet (HFD) induced global changes.

2 Materials and methods

2.1 Animals and dietary supplementation

The care and use of mice were in accordance with the French guidelines and approved by the experimental animal ethic local committee. Eight weeks old male C57BL/6J mice were obtained from Janvier (Le Genest Saint Isle, France) and were acclimatized to the experimental facility for 1 week. The mice were kept in a temperature and humidity controlled room and fed with water and food ad libitum. The mice were randomly divided into four groups, as follows: 1. CD: $n = 10$, control group given a control diet, 2. HFD: $n = 10$, given a high-fat diet (DIO Rodent Purified Diet with 45% Energy from Fat, 58V8, TestDiet). 3. HFD+lycopene: $n = 10$, mice given a high fat diet supplemented with lycopene and (4) HFD+TP: $n = 10$ given a high fat diet supplemented with tomato powder for 12 weeks. Purified lycopene (Oversal, 10% lycopene beadlets, Naturex, Avignon, France) and TP (Tomato 404, Naturex, Avignon, France; lycopene concentration: 214 mg/kg of dry tomato powder) were generously provided by NATUREX. The dose used for lycopene was 10 mg/kg food per day. The quantity of TP was calculated to provide the same amount of lycopene (10 mg/kg food/day). For all protocols, diets were made once a week, blended to ensure adequate distribution of the supplement and stored in opaque bottles at 4°C. During the experiment, animal growth was monitored via weekly weighing and dietary intake was assessed daily. At the end of treatment period, mice were fasted overnight and blood was collected by retro-orbital puncture under anesthesia. After sacrificing the animals, liver, muscle, epididymal, and perirenal adipose depots were collected then weighted and snap frozen in liquid nitrogen and stored at –80°C until use.

2.2 Biochemical analysis

Blood samples were obtained after 12 hours of overnight fasting, then centrifuged (3500 rpm, for 15 min, 4°C), serum total cholesterol (TC), triglyceride (TG), and glucose were determined by enzymatic methods (Spinreact, Esteve De Bas, Spain), nonesterified fatty acids (NEFA) were measured by colorimetric method (RANDOX, Crumlin, Co. Antrim, UK). Insulin was measured using an ELISA (ALPCO Diagnostics, New Hamshire, USA) and 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) known as an oxidative stress marker was quantified using the ELISA kit (ENZO Life Sciences, Farmingdale, USA) according to the manufacturer's instructions. The HOMA-IR index was calculated according to the following formula: fasting insulin (microU/L) × fasting glucose (nmol/L)/22.5.

2.3 Histological analysis

Liver and epididymal adipose 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) [21] the images were captured by a light microscope (EZAD, Leica, Germany). Steatosis was quantified as a grading system based on the percentage of liver section that is occupied by fat vacuoles. The grading system is defined as follows: grade 0 < 6%; grade 1 = 6–33%; grade 2 = 33–66%, and grade 3 > 66% as reported [22]. The numbers of lipid droplet in liver and adipocyte area (μm^2) were determined using (Image J) software.

2.4 RNA isolation and qPCR

Total cellular RNA from liver and epididymal adipose tissue was extracted using TRIzol reagent according to the manufacturer's instructions. The cDNA was synthesized from 1 μg of total RNA in 20 μL using random primers and Moloney murine leukemia virus reverse transcriptase. Real-Time Quantitative RT-PCR analyses were performed using the Mx3005P Real-Time PCR System (Stratagene, La Jolla, CA) as previously described [23]. For each condition, expression was quantified in duplicate, and 18S rRNA was used as the endogenous control in the comparative cycle threshold (CT) method [24].

2.5 Cytokines and chemokines quantification in adipose tissue

Tissue homogenates were prepared from 25 mg of epididymal adipose tissue homogenized in 250 μL of buffer (KH_2PO_4, 100 mM, NaH_2PO_4, 50 mM, pH 7). Protein degradation is inhibited by adding protease inhibitors (Complete cocktail, Roche, Basel, Swiss) to the buffer (9:1, V:V). After centrifugation at 13 000 g at 4°C for 30 minutes the aqueous phase was separated from the pellet and the lipid phase. The secretion of CCL2, CCL5, TNFα, and IL-6 were quantified in the aqueous phase by ELISA, using Ready-SET-Go mouse kits (TNFα and IL-6 eBiosciences SAS, Paris, France) and (CCL2, CCL5 DuoSet ELISA, R&D systems, Paris, France).

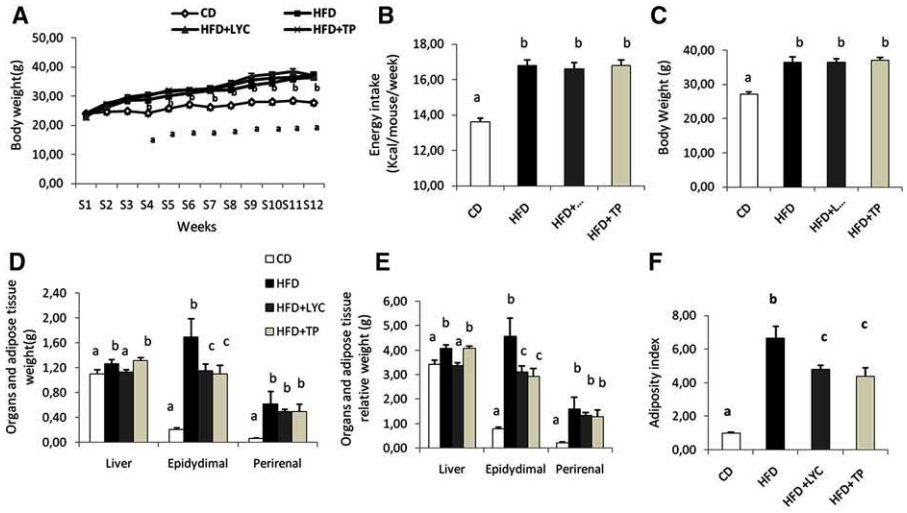

Figure 1. Effects of lycopene and TP on body composition. (A) Body weight evolution curve. (B) The energy intake was quantified by measuring food intake every day for a period of 12 week. (C) Before each sacrifice, animal weight was established. (D) During the sacrifice, organs, and adipose tissues were weighed. (E) Relative organs weight (organ weight/final body weight). (F) An adiposity index was calculated by calculating the ratio between total fat mass and body weight of animal. Values are presented as mean ± SEM. Values not sharing the same letter are significantly different < 0.05.

2.6 NF-$_K$B activation

To examine the involvement of NF-$_K$B signaling pathway the levels of p65 (Ser) and $I_K B\alpha$ (Ser32/36) phosphorylation were quantified in adipose tissue homogenates using the ELISA Instant One Kit according to the manufacturer's instructions (eBiosciences SAS, Paris, France), as previously described [25]

3 Statistical analysis

Data are expressed as the mean ± SEM. Significant differences between control and treated groups were determined using ANOVA, followed by the PLSD Fischer post hoc test using Statview software. Values of p <0.05 were considered significant.

4 Results

4.1 Lycopene or tomato powder supplementation prevents alterations in body composition mediated by HFD and improves metabolic parameters

To evaluate the phenotypic effect of lycopene or TP supplementation, C57BL/6J mice were fed a CD, HFD, supplemented with lycopene or TP for 12 weeks. As shown in Fig. 1A, mice consuming HFD have significant progressive body weight increase, compared to mice in the CD group ($p < 0.05$), and supplementation with lycopene or TP has no effect on the mice body mass compared to HFD fed mice. It is noteworthy that energy intake in HFD fed mice was higher compared to CD fed mice where no significant difference of energy intake was observed between HFD fed mice and HFD supplemented with lycopene or TP (Fig. 1B). Our results also showed that HFD consumption significantly increased liver, epididymal, and peri-renal adipose tissue (both absolute and relative mass) but had no effect on peri-renal adipose tissue weight (Fig. 1C). Compared to HFD group, the supplementation with lycopene decreased significantly the weight and relative weight of liver, epididymal adipose tissue, whereas the supplementation with TP decreased significantly the weight and relative weight of epididymal adipose tissue ($p < 0.05$) and has no effect on liver and peri-renal adipose tissue (Fig. 1D and E). The calculated adiposity index (sum of epididymal, peri-renal adipose tissue mass relative to total body mass) increased by 85% in the HFD group compared to the CD group, and it was significantly reduced by supplementation with lycopene or TP (27% and 34%, respectively; Fig. 1F).

In order to study more deeply the effect of the different diets, several biochemical, and antioxidant parameters were measured. As reported in Table 1, HFD fed mice displayed an increase in serum of total cholesterol (TC), triglycerides (TG), NEFA, and 8-iso-PGF2α concentrations in blood (23, 14, 45, and 54%, respectively) compared to CD fed mice. Supplementation with lycopene or TP strongly decreased the TG concentration ($p < 0.01$ and $p < 0.05$, respectively) as well as 8-iso-PGF2α concentration ($p < 0.01$ and $p < 0.001$, respectively) but had no effect on TC levels. The NEFA concentrations were significantly decreased by supplementation with lycopene ($p < 0.01$) and did not change with TP supplementation. We next evaluated the effect of lycopene and TP supplementation on glucose homeostasis by quantifying fasting glycemia and insulinemia. As expected, these parameters were strongly increased in HFD fed mice compared to CD fed mice (35% for glycemia and 26% for insulinemia), and significantly decreased in lycopene ($p < 0.0001$) and TP ($p < 0.01$) supplemented mice. Consequently, HOMA-IR index was increased in HFD fed mice (5.4 ± 0.4) compared to CD fed mice (2.13 ± 0.03) and decreased with lycopene and TP supplemented mice (3.2± 0.2 and 3.4± 0.4, respectively; Table 1).

Table 1. Effect of lycopene and TP on metabolic parameters and glucose homeostasis

	CD	HFD	HFD+LYC	HFD+TP
Total cholesterol (ng/dL)	45.9 ± 2.2^{a}	60.3 ± 2.9^{b}	54.2 ± 4.1^{a,b}	60.8 ± 3.9^{b}
Triglyceride (ng/dL)	79.4 ± 3.9^{a}	93.1 ± 3.2^{b}	81.1 ± 3.4^{a}	83.3 ± 3.0^{a}
Non esterified fatty acids (mmol/L)	0.83 ± 0.05^{a}	1.52 ± 0.32^{b}	0.80 ± 0.12^{a}	1.13 ± 0.08^{b}
8-iso-PGF2α (pg/mL)	231.4 ± 35.8^{a}	510 ± 21.9^{b}	303 ± 20.2^{c}	412 ± 19.4^{d}
Glycemia (mg/dL)	102.9 ± 32^{a}	158.1 ± 56.1^{b}	129 ± 41^{c}	137 ± 43.5^{c}
Insulinemia (ng/mL)	0.37 ± 0.01^{a}	0.50 ± 0.03^{b}	0.14 ± 0.02^{a}	0.42 ± 0.04^{a}
HOMA-IR index	2.13 ± 0.03^{a}	5.4 ± 0.4^{b}	3.2 ± 0.2^{c}	3.4 ± 0.4^{c}

CD: control diet, HFD: high fat diet, HFD + LYC, high fat diet supplemented with lycopene, HFD + TP, high fat diet supplemented with tomato powder. HOMA-IR index was calculated according to the following formula: fasting insulin (microU/L) × fasting glucose (nmol/L)/22.5. Values are presented as mean ± SEM. Values not sharing the same letter are significantly different, $p < 0.05$.

4.2 Lycopene or tomato powder supplementation improves hepatic steatosis and pro-inflammatory genes expression

The effects of lycopene and TP supplementation on hepatic steatosis in C57BL/6J mice were examined at histological level by (H&E) staining. Our results showed that HFD fed mice exhibited hepatic steatosis grade 2 compared to CD fed mice (Fig. 2A). Lycopene or TP supplementation effectively ameliorated the hepatic steatosis from grade 2 to grade 1 (number of lipid droplet: 6–33%) in a significant way ($p < 0.001$) (Fig. 2B). To examine the effect of lycopene or TP supplementation on hepatic lipid metabolism, hepatic genes expression analysis was undertaken (Fig. 2C). Genes coding for enzymes (acetyl-CoA carboxylase 1 (Acaca), fatty acid synthase (Fasn), and the transcriptional regulator (sterol-regulatory element binding protein 1c (Srebp-1c)) involved in hepatic de novo lipogenesis were quantified. The mRNA levels of Acaca, Fasn, and Srebp-1c were significantly increased in HFD fed mice, whereas the supplementation with lycopene or TP led to a significant decrease in the expression of Acaca, Fasn, and Srebp-1c. Furthermore, the expression of genes involved in the oxidation of fatty acids (Carnitine palmitoyl transferase (Cpt-1), Acetyl CoA oxidase (Acox), and the transcriptional regulator peroxisome proliferator activated protein α (Ppara) was quantified. Cpt-1, Acox, and Ppara mRNA levels were upregulated in HFD-fed animals as compared with CD, and were further upregulated by supplementation with lycopene and TP (Fig. 2C). To evaluate the effect of lycopene and TP supplementation on inflammation of liver, we analyzed the mRNA expression of different markers of inflammation such as tumoral necrosis factor alpha (Tnfa), monocyte chemoattractant protein 1 (Mcp-1), and Il-6. As expected, HFD induced a strong increase of Tnfa and Mcp-1 mRNA levels

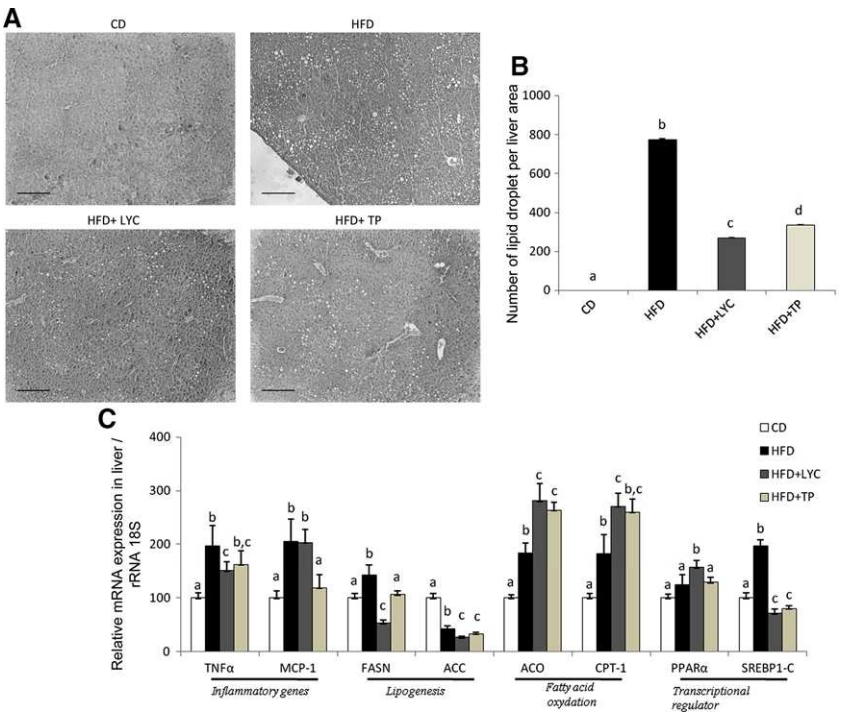

Figure 2. Effects of lycopene and TP supplementation on different parameters in liver. (A) Representative histological images of liver tissue after H&E stained, taken at 10X magnification (scale bar represent 200 μm). CD: control diet (grade 0 of steatosis), HFD: high fat diet (grade 2 of steatosis), HFD + LYC, high fat diet supplemented with lycopene (grade 1 of steatosis), HFD + TP, high fat diet supplemented with tomato powder (grade 1 of steatosis). (B) Number of accumulated lipid droplets per liver area determined by Image J software. (C) Relative expression of mRNA inflammatory genes and genes related to lipid metabolism measured through qPCR and expressed relative to 18S ribosomal RNA. Values are presented as mean ± SEM. Values not sharing the same letter are significantly different, $p < 0.05$.

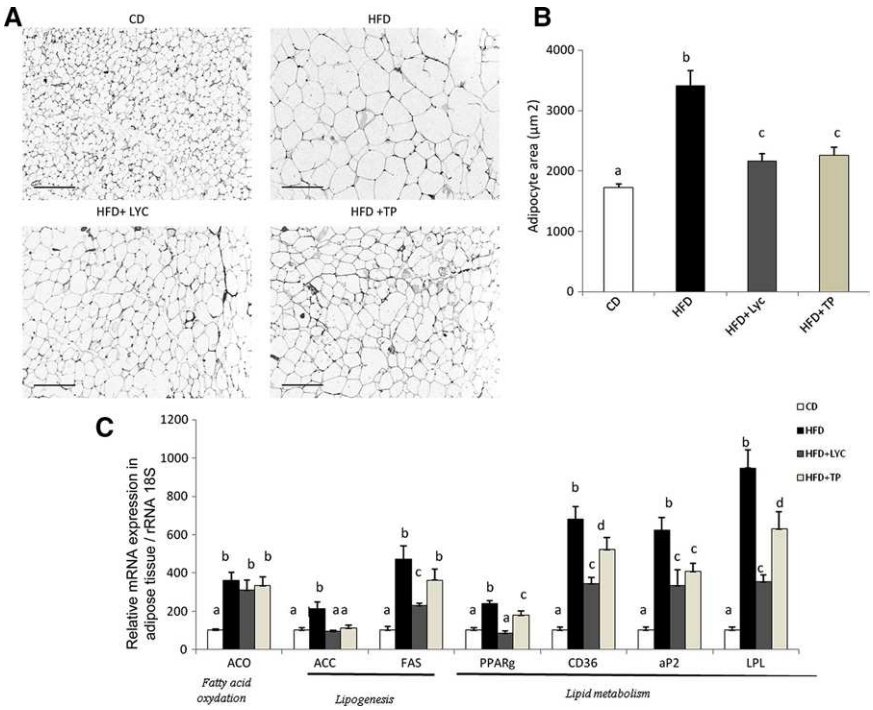

Figure 3. Effects of lycopene and TP supplementation in mice epididymal adipose tissue. (A) Representative histological images of epididymal fat pads H&E stained, taken at 10X magnification (scale bar represents 200 µm). CD: control diet, HFD: high fat diet, HFD + LYC: high fat diet supplemented with lycopene, HFD + TP: high fat diet supplemented with tomato powder. (B) Adipocyte area, determined using Image J software. (C) Relative expression of mRNA inflammatory genes and genes related to lipid metabolism measured through qPCR and expressed relative to 18S ribosomal RNA. Values are presented as mean ± SEM. Values not sharing the same letter are significantly different <0.05.

(50 and 51%, respectively) compared to the CD. In addition, supplementation with lycopene decreased significantly the expression of Tnfa ($p < 0.002$) but had no effect on Mcp-1 mRNA expression, whereas TP supplementation decreased significantly mRNA levels of Mcp-1 ($p < 0.05$) but had no effect on Tnfa expression (Fig. 2C). No impact of lycopene or TP supplementation was observed on Il-6 expression (data not shown).

4.3 Lycopene or tomato powder supplementation modifies lipid metabolism and decreases HFD induced proinflammatory cytokine and chemokine expression in adipose tissue

To evaluate the effect of lycopene or TP supplementation on the phenotype of adipose tissue, epidydimal adipose tissue was used for histological analysis. HFD promoted adipocyte hypertrophy with a significant increase of adipocyte area (Fig. 3A and B) compared to CD group. Interestingly, this effect was prevented by lycopene or TP supplementation (Fig. 3A and B). Furthermore, Acaca and Fasn mRNA levels were increased in HFD-fed group (52 and 78%, respectively) as compared with CD group (Fig. 3C). Lycopene supplementation significantly prevented these upregulations, however only TP improved significantly the Acaca increase but had no effect on FAS mRNA expression. The effects of lycopene or TP supplementation was also evaluated by measuring the mRNA level of peroxisome proliferator-activated receptor-γ (Pparg), a major transcription factor involved in lipid metabolism. Our results indicated that the expression of Pparg was significantly increased by 85% in mice receiving HFD relative to the CD group while lycopene or TP supplementation led to a significant decrease of Pparg mRNA levels (Fig. 3C). The expression of several Pparg target genes were evaluated, including cluster of differentiation (Cd36), adipocyte fatty acid-binding protein (aP2), and lipoprotein lipase (Lpl). Their expression was significantly induced by HFD (85, 83, and 89%, respectively) and reduced by lycopene or TP supplementation. Expression of Acox was significantly increased in HFD fed group but was not modified by lycopene or TP supplementation (Fig. 3C)

To evaluate the effect of lycopene and TP on inflammation in adipose tissue, the expression of different markers of inflammation such as cytokines (Il-6, Tnfα, Mcp-1), chemokines (Chemokine C-C motif ligand 5 (Ccl5), chemokine C-X-C ligand motif 10 (Cxcl10)) acute-phase proteins (haptoglobin, serum amyloid A 3(Saa3)), adipokines (resistin, visfatin, leptin), and matrix metalloproteinases 3 (Mmp3) and 9 (Mmp9) was measured. As expected, the HFD induced a strong increase of Il-6, Tnfα, Mcp1, Ccl5, Cxcl10, Saa3, leptin, haptoglobin, resistin, visfatin, Mmp3, and Mmp9 mRNA compared to the CD (Table 2). Interestingly, lycopene or TP supplementation decreased the expression of most inflammatory markers, however, the expression of Tnfα and Cxcl10 was not affected (Table 2). The expression of Ccl5 decreased significantly with TP supplementation but was not modified with lycopene. In contrast mRNA levels of visfatin decreased strongly with lycopene supplementation and was not modified by TP.

The expression of anti-inflammatory genes such as Il-10 and transforming growth factor β (Tgf-β) was also evaluated. Interestingly, lycopene and TP supplementation strongly

Table 2. Relative values of mRNA expression in the epididymal adipose tissue

	CD	HFD	HFD+ LYC	HFD + TP
IL-6	100 ± 18[a]	394 ± 50[b]	244 ± 35[c]	185 ± 22[d]
TNFα	100 ± 28[a]	344 ± 46[b]	341 ± 44[b]	313 ± 30[b]
MCP-1	100 ± 17[a]	810 ± 11[b]	656 ± 75[c]	582 ± 83[c]
CCL5	100 ± 30[a]	530 ± 64[b]	441 ± 57[b,c]	382 ± 74[c]
CXCL10	100 ± 20[a]	210 ± 42[b]	176 ± 21[b]	171 ± 37[b]
MMP3	100 ± 19[a]	685 ± 88[b]	498 ± 118[c]	607 ± 94[c]
MMP9	100 ± 12[a]	185 ± 40[b]	84 ± 10[c]	107 ± 18[c]
SAA3	100 ± 8[a]	22 369 ± 9943[b]	7449 ± 2497[c]	8258 ± 2356[c]
Leptin	100 ± 23[a]	3175 ± 494[b]	1765 ± 194[c]	1628 ± 336[b]
Haptoglobin	100 ± 17[a]	1521 ± 316[b]	594 ± 85[c]	921 ± 174[d]
Resistin	100 ± 20[a]	1273 ± 256[b]	363 ± 79[c]	668 ± 119[d]
Visfatin	100 ± 19[a]	213 ± 38[b]	147 ± 14[c]	203 ± 28[b]
IL-10	100 ± 24[a]	83 ± 9[b]	234 ± 29[c]	378 ± 67[d]
TGFβ	100 ± 25[a]	219 ± 22[b]	501 ± 62[c]	302 ± 43[d]

CD, control diet; HFD, high fat diet; HFD + LYC, high fat diet supplemented with lycopene; HFD + TP, high fat diet supplemented with tomato powder. Values are presented as mean ± SEM. Values not sharing the same letter are significantly different, $p < 0.05$.

increased the expression of Il-10 (64 and 78%, respectively) and Tgf-β (56 and 30%), respectively (Table 2).

To confirm the anti-inflammatory effect of lycopene or TP supplementation at the protein levels, epidydimal adipose tissue homogenates were prepared to quantify proinflammatory cytokines. An increase of IL-6, TNFα, CCL2, and CCL5 protein levels was observed in the tissue homogenates from HFD mice compared to CD. As reported at the mRNA level, lycopene or TP supplementation decreased the amount of TNFα, IL-6, CCL2, and CCL5 proteins in adipose tissue ($p < 0.001$) (Fig. 4C, D, E, F).

4.4 Lycopene and tomato powder limit NF-κB activation in epididymal adipose tissue

NF-κB is a well-known transcription factor involved in the regulation of proinflammatory cytokines and chemokines [26]. Thus, we examined the involvement of NF-κB signaling pathway in our adipose tissue inflammatory model and the ability of lycopene and TP to modulate them. To this purpose, the phosphorylation of p65 and I_KB were quantified through ELISA. Expectedly, the phosphorylation levels of p65 and IκB were significantly increased with HFD compared to CD whereas HFD + lycopene and HFD + TP strongly limited the phosphorylation of p65 and IκB, suggesting that lycopene and TP reduced NF-κB activation in epididymal adipose tissue (Fig. 4A and B).

5 Discussion

In the present study, we used a model of obesity induced by a HFD to examine the metabolic effect of lycopene or TP supplementation. In addition, we aimed at comparing the effect of purified lycopene versus TP, which contained lycopene included in a natural matrix (TP) on metabolic parameters. As expected, the HFD diet led to a significant increase in the total mass and adiposity index of mice, modified lipidic, and glucidic serum parameters, as well as cell morphology and gene expression in adipose tissue and in the liver. Interestingly, lycopene or TP supplementation improved most of these phenotypical perturbations except total body weight, which was not modified. Similar lack of effect of lycopene supplementation on body weight has already been reported in rats [13], contrarily to the recently reported data in mice [12]. The origin of this discrepancy is presently unresolved and could be due to species or to genetic discrepancies between animal models used.

Even if total body mass was not affected by lycopene or TP supplementation, HFD-mediated adiposity was clearly improved, as reported by Singh et al. [12], as well as several related paramaters including lipidic (TG and NEFA) and glucidic parameters (glycemia, insulinemia, and HOMA-IR). Most of these results are in agreement with the literature that suggests a role of lycopene or tomato product on various biochemical parameters such as lipemia [27], or glucose homeostasis [28]. In addition, we also reported a decrease of circulating 8-iso-PGF 2α, a well-established marker of oxidative stress, which is in agreement with the antioxidant effect of lycopene or TP supplementation [29] and the subsequent protective effect against cell oxidative damages associated to obesity [30].

We next investigated the effect of lycopene or TP supplementation on hepatosteatosis and lipid metabolism in liver. Hepatosteatosis is defined as the presence of diffuse infiltration in the liver and is characterized by ballooning hepatocytes injury and inflammation in the hepatocytes [31, 32]. Our results showed that supplementation with lycopene or TP for 12 weeks ameliorated the hepatic steatosis induced by HFD, which is consistent with several studies that demonstrated the amelioration of hepatosteatosis under lycopene

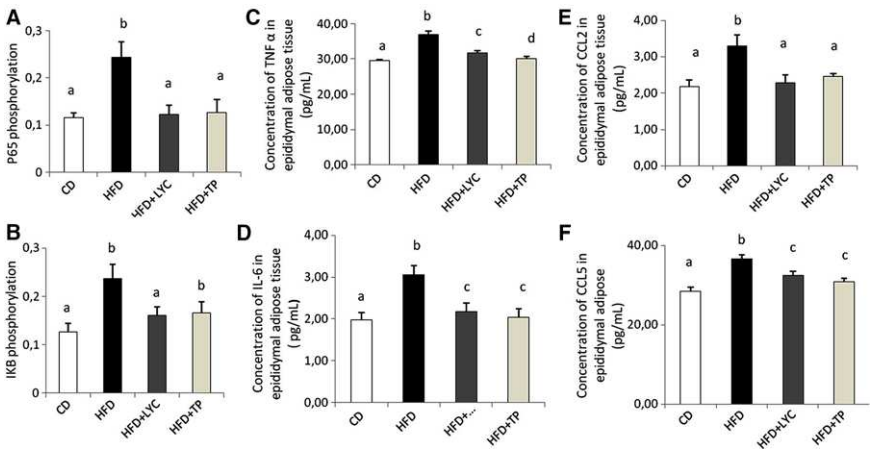

Figure 4. Effects of LYC and TP on the phosphorylation levels of the NF-κB subunits (p65 and IκB) evaluated using ELISA. CCL2, CCL5, TNFα, and IL-6 secretion in epididymal adipose tissue was quantified using ELISA. Values are presented as mean ± SEM. Values not sharing the same letter are significantly different, $p < 0.05$.

or tomato products effect [14, 15, 33–37]. The protective effects of lycopene and TP in hepatosteatosis may be related to multiple mechanisms [15, 34], including anti-oxidant/anti-inflammatory effects [38, 39] and lipid metabolism modulation [34, 40–42]. In agreement, we reported an anti-inflammatory effect of lycopene or TP in the liver but also an impact on lipid metabolism. Based on our gene expression analysis, we postulated that lycopene or TP supplementation affected both lipogenesis and β-oxidation. Indeed, a strong downregulation of the Srebp-1c expression was observed under lycopene or TP supplementation, as well as a reduction of Fasn expression compared to HFD condition. SREBP-1c is a membrane-bound transcription factor that regulates key genes involved in the regulation of lipid metabolism [43]. The role of SREBP-1c in lipogenesis has been demonstrated in mice overexpressing the active form of this factor in the liver. These mice developed massive hepatic steatosis due to activation the expression of the lipogenic genes [44], including Fasn gene that encode for a multienzyme that catalyses fatty acid synthesis [45]. In addition, lycopene or TP supplementation upregulated genes involved in fatty acids (FA) β-oxidation. Indeed, Cpt-1 and Acox were induced as well as Ppara. It is noteworthy that these three genes encode for major actors of the beta-oxidation pathway [43]. PPARα is considered as the master regulator of hepatic FA oxidation [46], CPT-1 facilitates FA translocation into mitochondria [43] and ACOX catalyses the peroxisomal FA oxidation [47]. Altogether, our results support the idea that hepatosteatosis was reduced in our condition via a limitation of lipogenesis associated to an improvement of FA β-oxidation resulting in a limitation of lipid accumulation in the liver as suggested by histological data. Similar results have already been reported with apo-10-lycopenoic acid, a lycopene metabolite that counteracts the hepatic steatosis elicited by a HFD in C57BL/6J mice via a Sirt1-mediated suppression lipogenesis and stimulation of lipid catabolism [34].

We investigated the effect of lycopene or TP supplementation on epididymal adipose tissue. First, we observed that the HFD-mediated adipocyte hypertrophia (quantified from histological analysis) was prevented by supplementation with lycopene or TP. Concerning the effect of lycopene, this observation is in agreement with Singh et al. who demonstrated that oral supplementation with lycopene prevents adipocyte hypertrophy in mice [12]. In addition, we demonstrated that the impact on adipose tissue cellularity was associated with modifications of expression of genes coding for protein involved in lipogenesis and lipid metabolism. Indeed, lycopene or TP supplementation reduced the Fasn and Acaca compared to HFD condition. These supplementations also reduced Pparg mRNA expression as well as many target genes of PPARγ such as aP2, Cd36, Lpl. Interestingly, since PPARγ is considered as the master regulator of adipogenesis, its reduced expression and the downregulation of its target genes likely explain the reduced adiposity of mice upon lycopene or TP supplementation.

We also investigated the impact of lycopene and TP supplementation on adipose tissue inflammatory status, and we reported an overall downregulation of genes encoding proinflammatory markers such as cytokines (Il-6, Tnfα), adipokines (resistin, visfatin, leptin), acute phase proteins (Saa3, haptoglobin), and chemokines (Ccl5, Mcp-1, Cxcl10), a downregulation of genes encoding metalloproteinases (Mmp3 and Mmp9), and an upregulation of genes encoding anti-inflammatory proteins such as Il10 and Tgf-β. In addition, we confirmed at protein level that some of these proinflammatory markers (IL-6, TNFα, MCP-1, CCL5) were reduced compared to HFD conditions. Most of these genes are deeply involved in the genesis of obesity-associated pathologies, such as insulin resistance and type II diabetes. Indeed, IL-6 and TNFα are major obesity-related proinflammatory cytokines [48, 49], and chemokines (MCP-1, CCL5, or CXCL10) induce macrophage infiltration, which largely participates in the low-grade inflammation of adipose tissue [2, 50–52]. Anti-inflammatory cytokines are largely upregulated in adipose tissue during obesity to compensate the massive production of proinflammatory mediators [53]. Acute phase proteins (SAA3 and haptoglobin) are also strongly related to metabolic inflammation [54]. Adipokines (leptin, resistin, and visfatin) are known to be deregulated

in obesity and have systemic impact on insulin sensitivity [55] and metalloproteinases participate to adipose tissue remodeling observed during obesity [56]. Altogether, the regulations depicted in the present article are clearly in favor of an improvement of inflammatory tone in adipose tissue and an improvement of adipose tissue function under lycopene or TP supplementation. Our results are fully consistent with previously reported anti-inflammatory effects of lycopene on adipose tissue, preadipocytes and adipocytes [10–13]. Interestingly an anti-inflammatory effect of TP on adipose tissue has never been reported yet, but similar results, i.e. decrease of proinflammatory adipokines, have already been reported after consumption of tomato juice in healthy women [57].

To identify the molecular mechanisms involved in this reduction of the inflammatory tone in adipose tissue, we evaluated the effect of lycopene and TP on the phosphorylation of p65 and IκB, two actors of the NF-κB signaling pathway [1, 58]. We observed that lycopene and TP strongly reduced the phosphorylation levels of p65 and IκB. These results are highly consistent with several other studies suggesting the potent effect of lycopene on inhibition of NF-κB signaling through multiple mechanisms [59, 60], and suggest that the anti-inflammatory effect of lycopene and TP on adipose tissue results from their ability to inhibit NF-κB signaling in adipose tissue. Despite this clear impact of lycopene or TP on NF-κB signaling pathway, we cannot exclude that this effect is strictly due to the reduced adiposity, which could result in a deactivation of this signaling pathway. However, it is noteworthy that we already reported the per se anti-inflammatory effects of lycopene in adipocytes, preadipocytes [10], and macrophages [11], suggesting that both direct and indirect effects are probably responsible of the overall anti-inflammatory effect reported.

In the present study, similar effects were observed for lycopene and TP supplementation regarding improvement of metabolic parameters. These observations are noteworthy since in the literature the effects of tomato products (extract or powder) are globally more efficient to drive health effects at both epidemiological and preclinical level. This has notably been highlighted in the field of cancer [61], cardiovascular diseases [62], or hepatosteatosis [22]. Indeed, Stice et al. recently reported the superior protective effects of TP when compared to partial tomato extracts, and purified lycopene against alcohol-induced hepatic injury in rats, where only TP reduced severity of alcohol-induced disorders [22]. However, the superior effect of tomato product is not the absolute rule since the effect of lycopene is sometime higher, notably in the management of the blood pressure [62].

As a conclusion, we show for the first time that TP supplementation was able to improve obesity-associated disorders, similarly to the lycopene effect that has already been depicted. Since similar amounts of lycopene were brought by TP and lycopene supplementation and resulted to similar effects, it strongly suggests that the TP supplementation effects were probably related to lycopene content. However, we cannot exclude that other components naturally found in tomatoes (α- and β-carotene, lutein, zeaxanthin, phytoene, phytofluene, ascorbic acid, folate, α-tocopherol, quercetin, and other polyphenols) could be responsible via isolated, additive, or synergic effects. Altogether, our data clearly highlight the potential beneficial effect of TP as an effective whole food intervention against obesity associated comorbidities.

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H.H. and J.F.L. designed the experiments. S.F., J.A., L.B., E.K., and F.T. performed the experiments and analyzed the results. S.F. and J.F.L. wrote the manuscript.

No conflict of interest has to be disclosed.

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