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Effets des métabolites d'acides gras sur la formation et la fonction des adipocytes thermogéniques

Cécilia Colson

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THÈSE DE DOCTORAT

Effets des métabolites d'acides gras sur la
formation et la fonction des adipocytes
thermogéniques

Cécilia COLSON

Institut de Biologie Valrose – CNRS UMR7277 Inserm U1091

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Dirigée par : Dr. Ez-Zoubir AMRI

Soutenue le : Mardi 24 Novembre 2020

Devant le jury, composé de :

Pr. Isabelle NIOT, PU, AgroSup Dijon

Pr. Bruno FEVE, PUPH, Paris Saint Antoine

Dr. Isabelle DUGAIL, DR, Paris Sorbonne

Dr. Patricia LEBRUN D'AMBROSIO, MCU, IPMC

Dr. Ez-Zoubir AMRI, DR, iBV

Effets des métabolites d'acides gras sur la formation et la fonction des adipocytes thermogéniques

Jury :

Présidente du jury:

Pr. Isabelle NIOT, Professeur d'Université, AgroSup Université Franche-Comté Bourgogne, DIJON

Rapporteurs :

Dr. Isabelle DUGAIL, Directrice de Recherche, Sorbonne Université, PARIS

Pr. Bruno FEVE, Professeur d'Université Praticien Hospitalier, Centre de Recherche Saint Antoine, PARIS

Examinatrice :

Dr. Patricia LEBRUN D'AMBROSIO, Maître de Conférence Universitaire, IPMC, SOPHIA ANTIPOLIS

Directeur de thèse :

Dr. Ez-Zoubir AMRI, Directeur de Recherche, institut de Biologie Valrose, NICE

Effets des métabolites d'acides gras sur la formation et la fonction des adipocytes thermogéniques

L'obésité est devenue épidémique et est associée à de sérieuses comorbidités comme le diabète de type 2, les problèmes cardio-vasculaires et certains cancers. A ce jour, il n'existe pas de traitement pharmacologique efficace contre l'obésité sévère. Seule la chirurgie bariatrique est à même d'entraîner une rapide perte de poids et améliorer la survie des obèses morbides. L'obésité est due à un développement excessif du tissu adipeux blanc (WAT). Le tissu adipeux peut également être de couleur brune (tissu adipeux brun, BAT), responsable de la thermogenèse et qui participe ainsi à la dépense énergétique. Cependant, les capacités du BAT sont altérées chez les individus obèses. Une perspective de lutte contre l'obésité serait alors de développer des stratégies pour favoriser la formation et augmenter l'activité de ce tissu adipeux thermogénique. Les acides gras alimentaires sont stockés dans les adipocytes sous forme de triglycérides et participent au métabolisme. Ils sont également les substrats de la thermogenèse. De plus en plus d'études, dont certaines proviennent de notre laboratoire, mettent en évidence que les acides gras polyinsaturés (AGPI) et leurs métabolites sont impliqués dans la régulation de la thermogenèse.

Mes travaux de thèse concernent l'identification des métabolites d'acides gras impliqués dans la formation et l'activité d'adipocytes thermogéniques. Dans un premier temps, nous avons mis en évidence l'importance de l'équilibre entre les AGPI $\omega 6$ et $\omega 3$ dans l'alimentation. Nous avons montré que l'inhibition de la thermogenèse par les AGPI $\omega 6$ était principalement causée par la présence de l'acide arachidonique (ARA) et d'un de ses métabolites, la Prostaglandine F 2α (PGF 2α). En étudiant cette fois des biopsies de tissus adipeux humains, j'ai identifié des candidats potentiels que j'ai validés *in vitro* sur notre modèle adipocytaire, les cellules hMADS. Mes résultats mettent en évidence l'implication d'un autre métabolite de l'ARA, dans la régulation négative de la formation des adipocytes thermogéniques : la Thromboxane A 2 (TxA 2). La TxA 2 inhibe l'expression du marqueur essentiel de l'adipogenèse qui est l'Uncoupling protein 1 (UCP1) alors que la prostacycline, un autre métabolite de l'ARA, induit l'expression d'UCP1. Par la suite, j'ai comparé les profils de métabolites retrouvés chez l'Homme et chez la souris exposée à différentes températures. Nos résultats montrent que les profils de métabolites ne permettent pas de distinguer le BAT et WAT chez l'Homme contrairement à la souris. Ces résultats confirment les hypothèses énoncées dans la littérature qui suggèrent que le BAT humain est un tissu mixte d'adipocytes thermogéniques et non thermogéniques. Dans un second temps, j'ai identifié et caractérisé un antagoniste des marqueurs clés de l'adipogenèse et de la thermogenèse *Peroxisome Proliferator-activated receptor α* (PPAR α) et PPAR γ : l'acide carnosique, extrait du romarin et utilisé dans la médecine traditionnelle. De plus, mes travaux m'ont permis de montrer l'importance de 2 métabolites, 9- et 13-hydroxyoctadécadiénoïques (9-/13-HODEs), dans le maintien du phénotype anti-inflammatoire des macrophages résidents du tissu adipeux.

Mes résultats de thèse ont donc mis en évidence de nouveaux métabolites jouant un rôle dans la régulation de la thermogenèse. D'autres études sont néanmoins nécessaires pour déterminer leurs mécanismes d'action. De plus, l'identification d'un nouvel antagoniste de PPAR α et PPAR γ issu d'une plante permet d'entrevoir de nouvelles stratégies thérapeutiques chez des patients souffrants d'une sur-activation du métabolisme comme chez les grands brûlés et les patients ayant développé une cachexie induite par le cancer.

Mots clés : Obésité, Tissu adipeux, Thermogenèse, PPAR γ , Acides gras, Oxylipines

Effects of fatty acids metabolites on development and function of thermogenic adipocytes

Obesity has become epidemic and is associated with serious comorbidities such as type 2 diabetes, cardiovascular diseases and certain cancers. So far, there is no effective pharmacological treatment for severe obesity except bariatric surgery which may lead to rapid weight loss and improve survival in morbidly obese patients. Obesity is caused by an excessive development of white adipose tissue (WAT). Adipose tissue can also be brown (brown adipose tissue, BAT), responsible for thermogenesis and thus participating in energy expenditure. However, the capabilities of BAT are impaired in obese individuals. One perspective in the fight against obesity would be to develop strategies to promote the formation and the activity of this thermogenic adipose tissue. Dietary fatty acids are stored in adipocytes in the form of triglycerides and participate in metabolism and are also the substrates of thermogenesis. Several studies, some of which came from our laboratory, show that polyunsaturated fatty acids (PUFAs) and their metabolites are involved in the regulation of thermogenesis.

My Ph.D. thesis work concerns the identification of fatty acid metabolites involved in the formation and function of thermogenic adipocytes. First, we highlighted the importance of the balance between $\omega 6$ and $\omega 3$ PUFAs in the diet. We have shown that the inhibition of thermogenesis by $\omega 6$ PUFAs is mainly due to the presence of arachidonic acid (ARA) and one of its metabolites, prostaglandin F 2α (PGF 2α). In human adipose tissue biopsies, I identified potential candidates that I validated in vitro on our adipocyte model, hMADS cells. My results highlight the involvement of another ARA-derived metabolite in the downregulation of thermogenic adipocyte formation: Thromboxane A 2 (TxA 2). TxA 2 inhibits the expression of the main marker of adipogenesis, Uncoupling protein 1 (UCP1), while prostacyclin, another ARA-derived metabolite, induces its expression. Subsequently, I compared the metabolite profiles found in humans and in mice exposed to different temperatures. Our results show that the metabolite profiles do not allow to distinguish between BAT and WAT in humans, unlike in mice. These results are in agreement with the reported hypotheses suggesting that human BAT is composed with a mixture of thermogenic and non-thermogenic adipocytes. Second, I identified and characterized an antagonist of the key markers of adipogenesis and thermogenesis Peroxisome Proliferator-activated receptor α (PPAR α) and PPAR γ : carnosic acid, extracted from rosemary and used in traditional medicine. In addition, my work showed the importance of 2 metabolites, 9- and 13-hydroxyoctadecadienoic acid (9- / 13-HODEs), in maintaining the anti-inflammatory phenotype of resident macrophages in adipose tissue.

Therefore, my Ph.D. thesis data revealed new metabolites playing a role in the regulation of thermogenesis. However, further studies are needed to determine their mechanisms of action. Furthermore, the identification of a novel PPAR α and PPAR γ antagonist derived from a plant might allow the development of new therapeutic strategies for patients suffering from metabolism over-activation such as in severely burned patients or with cancer-induced cachexia.

Key words: *Obesity, Adipose tissue, Thermogenesis, PPAR γ , Fatty acids, Oxylipins*

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ABBREVIATIONS

ABDH	<i>Abhydrolase Domain Containing 5</i>
AC	Acide Carnosique
ACS	Acyl coenzyme A (CoA) synthétase
ADP	Adénosine DiPhosphate
ADN	Acide DésoxyriboNucléique
AG	Acide gras
AGPI	Acide gras polyinsaturé
ALA	Acide α -linoléique
AMPc	AMP cyclique
ARA	Acide Arachidonique
ARN	Acide RiboNucléique
ATP	Adénosine TriPhosphate
ATGL	<i>Adipose TriGlyceride Lipase</i>
BAT	<i>Brown Adipose Tissue</i>
BMP8b	<i>Bone Morphogenic Protein 8b</i>
C/EBP	<i>CCAAT/Element Binding Protein</i>
CD36	<i>Cluster of Differentiation 36</i>
CHOP10	<i>C/EBP Homologous Protein 10</i>
COX	Cyclooxygénase
CPT1M	<i>Carnitine Palmitoyltransferase1M</i>
CRE	<i>C/EBP Response Element</i>
CREB	<i>cAMP Response Element Binding</i>
DHA	Acide Docosahexaénoïque
diHOME	Acide dihydroxyoctadécénoïque
Dlk1	Delta Like Non-Canonical Notch Ligand 1
EET	Acide Epoxyeicosatriénoïque
EPA	Acide Eicosapentaénoïque
FABP	<i>Fatty Acid Binding Protein</i>
FAHFA	<i>Fatty Acid Hydroxylated Fatty Acid</i>
FATP	<i>Fatty Acid Transfer Protein</i>
FADH	Flavine Adénine Dinucléotide
FGF	<i>Fibroblast Growth Factor</i>
FTO	<i>Fat mass and Obesity associated gene</i>
GCPR	<i>G-coupled Receptor Protein</i>
HEPE	Acide Hydroxy-eicosapentaénoïque
HETE	Acide Hydroxy-eicosatétraénoïque
HODE	Acide Hydroxy-octadécadiénoïque
hMADS	<i>human Mesenchymal-Adipose Derived Stem cells</i>
HSL	Hormone-Sensitive Lipase
IBMX	3-isobutyl-1-méthylxanthine
IGF1	<i>Insulin-Growth factor 1</i>
IL-6	Interleukine 6
IL-1 β	Interleukine 1 β

IMC	Indice de Masse Corporelle
KLF	<i>Kruppel Like Factor</i>
LA	Acide Linoléique
LAP	<i>Liver Activator Protein</i>
LIP	<i>Liver Inhibitor Protein</i>
LOX	Lipoxygénases
LT	Leucotriènes
MCR	<i>Melano-Cortin Receptor</i>
MCP-1	<i>Monocyte Chemoattractant Protein 1</i>
MGL	<i>MonoGlyceride Lipase</i>
NADH	Nicotinamide adénine dinucléotide
NF- κ B	<i>Nuclear Factor κB</i>
NP	Natriuretic Peptide
PAHSA	<i>Palmitic Acid 9-hydroxy Stearic Acid</i>
PCSK1	<i>Proprotein Convertase Subtilisin/Kexin Type 1</i>
PG	Prostaglandines
PGC1 α	<i>PPARγ Cofactor 1α</i>
PKA	Protéine Kinase A
PLIN	Périlipines
PLTP	<i>PhosphoLipid Transfer Protein</i>
POMC	Proopiomélanocortine
PPAR	<i>Peroxisome Proliferator Associated Receptor</i>
PPRE	<i>PPAR Response Element</i>
PRDM16	<i>PR domain containing 16</i>
SREBP	<i>Sterol regulatory element-binding protein</i>
TASK	<i>TWIK-related acid-sensitive K</i>
TG	Triglycéride
TNF α	<i>Tumor Necrosis Factor α</i>
TX	Thromboxane
UCP1	<i>Uncoupling Protein 1</i>
RXR	<i>Retinoic X Receptor</i>
WAT	<i>White Adipose Tissue</i>

INTRODUCTION GENERALE

Partie I : L'obésité

1. Définition et prévalence

Selon l'Organisation Mondiale de la Santé, l'obésité est une pathologie caractérisée par un excès de masse grasse corporelle. De plus en plus d'études épidémiologiques comparent même l'obésité à une pathologie dont la nourriture serait l'agent pathogène (Bray et al., 2017).

L'état de surpoids et d'obésité se mesure par le calcul de l'Indice de Masse Corporelle (IMC), correspondant au poids en kilogrammes divisé par la taille de l'individu en mètres et au carré. Lorsque l'IMC est supérieur à 25, on considère que l'individu est en surpoids et l'individu est obèse au-delà d'un IMC de 30 (**Figure 1A**). L'obésité est considérée comme morbide au-delà d'un IMC de 40, c'est-à-dire que l'état d'obésité entraîne un risque de décès prématuré. Le calcul de cet indice ne concerne pas les individus sportifs ainsi que les enfants dont la courbe de croissance suit d'autres règles (**Figure 1B et C**).

Selon les études les plus récentes, l'obésité à l'échelle mondiale a pratiquement triplé ces 40 dernières années avec aujourd'hui près de 2 milliards d'adultes en surpoids dont 700 millions d'obèses (The GBD 2015 Obesity Collaborators, 2017)

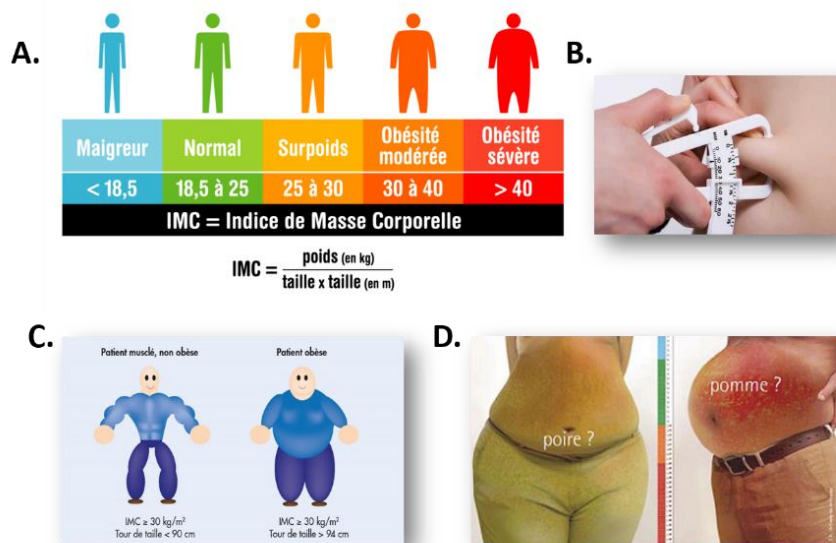


Figure 1 : L'obésité se mesure en calculant l'indice de masse corporelle (IMC) (A). Le taux de masse grasse (B) est une information complémentaire puisque l'IMC n'utilise que la taille et le poids, faussés chez les sportifs par exemple (C). Le type d'obésité est caractérisé de gynoïde ou d'androïde si la masse est répartie selon une forme de poire ou de pomme respectivement (D), la masse grasse abdominale (Androïde, pomme) étant un facteur de risque important pour les maladies métaboliques et cardio-vasculaires.

2. Comorbidités associées

L'obésité représente un réel problème de santé publique car elle entraîne de nombreuses pathologies associées à l'excès de masse grasse, appelées comorbidités (**Figure 2**), et dont l'impact sur la santé publique est important. On considère notamment tous les problèmes métaboliques tels que le diabète de type 2 et la stéatose hépatique non alcoolique (Chadt et al., 2000). L'excès de poids entraîne également de nombreuses pathologies cardio-vasculaires avec la formation de plaques athéro-sclérotiques dans les vaisseaux sanguins et la cause d'accidents vasculaires cérébraux (The GBD 2015 Obesity Collaborators, 2017). Toutes ces comorbidités sont également associées à la présence d'une inflammation chronique dite de bas grade. De très récentes études ont d'ailleurs mis en évidence que cette inflammation chronique de bas grade est associée à d'une inflammation cérébrale hypothalamique (Tran et al., 2016) ainsi que de douleurs articulaires chroniques (Hawker, 2019).

L'obésité est également associée à l'apparition de cancers, les organes concernés étant ceux du métabolisme énergétique tels que le foie, le pancréas, l'estomac, le colon ou encore les organes dits hormono-sensibles comme les seins ou l'utérus. Ces cancers sont liés à la surcharge énergétique, à l'inflammation chronique de bas grade suscitée ou encore à la mauvaise gestion des ressources lipidiques, notamment le cholestérol, à la base de la synthèse des hormones stéroïdiennes (Deng et al., 2016). En outre, le tissu adipeux est le siège d'une forte production

d'estrogènes car il exprime l'enzyme CYP450 aromatasase, responsable de l'aromatation de la testostérone en œstradiol (Siiteri, 1987).

3. Traitement

La plupart des traitements pharmacologiques testés utilisaient des amphétamines anorexigènes, communément connues comme des « coupe-faim » mais entraînaient de sérieux effets secondaires, notamment cardio-vasculaires (Müller et al., 2018). Aujourd'hui, la seule prescription sans danger que les patients en surpoids ou en obésité légère peuvent recevoir est une molécule qui empêche l'absorption intestinale des graisses et connue sous le nom d'Orlistat (Kim et al., 2020; Zhi et al., 1995). Son efficacité reste limitée et son utilisation doit être accompagnée d'un régime hypocalorique modéré. La mise en place d'une meilleure hygiène de vie et d'une diététique adaptée n'est cependant pas suffisante pour les patients dont l'obésité est associée à de graves comorbidités (obésité dite morbide). La chirurgie bariatrique, qui est une intervention consistant à réduire le volume (sleeve gastrectomie) ou la capacité d'absorption de l'estomac (bypass et anneau gastrique), reste actuellement leur seule alternative. Ces types d'interventions chirurgicales montrent de nombreux effets positifs et rapides sur la perte de poids ainsi que l'amélioration des comorbidités, notamment le diabète. Ce sont néanmoins des actes invasifs pouvant entraîner des carences vitaminiques et des désordres psychologiques (Alligier et al., 2020).

4. Etiologie de l'obésité

L'obésité peut avoir plusieurs causes, génétiques monogéniques (exemple de la mutation du récepteur de la leptine) et polysyndromiques (Exemple du syndrome de Prader-Willi) ou médicamenteuses (traitement chronique aux corticoïdes) mais qui restent rares par rapport aux obésités liées au mode de vie, c'est-à-dire avec une alimentation hypercalorique et peu de dépenses énergétiques (**Figure 2**).

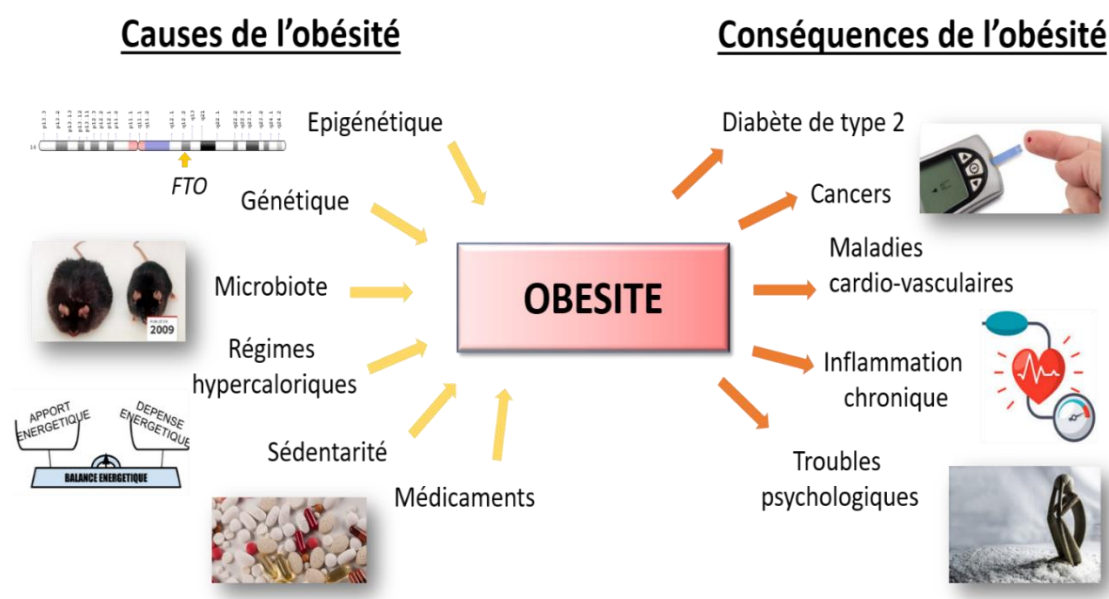


Figure 2: L'obésité est une maladie multifactorielle aux conséquences graves et multiples.

a. L'héritabilité génétique

De nombreuses études sur les jumeaux ont été réalisées afin de déterminer l'importance du patrimoine génétique ainsi que de l'environnement sur l'apparition de l'obésité. Les conclusions de ces études sont contradictoires car les résultats sont souvent hétérogènes, notamment à cause des différences entre les critères d'inclusion, les modes d'évaluation tel que le questionnaire dans le cadre d'études rétrospectives. La plupart de ces études mettent néanmoins en avant que l'héritabilité génétique ne constitue pas un déterminant à l'apparition de l'obésité infantile ou adulte mais plutôt un facteur de risques (Börjeson, 1976; Elks et al., 2012; Silventoinen et al., 2017)

b. Les polymorphismes et l'épigénétique

Des études d'association pangénomique (ou GWAS pour Genome-Wide Association Study), étudiant les corrélations entre des traits phénotypiques et des polymorphismes génétiques, montrent également la présence d'une mutation dans le gène FTO (Fat mass and obesity associated) qui est associée à un risque 1,67 plus élevé d'être obèse avec un poids moyen plus élevé (Frayling et al., 2007; Mehrdad et al., 2020; Scuteri et al., 2007).

Il existe en outre de plus en plus d'études pointant l'implication des modifications épigénétiques dans la régulation du métabolisme énergétique (**Figure 3**). L'épigénétique correspond à un niveau supplémentaire de régulation de l'expression des gènes puisque, sans modifier le gène

lui-même, des modifications des histones et de la compaction de l'ADN vont entraîner des régulations différentes de l'expression du gène selon son accessibilité à la machinerie de transcription et aux différents cofacteurs ou corépresseurs. Les études réalisées à grande échelle montrent l'existence de nombreuses modifications épigénétiques corrélées au volume de tissu adipeux, à la circonférence de la taille caractéristique de l'obésité viscérale ou à l'IMC (Dhana et al., 2018). De nombreux gènes impliqués dans le métabolisme énergétique semblent pouvoir être impactés par les modifications épigénétiques (Ling and Rönn, 2019). De récentes études suggèrent également le rôle de l'alimentation, notamment avec la présence de méthionine et de folate qui se retrouvent être des donneurs de méthyls, et ce sont les groupements méthyls qui modifient les histones et ainsi la régulation de la transcription (Ducker and Rabinowitz, 2017).

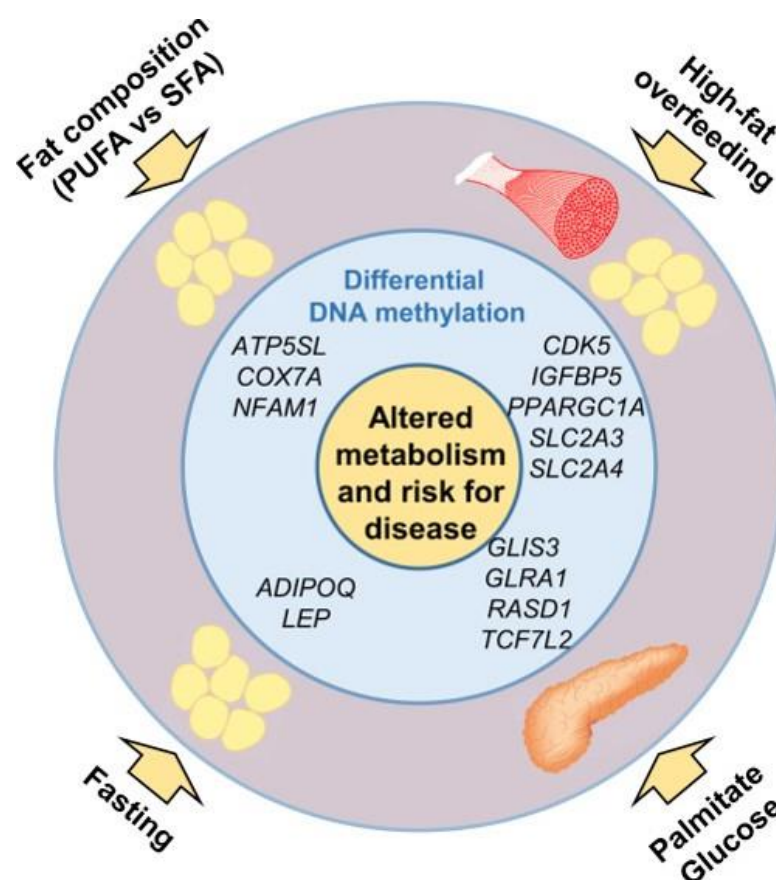


Figure 3: L'alimentation entraîne des modifications épigénétiques des gènes impliqués dans le métabolisme énergétique et dans les différents organes impliqués comme le tissu adipeux, le muscle et le pancréas. Schéma d'après (Ling and Rönn, 2019)

Ces études mettent en évidence l'existence de polymorphismes génétiques augmentant le facteur de risque de développer une obésité.

c. L'obésité monogénique

Il existe néanmoins des obésités dites monogéniques, c'est à dire dues à la mutation d'un seul gène. Chez l'humain, on distingue notamment des mutations dans le gène codant pour une hormone sécrétée par le tissu adipeux et impliquée dans la prise alimentaire et notamment la sensation de satiété, la leptine, ou son récepteur (Farooqi and O'Rahilly, 2014). On peut également citer les altérations de la voie de signalisation de la mélanocortine, au niveau des neurones hypothalamiques POMC, exprimant le récepteur à la leptine et régulant la prise alimentaire. On observe à ce niveau des mutations sur les gènes codants pour les récepteurs à la mélanocortine (MCR3 et MCR4), des mutations du gène codant pour le gène POMC ou encore codant pour l'enzyme de conversion de la prohormone (*prohormone convertase subtilisin/kexin type 1*, PCSK1) (Clément et al., 2020).

La majorité des cas d'obésités provient d'un déséquilibre de la balance énergétique, c'est-à-dire l'équilibre entre l'énergie ingérée, représentée par l'alimentation, et l'énergie dépensée, représentée par l'exercice physique et le métabolisme de base, qui comprend toute l'énergie nécessaire à l'organisme pour maintenir ses fonctions physiologiques. Lorsqu'il y a un défaut de dépenses énergétiques au profit des apports, l'énergie excédentaire est alors stockée dans la masse grasse ou tissu adipeux.

Partie II : Le tissu adipeux

Le tissu adipeux apparaît au 3^{ème} trimestre de gestation chez l'Homme et représente de 15 à 30% chez les femmes et de 10 à 15% chez les hommes de la masse totale du corps à l'âge adulte. Il se décline sous deux couleurs : les tissus adipeux blanc (**WAT** pour *White Adipose Tissue*) et brun (**BAT** pour *Brown Adipose Tissue*).

Le WAT est le tissu majeur du stockage et de libération de l'énergie tandis que le BAT participe à la dépense énergétique en réalisant la thermogénèse, c'est à dire en utilisant les lipides stockés pour produire de la chaleur et maintenir la température corporelle. Le WAT et le BAT sont également des organes sécréteurs d'hormones contribuant à l'homéostasie énergétique. Outre les adipocytes du WAT (adipocytes blancs) et du BAT (adipocytes bruns), il existe l'adipocyte beige ou brite, pour "*brown-in-white*", un adipocyte thermogénique retrouvé au sein du WAT après certains stimuli (Guerra et al., 1998; Petrovic et al., 2010).

Les tissus adipeux WAT et BAT sont donc des acteurs majeurs du métabolisme énergétique puisqu'ils possèdent des capacités de stockage, de libération et de dépense d'énergie et qu'ils dialoguent avec les autres organes du métabolisme ainsi qu'avec le cerveau *via* des sécrétions endocrines. De plus en plus d'études parlent d'ailleurs d'un organe adipeux unique malgré ses différentes localisations anatomiques ainsi que ses multiples fonctions (Cinti, 2018a).

Le tissu adipeux contient plusieurs types cellulaires dont les adipocytes matures et les précurseurs adipocytaires mais également des fibroblastes, des cellules endothéliales et des cellules immunitaires résidentes comme les macrophages et les lymphocytes T. Les cellules non adipocytaires sont appelées les cellules de la fraction stroma-vasculaire (**Figure 4**).

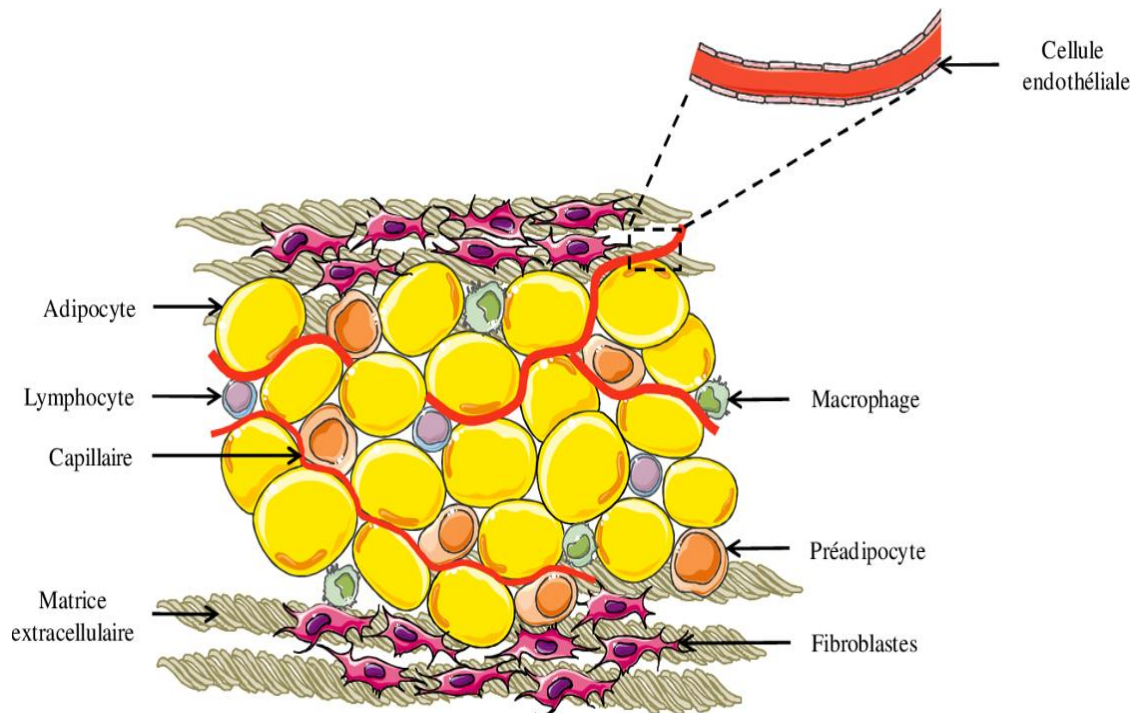


Figure 4 : Le microenvironnement adipeux contient des adipocytes matures et des précurseurs, des fibroblastes et des cellules immunitaires (macrophage et lymphocyte). Schéma d'après la thèse de Marie-Amélie LUKASZEWSKI.

1. Le tissu adipeux blanc

Le WAT représente la majorité de la masse grasse totale. Il est retrouvé au niveau viscéral et essentiellement en sous cutané, recouvrant toute la surface du corps à l'exception des muqueuses. Au niveau intra-abdominal on le retrouve au niveau viscéral en position intrapéritonéale (WAT omental et mésentérique), rétro-péritonéale et péri-rénale (**Figure 5**). Le rôle principal du WAT est le stockage de l'énergie sous forme de triglycérides à partir des apports nutritionnels en glucides et lipides. Lorsque l'organisme a besoin d'énergie, entre deux repas par exemple, le WAT est capable d'hydrolyser les triglycérides et de libérer les acides gras dans la circulation sanguine. Ces acides gras sont alors pris en charge par des protéines de transport des lipides (Albumine, vésicules lipidiques) et amenées aux organes réclamant du substrat énergétique.

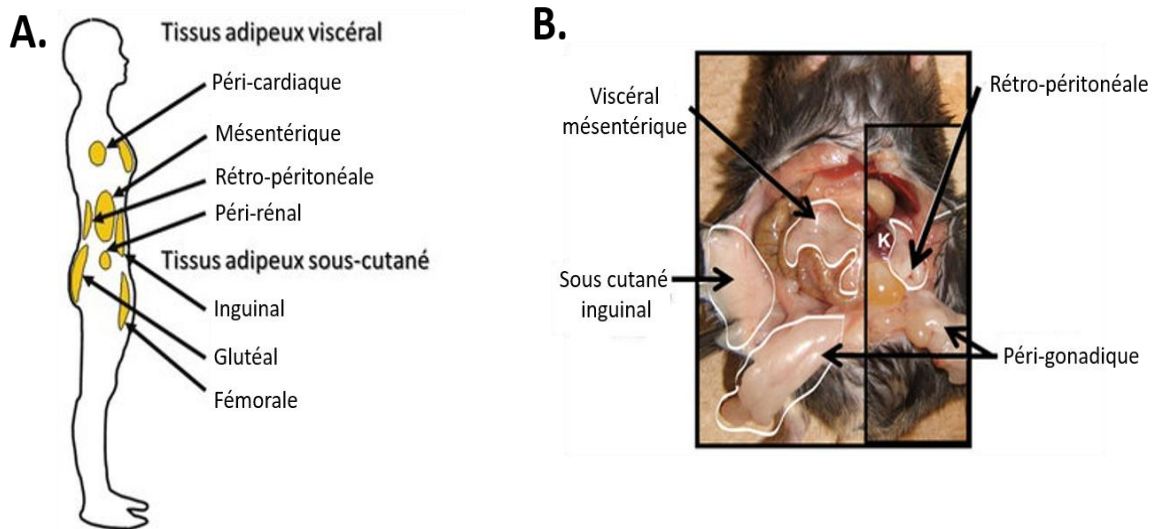


Figure 5 : Les dépôts de tissu adipeux blancs chez l'humain (A) et chez la souris (B). Photo (B) d'après (Berryman and List, 2017).

a. L'adipocyte blanc

L'adipocyte blanc est une cellule sphérique de 40 à 115 μm environ de diamètre qui se caractérise par une gouttelette lipidique occupant 90% de l'espace cytoplasmique (Le Lay et al., 2015). Le noyau ainsi que les organelles sont repoussées à la membrane plasmique de l'adipocyte (**Figure 6**). L'adipocyte blanc possède également peu de mitochondries. La gouttelette lipidique de l'adipocyte blanc est constituée de triglycérides (TG) et d'esters de cholestérol entourés d'une monocouche de phospholipides (micelle) et de protéines nécessaires pour le transport et le métabolisme des acides gras. Parmi ces protéines, les périlipines (PLIN) régulent l'accès des enzymes cytoplasmiques aux TG (**Figure 7B**). On distingue 5 périlipines (PLIN1 à PLIN5) réparties dans différents organes : la PLIN2 (aussi appelée ADRP ou adipophiline) et la PLIN3 (aussi appelée Tip-47) sont ubiquitaires. La PLIN4 est essentiellement retrouvée dans le WAT tandis que la PLIN5 est uniquement présente dans les tissus où la lipolyse sert à libérer des acides gras pour la β -oxydation (BAT, muscles, cœur) (Sztalryd and Brasaemle, 2017). La PLIN1 est spécifique du tissu adipeux et surtout majoritaire à la surface de la gouttelette lipidique. En conditions basales, elle protège les TG de la machinerie lipolytique cytoplasmique. En cas de jeûne ou d'exercice physique, la signalisation cellulaire induite par les agonistes β -adrénergiques entraîne l'activation de la PKA qui va à son tour phosphoryler la PLIN1 et permettre aux lipases d'avoir accès aux substrats (Sztalryd and Brasaemle, 2017).

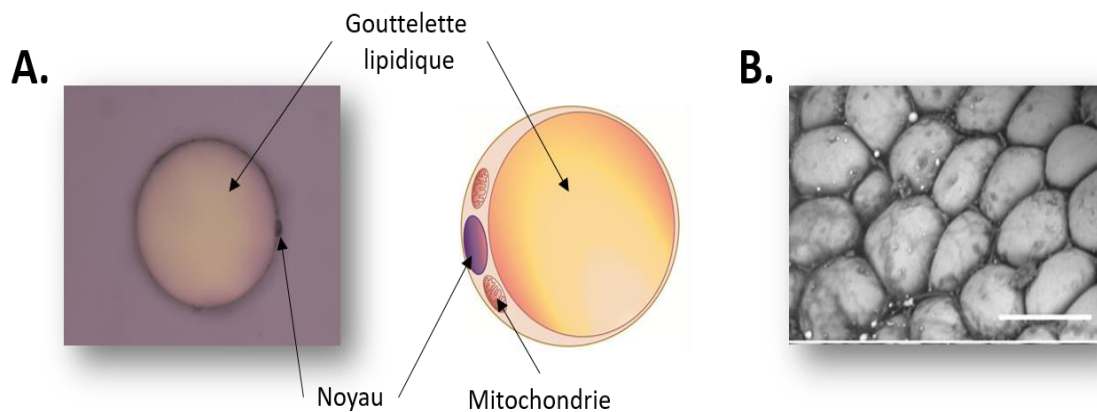


Figure 6 : L'adipocyte blanc observé en microscopie inversée (A) et au sein du tissu adipeux blanc (B). Schéma (A) d'après la thèse d'Alexandra Paul et photos (A et B) d'après (Cinti, 2018a) Echelle 50 μm .

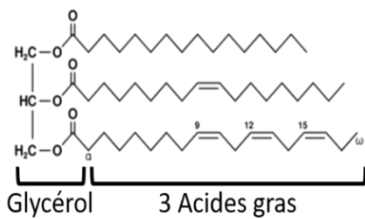
b. Le stockage de l'énergie dans l'adipocyte blanc

Les triglycérides sont la principale forme de stockage du substrat énergétique par les adipocytes. Leur stockage représente une réserve énergétique estimée à 2 mois chez un adulte sain avec 90 000 à 180 000 kcal contre 2 000 kcal pour les protéines et quelques centaines seulement de réserve glucidique (Foufelle and Ferré, 2013). Les TG sont constitués d'une molécule de glycérol sur laquelle sont estérifiés 3 AG (**Figure 7A**). La majorité des AG alimentaires arrivent dans l'intestin sous forme estérifiés. Au niveau du duodénum, les TG sont hydrolysés par la lipase pancréatique afin de former des monoglycérides et 2 AG, le tout va être enveloppé dans des acides biliaires afin de former des micelles qui vont entrer dans les entérocytes, les cellules spécialisées de l'intestin grêle, soit par diffusion passive à travers la membrane plasmique soit à l'aide d'un transporteur spécifique tels que *Fatty Acid Transport Protein 4* (FATP4) ou *Fatty Acid Transporter* (FAT)/ *Cluster of Differentiation 36* (CD36). Les AG sont ensuite dirigés vers le réticulum endoplasmique où ils vont de nouveau être estérifiés sur une molécule de glycérol et incorporés avec des esters de cholestérol dans des vésicules de transport, les chylomicrons. Les chylomicrons vont être sécrétés dans la circulation pour acheminer les TG et le cholestérol jusqu'au tissu adipeux puis vont être re-captés par le foie afin d'être réutilisés (Hussain, 2014). Lorsque les chylomicrons arrivent au niveau du tissu adipeux, les TG contenus sont de nouveaux hydrolysés en diglycérides et AG par la Lipoprotéine Lipase (LPL), une enzyme exprimée par les adipocytes sous le contrôle des facteurs *Sterol Regulator Element Binding Protein* (SREPB) 1 et 2 et *Peroxisome Proliferator Activated Protein γ* (PPAR γ) (Wang and Eckel, 2009). L'activité de la LPL est régulée par la signalisation insulinique, activée par l'insuline en période post-prandiale lorsqu'il est nécessaire de stocker les AG ou inhibée en période de jeûne par les protéines de la famille des *Angiopoietin like protein* (Angptl), notamment les Angptl 3 et 4 produites par l'adipocyte et l'Angptl 8 produite par le foie (Mohamed et al., 2020). L'entrée des

AG dans l'adipocyte est permise par la présence de transporteurs similaires à ceux présents dans les entérocytes : la famille des FATP et FAT/CD36. Les FATPs sont représentés par 6 isoformes dont notamment FATP1 dans l'adipocyte et sont régulés par la signalisation insulinique. Les animaux dont l'expression de la FATP1 a été invalidée sont résistants à l'obésité induite par la nutrition et restent sensibles à l'insuline (Kazantzis and Stahl, 2012). La FATP1 est cependant nécessaire à la thermogénèse et les animaux invalidés pour l'expression de la protéine sont incapables de s'adapter au froid (Wu et al., 2006). Le CD36 est un récepteur glycoprotéique transmembranaire appartenant à la famille des récepteurs scavenger B (Puchałowicz and Rać, 2020). Le CD36 est exprimé dans de nombreux types cellulaires, notamment les membres des cellules immunitaires, les adipocytes et dans les récepteurs gustatifs sur la langue où certaines études montrent son implication dans la perception du goût des graisses et l'obésité (Chevrot et al., 2013). Les ligands pris en charge par CD36 peuvent être protéiques, lipidiques comme les AG mais également un ensemble de protéines et lipides comme les lipoprotéines oxydées à faible densité (*oxLDL*). Le CD36 est un marqueur de la différenciation adipogénique (Puchałowicz and Rać, 2020).

A l'intérieur de l'adipocyte, les AG sont pris en charge par des protéines de transport intracellulaire, les *Fatty Acid Binding Protein* (FABP). La famille des FABP comprend 9 membres avec des spécificités tissulaires précises dans le foie, le muscle, l'intestin, l'iléon, l'épiderme, le testis, les cellules myélinisées, le cerveau et bien sûr l'adipocyte (Storch and Thumser, 2010). Dans l'adipocyte, FABP4, aussi appelée aP2 ou FABP-A, est la protéine majoritaire. Son expression est sous le contrôle du facteur de transcription PPAR γ , de l'insuline et des AG. L'invalidation de FABP4 entraîne une protection contre le développement de la résistance à l'insuline mais une élévation des AG libres dans le cytoplasme (Floresta et al., 2017).

A. Un triglycéride



B.

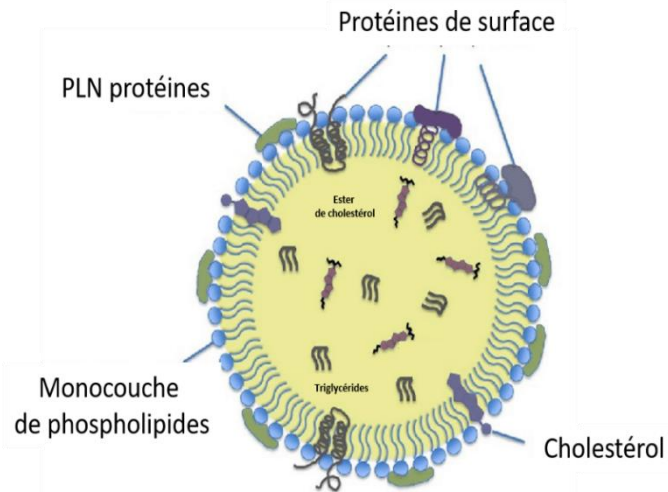


Figure 7 : La gouttelette lipidique. Le contenu de la gouttelette lipidique est constitué de triglycérides (A) et d'esters de cholestérol. Ce corps est entouré d'une monocouche de phospholipides provenant de sa synthèse dans le réticulum endoplasmique, de protéines dont les périlipines et de quelques molécules de cholestérol. Schéma d'après (Onal et al., 2017)

c. La lipolyse, libération de l'énergie

L'hydrolyse des lipides présents dans la gouttelette lipidique est le processus de lipolyse. Il est caractérisé par l'activité de 3 lipases : *Patatin-like phospholipase domain containing 2* (PNPLA2)/*Adipocyte Triglyceride Lipase* (ATGL), *Hormone-sensitive Lipase* (HSL) et *Monoacylglycerol Lipase* (MGL).

La lipolyse est induite essentiellement par l'adrénaline et la noradrénaline, des catécholamines agonistes des récepteurs β -adrénergiques qui vont entraîner une activation de l'adénylate cyclase et une augmentation de la concentration d'AMP-cyclique. La protéine kinase A (PKA) va ainsi être activée et va phosphoryler la PLIN1, ce qui va permettre la libération d'ABDH5, une protéine co-activatrice de la lipase PNPLA2/ATGL (**Figure 8**). L'ATGL est la première lipase à intervenir lors de la lipolyse des TG, elle hydrolyse les triglycérides en diglycérides et permet la libération d'un AG. Les diglycérides sont ensuite hydrolysés en monoglycérides par l'HSL également phosphorylée, ce qui permet la libération d'un autre acide gras et enfin la MGL catalyse la dernière étape en libérant le dernier AG et le glycérol (**Figure 8**). Le glycérol est libéré dans la circulation pour être utilisé par le foie ou les muscles squelettiques dans la synthèse *de novo* de glucose (gluconéogenèse) ou recapté par l'adipocyte pour être réutilisé dans la synthèse de TG. Les AG vont être utilisés comme substrat énergétique, notamment par le cœur.

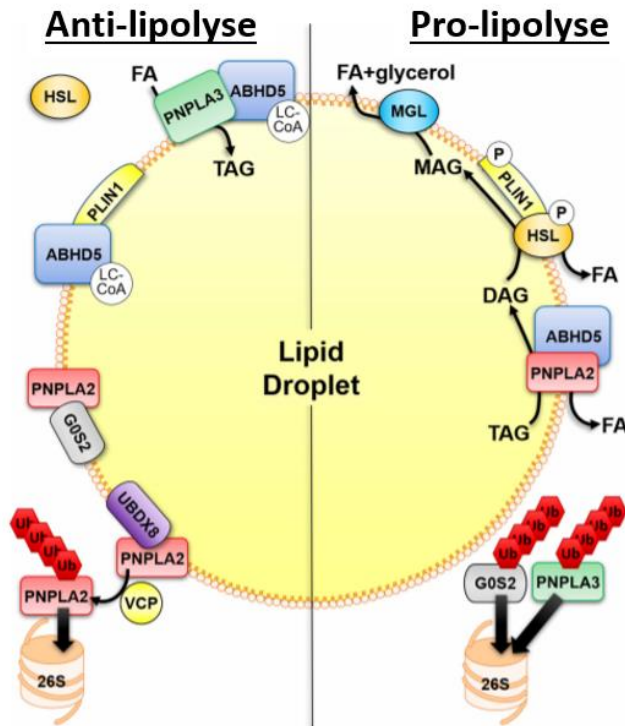


Figure 8: Le processus de lipolyse. En conditions basales (anti-lipolytique), la lipase PNPL2/ATGL est maintenue inactive grâce à l'interaction entre son co-activateur ABHD5 avec la PLIN1 ou la PNPL3/Adiponutrine. Par ailleurs, PNPL2/ATGL est inhibée par la présence de G0S2 et peut être liée par UBDX8 pour être ubiquitinylée (Ub) et dégradée par le protéasome (26S). Lorsque la lipolyse est stimulée (Pro-lipolyse), la phosphorylation (P) de la PLIN1 permet la libération d'ABHD5 qui se lie alors avec la lipase PNPL2/ATGL. La lipase HSL est également phosphorylée, ce qui entraîne sa liaison à la gouttelette lipidique et son interaction avec la PLIN1. Les triglycérides (TAG, TriAcyl Glycerides) sont alors hydrolysés d'abord par la lipase PNPL2/ATGL en diglycérides (DAG, DiAcyl Glycerides) puis en monoglycérides (MAG, MonoAcyl Glycerides) par la lipase HSL et enfin par la lipase MGL pour la libération de 3 acides gras (FA, Fatty Acids) et une molécule de glycérol. En parallèle, les facteurs inhibiteurs G0S2 et PNPL3 sont ubiquitinylés et dégradés par le protéasome. Schéma d'après (Yang and Mottillo, 2020)

Glycerides) par la lipase HSL et enfin par la lipase MGL pour la libération de 3 acides gras (FA, Fatty Acids) et une molécule de glycérol. En parallèle, les facteurs inhibiteurs G0S2 et PNPL3 sont ubiquitinylés et dégradés par le protéasome. Schéma d'après (Yang and Mottillo, 2020)

2. Le développement du tissu adipeux ou adipogénèse

Le développement de l'adipocyte se déroule en 3 étapes : i) l'induction des cellules souches adipogéniques qui deviennent des précurseurs adipocytaires ou pré-adipocytes, ii) l'engagement des pré-adipocytes et iii) la différenciation en adipocytes matures fonctionnels. *In vitro*, la majorité des études utilisent des pré-adipocytes. Ces étapes nécessitent l'action d'un réseau complexe de facteurs de transcription et d'activateurs dont les principaux seront décrits ici.

a. L'origine de l'adipocyte

La nature et l'origine des cellules souches adipogéniques est toujours débattue. Au niveau embryonnaire, la multitude de localisation des dépôts de tissus adipeux met en évidence les origines embryonnaires différentes, le WAT ayant une origine mésenchymateuse tandis que le BAT proviendrait du mésoderme comme le muscle. Ces hypothèses sont supportées par la présence de certains marqueurs, notamment Myf5 dans le BAT, caractéristiques de la cellule musculaire (Berry et al., 2013; Nascimento et al., 2018).

De nombreuses études s'accordent sur la présence de précurseurs dans les parois des capillaires sanguins vascularisant le tissu adipeux (Tang et al., 2008), ce seraient les péricytes car ils expriment des marqueurs de l'engagement adipocytaire. Certaines études semblent supposer

également que des cellules endothéliales peuvent devenir des précurseurs. Il est cependant difficile *in vivo* de déterminer les localisations des cellules souches vu la profusion de dépôts de tissu adipeux, la difficulté de prélever les zones présumées chez les embryons et le manque de marqueurs spécifiques. Ces difficultés ont mené à la mise au point par Green en 1974 des cellules immortalisées de fibroblastes de souris appelées les cellules 3T3-L1 et 3T3-F442A (Russell and Ho, 1976). Ces cellules sont considérées comme des précurseurs prêts à s'engager dans l'adipogenèse grâce à un traitement hormonal adéquat incluant insuline, glucocorticoïdes et inducteurs d'AMP cyclique (AMPC). En parallèle, d'autres lignées similaires pour étudier l'adipogenèse *in vitro* sont mises au point, dont les cellules clonales du WAT épидidymaire de souris obèses ob/ob, les cellules Ob17, par l'équipe de Gérard Ailhaud (Négrel et al., 1978). Chez l'humain, de nombreux modèles ont été décrits comme outils pour étudier l'adipogenèse *in vitro* comme PAZ6 (Zilberfarb et al., 1997) ou encore les cellules SGBS (Wabitsch et al., 2001), et plus récemment les cellules hMADS (Rodriguez et al., 2004) dont les caractéristiques seront développées un peu plus loin dans ce manuscrit.

b. L'adipogenèse in vitro

L'adipogenèse *in vitro* suit une séquence temporelle très organisée impliquant les deux isoformes du facteur de transcription majeur du *Peroxisome Proliferator-Activated Receptor* (PPAR γ 1 et 2) formant un hétérodimère avec le facteur *Retinoid X Receptor* (RXR) et 3 membres de la famille des *CCAAT/enhancer-binding protein* (C/EBPs) (**Figure 9A**). L'expression des C/EBP δ , C/EBP β et C/EBP α est activée de manière séquentielle (Kuri-Harcuch et al., 2019).

Les facteurs C/EBP δ et C/EBP β sont les premiers à être exprimés au cours des étapes précoces de la différenciation adipocytaire (**Figure 9B**). Ces deux facteurs coopèrent pour induire l'expression de C/EBP α et PPAR γ 2 qui contrôlent la différenciation terminale de l'adipocyte.

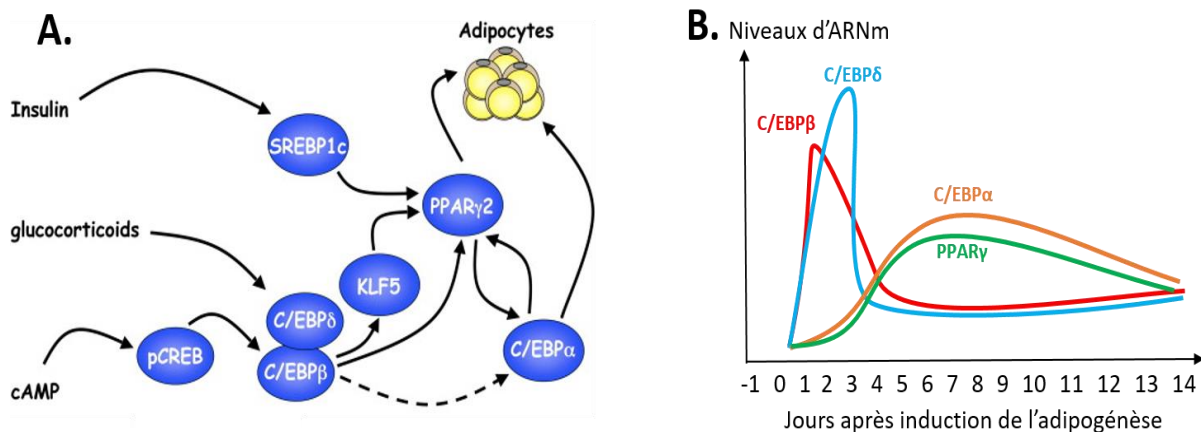


Figure 9: La cascade des facteurs de transcription de l'adipogenèse. L'ajout des inducteurs adipogéniques (insuline, glucocorticoïdes et inducteur d'AMPc) entraîne l'expression de C/EBPβ et C/EBPδ. L'hétérodimère induit l'expression de KLF5 et les 3 facteurs vont permettre l'expression de C/EBPα et PPARγ2 pour mener la différenciation adipocytaire à terme (A). L'expression de ces 4 facteurs de transcription est séquentielle durant le processus d'adipogenèse (B). Schéma (A) d'après (Farmer, 2006), schéma (B) inspiré de (Gaya et al., 2013).

L'inactivation des gènes codants pour C/EBPδ et C/EBPβ chez la souris entraîne 85% de mortalité à la naissance et les survivantes présentent un tissu adipeux presque inexistant (Tanaka et al., 1997).

In vitro, l'engagement adipocytaire nécessite l'ajout d'un inducteur d'AMPc (généralement *isobutyl methyl xanthine* ou IBMX), un glucocorticoïde (dexaméthasone) et une hormone lipogénique, l'insuline. La différenciation requiert la présence d'insuline, de l'hormone T3 et certains modèles cellulaires nécessitent l'ajout d'un agoniste de PPARγ de la famille des thiazolidinediones.

Les premiers modèles cellulaires d'adipocytes étaient cultivés dans un milieu contenant du sérum animal (sérum de veau fœtal) qui était capable d'induire l'adipogenèse donc il semble évident que le sérum contient des facteurs adipogéniques. Pour cultiver des pré-adipocytes dans un milieu sans sérum, il a fallu déterminer quels facteurs de différenciation étaient essentiels. Le potentiel adipogénique de plusieurs facteurs a été testé et les conditions de cultures *in vitro* de pré-adipocytes actuelles nécessitent un facteur de croissance (*insulin-like growth factor*), un inducteur d'AMPc ou inhibiteur de phosphodiesterase (IBMX) et un glucocorticoïde (dexaméthasone) pour augmenter le recrutement d'adipocytes (Kuri-Harcuch et al., 2019).

c. Les facteurs de transcription essentiels de l'adipogenèse

La famille des PPARs

Les PPARs sont des récepteurs nucléaires et facteurs de transcription contrôlant l'expression des gènes cibles possédant un site *PPAR Response Element* (PPRE) sur leur promoteur, sur lequel ils se lient grâce à leurs 2 motifs en doigts de zinc et en collaboration avec le facteur de transcription RXR (**Figure 10**). Les PPARs sont responsables de l'expression de nombreux gènes et tout particulièrement ceux impliqués dans l'adipogenèse et le maintien de la fonction adipocytaire (Spiegelman and Flier, 1996).

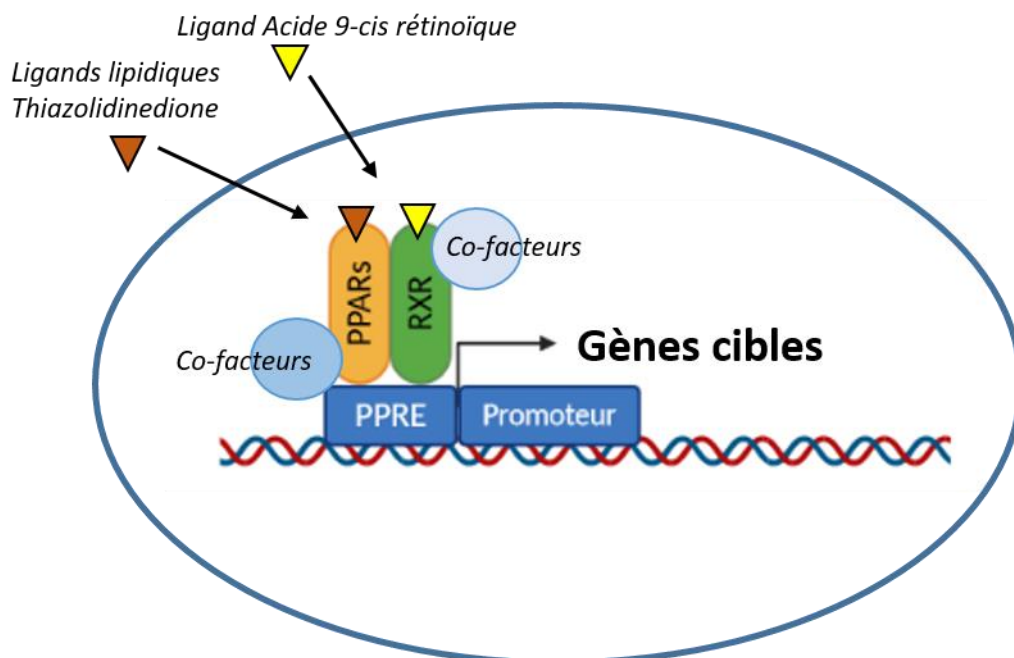


Figure 10: Les PPARs se lient à un PPRE situé sur le promoteur de leurs gènes cibles afin d'induire leur transcription.

On distingue 3 types de PPARs chez les mammifères :

- i) PPAR α est exprimé essentiellement dans le foie mais également dans le tissu adipeux, le cœur et le muscle. Il joue un rôle particulièrement important dans le métabolisme énergétique et notamment dans la libération d'énergie en favorisant le catabolisme des AG et la β -oxydation mitochondriale (Lee et al., 2011). Les souris invalidées pour PPAR α vont présenter une hypertrophie et une hyperplasie adipocytaire ainsi qu'une hyper-sensibilité à l'insuline due à l'augmentation de l'expression du transporteur de glucose Glut4 (Knauf et al., 2006). Les activateurs de PPAR α les plus connus sont les fibrates et sont utilisés dans le traitement de certaines formes d'hypertriglycéridémie (**Figure 12**) (Kim and Kim, 2020; Staels et al., 2008).

- ii) PPAR β/δ est exprimé de manière ubiquitaire, cependant il est essentiellement actif dans les tissus métabolisant les lipides comme l'intestin, les muscles squelettiques, le cœur et le tissu adipeux. Une étude montre le rôle de PPAR β/δ dans la diminution de la voie inflammatoire *Nuclear factor kappa B* (NF/ κ B) dans les macrophages, lui conférant ainsi des propriétés anti-inflammatoires (Zingarelli et al., 2010). PPAR β/δ est exprimé dans les stades précoces de l'adipogenèse et régule l'expression de certaines protéines du métabolisme lipidique comme les FATPs, FABPs et l'ACS (Amri et al., 1995; Nakamura et al., 2014). PPAR β/δ semble jouer un rôle important dans l'angiogenèse (Du et al., 2020).
- iii) PPAR γ est la forme prédominante exprimée dans le tissu adipeux. Il est impliqué dans le stockage d'énergie, l'adipogenèse et la thermogenèse (Voir chapitre thermogenèse). PPAR γ existe sous deux isoformes majoritaires, PPAR γ 1 et 2 (**Figure 11**), générées par la présence de 2 promoteurs sur le même gène. Les deux protéines générées sont identiques mais PPAR γ 2 possède 28 (chez l'Homme) ou 30 (chez la souris) acides aminés supplémentaires du côté N-terminal (Fajas et al., 1997; Hallenborg et al., 2016; Zhu et al., 1995). PPAR γ 1 est exprimé dans de nombreux tissus tandis que PPAR γ 2 est exclusivement présent dans le tissu adipeux. La suppression totale de PPAR γ chez l'animal est létale (Barak et al., 1999). En revanche, la suppression spécifique de PPAR γ 2 dans le tissu adipeux n'empêche pas l'adipogenèse, suggérant que PPAR γ 1 peut compenser l'absence de PPAR γ 2 mais les animaux sont résistants à l'insuline (Rosen et al., 2002). Les expériences d'expression ectopiques de PPAR γ 2 montrent qu'il est capable d'induire la totalité du programme adipogénique et de mener au stockage des TG comme dans les adipocytes (Tontonoz et al., 1994) mais également dans le foie ou les muscles, ce qui est le cas dans l'obésité où PPAR γ 2 se met à être exprimé (Medina-Gomez et al., 2005).

Il existe deux autres isoformes de PPAR γ transcrites à partir du gène de PPAR γ 1 : PPAR γ 3 est transcrite à partir d'un promoteur différent et est exprimée essentiellement dans le tissu adipeux, le colon et les macrophages (Fajas et al., 1997) et PPAR γ 4, dont la transcription est initiée au début de l'exon 1 (Sundvold and Lien, 2001).

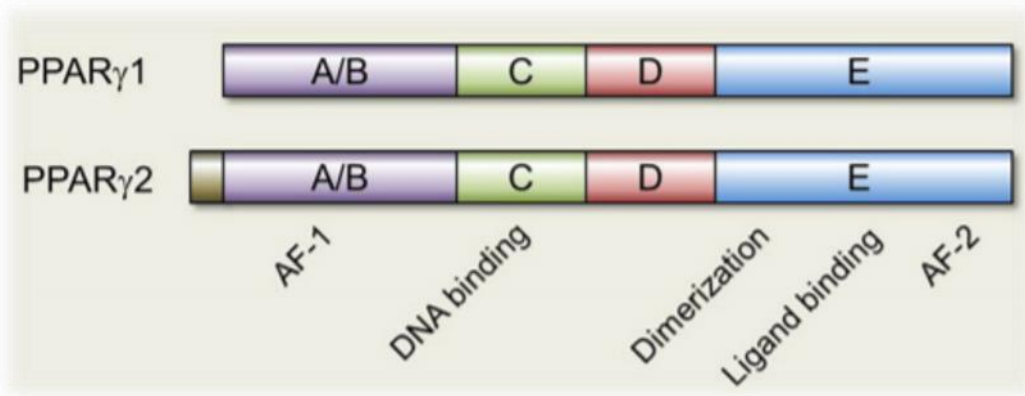


Figure 11 : PPAR γ 1 et 2 proviennent du même gène mais sont transcrites à partir de 2 promoteurs différents séparés de 90 nucléotides soit une protéine finale qui diffère de 30 acides aminés pour PPAR γ 2 à son extrémité N-terminale dans le domaine AF-1 (domaine d'activation indépendant de la présence d'un ligand). Schéma d'après (Hallenborg et al., 2016)

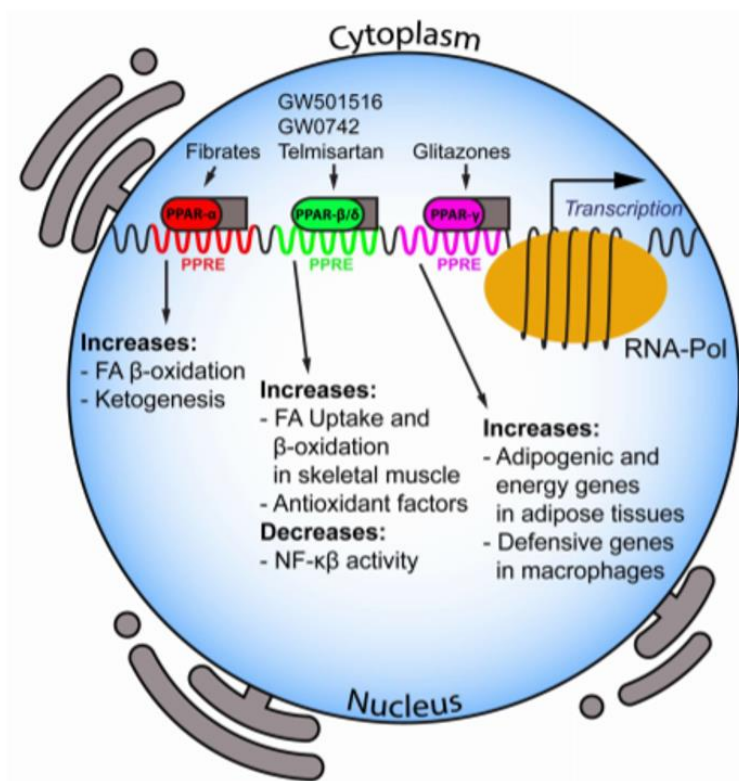


Figure 12 : Les ligands utilisés des PPARs. Schéma d'après (Botta et al., 2018)

Les ligands endogènes des facteurs de transcription PPARs n'ont pas été clairement mis en évidence même s'il semble avéré que ce sont des molécules lipidiques appartenant à la famille des acides gras et leurs métabolites. Des études ont décrit des ligands potentiels pour PPAR γ tels que le *15-deoxy-Delta12,14-prostaglandin J2* (15d-PGJ2) ou les 9- et 13-HODE qui seront détaillés plus loin dans la partie des métabolites des acides gras. *In vitro*, l'activation des PPARs (**Figure 12**) se fait de manière pharmacologique avec les thiazolidinediones pour PPAR γ , les fibrates pour PPAR α et par GW501516 ou GW0742 pour PPAR β/δ (Botta et al., 2018; Grygiel-Górniak, 2014).

La famille des C/EBPs

Les *CCAAT/enhancer binding protein* (C/EBPs) sont des facteurs de transcription se liant à la région CCAAT des promoteurs grâce à leur domaine de liaison contenant un motif riche en acides aminés basiques suivi d'une répétition de leucine (*basic-leucine zipper domain*). Ils forment des homo- ou des hétéro-dimères pour exercer leur action de transcription.

On distingue 5 isoformes (C/EBP α , β , δ , ϵ et *C/EBP Homologous Protein* ou CHOP10 appelé également C/EBP ζ) dont 3 sont essentielles à l'adipogenèse : C/EBP β et C/EBP δ sont exprimées de manière précoce durant l'adipogenèse sous les effets de l'IBMX et des glucocorticoïdes respectivement (Cao et al., 1991) et vont permettre l'expression de C/EBP α et de PPAR γ pour la différenciation terminale.

C/EBP α a d'abord été mise en évidence dans le foie, où l'inactivation totale du gène chez des souris est létale car C/EBP α est essentiel à la gluconéogenèse (Wang et al., 1995). Si le gène est supprimé de tous les tissus sauf du foie, le tissu adipeux ne se développe pas normalement (Linhart et al., 2001) et son expression ectopique dans des cellules fibroblastiques est capable d'induire l'adipogenèse (Freytag et al., 1994). Sa suppression de manière spécifique dans les adipocytes n'empêche pas l'adipogenèse mais on observe une résistance à l'insuline des adipocytes (Wu et al., 1999).

Il y a des éléments de réponses aux C/EBPs (CRE, *C/EBP Response element*) dans les promoteurs de C/EBP α et PPAR γ , ce qui signifie que C/EBP α est capable de contrôler sa propre transcription et que le duo active sa transcription mutuellement.

C/EBP β est exprimée sous 2 isoformes, une isoforme activatrice LAP (liver-enriched activating protein) et une isoforme inhibitrice LIP (liver-enriched inhibitory protein). C'est le ratio des deux formes qui détermine l'action activatrice ou inhibitrice de C/EBP β (Descombes and Schibler,

1991). L'expression de C/EBP β est induite par l'augmentation d'AMPc dans le pré-adipocyte (Cao et al., 1991; Zhang et al., 2004). La surexpression de l'isoforme LAP est capable d'induire les phases précoces de l'adipogenèse tandis que la surexpression de l'isoforme LIP inhibe la différenciation en inhibant l'expression de C/EBP α et PPAR γ (Lechner et al., 2013). Au cours de la différenciation, l'expression de CEBP β diminue au profit de celle de CEBP α .

Les autres facteurs de l'adipogenèse

La famille des *Kruppel-like factors* (KLF)

D'autres protéines interviennent dans la différenciation adipocytaire : on retrouve notamment des protéines de la famille des *Kruppel-like factor* (KLFs) dont KLF5, KLF6, KLF11 et KLF15. L'expression de KLF5 et KLF15 est induite par les C/EBP β et δ et agit de concert avec les deux CEBPs pour induire l'expression de PPAR γ (Mori et al., 2005; Oishi et al., 2005). KLF6 inhibe l'expression de Dlk1 (*Delta-like 1*, appelé également Pref1), une protéine transmembranaire inhibant l'adipogenèse (Li et al., 2005). L'expression de KLF11 est induite par PPAR γ et son invalidation empêche l'expression des marqueurs adipogéniques (Loft et al., 2015).

Les *Sterol Regulatory Element-Binding Proteins* (SREBP)

Un autre facteur de l'adipogenèse est la *Sterol Regulatory Element-Binding Protein 1c* (SREBP-1c). Les SREBP sont une famille de facteurs de transcription régulant l'expression de gènes impliqués dans la synthèse de lipides. On distingue 3 isoformes, SREBP-1a, SREBP-1c et SREBP-2. Les études réalisées *in vivo* chez l'animal laissent supposer que SREBP-1c est impliquée dans la synthèse d'acides gras tandis que SREBP-2 serait importante dans la synthèse de cholestérol. Enfin, SREBP-1a semble impliquée dans les deux voies de synthèse (Eberlé et al., 2004). L'expression d'une protéine SREBP-1c dominante négative inhibe l'adipogenèse (Kim and Spiegelman, 1996). En outre, son expression est augmentée par la présence d'insuline dans le milieu de culture des cellules 3T3-L1 (Kim et al., 1998a) et elle est impliquée dans l'activité de PPAR γ , certaines hypothèses lui accordent un rôle dans la production des ligands de PPAR γ (Kim et al., 1998b).

La voie Wnt

De même que nous venons de décrire certains régulateurs positifs de l'adipogenèse, il existe des protéines régulant négativement la différenciation. Il a notamment été mis en évidence que l'activation de la voie de signalisation Wnt inhibe l'adipogenèse en empêchant l'expression de C/EBP α et de PPAR γ (Ross et al., 2000). La voie Wnt est décrite comme impliquée dans le destin des cellules du mésoderme. D'autres protéines voient leur expression diminuer au cours de la différenciation et leur expression forcée inhibe l'adipogenèse, on distingue par exemple KLF2 qui

se lierait au promoteur de PPAR γ , empêchant ainsi KLF5 d'induire son expression (Wu et al., 2005). On peut également mentionner CHOP10 qui ne possède pas de domaine de liaison à l'ADN et viendrait former un dimère avec C/EBP β ou δ , empêchant le duo d'induire l'expression de C/EBP α et de PPAR γ (Batchvarova et al., 1995).

Les GATA-Binding Protein 2 et 3

Les facteurs de transcription de la famille des GATA se fixent sur des séquences d'ADN consensus contenant la séquence GATA. Dans les adipocytes, et plus particulièrement les pré-adipocytes, ce sont les facteurs GATA-2 et GATA-3 qui sont les plus exprimés sur les 6 membres de la famille. Les facteurs GATA-2 et GATA-3 sont des inhibiteurs de la différenciation adipocytaire, ils bloquent le pré-adipocyte dans son stade indifférencié en empêchant l'expression de PPAR γ (Tong et al., 2000). Cependant, une étude récente met en évidence le rôle de GATA-3 durant le processus de brunissage des adipocytes blancs en adipocytes thermogéniques. GATA-3 serait induit par l'exposition au froid et la stimulation par la noradrénaline (Son et al., 2020).

d. L'expansion clonale

Dans les études *in vitro*, l'adipogenèse est induite par un cocktail hormonal d'inducteurs lorsque les cellules ont atteint le stade de confluence. Il est néanmoins admis que les pré-adipocytes subissent une vague de mitoses obligatoires, une phase appelée expansion clonale. En effet l'inhibition pharmacologique de la prolifération cellulaire à ce moment empêche l'adipogenèse. Il est supposé que les pré-adipocytes ont besoin de réorganiser leur chromatine et permettre l'accès aux facteurs de transcription (Tang et al., 2003). Une autre hypothèse est que l'expansion clonale est nécessaire pour l'expression de certaines protéines du cycle cellulaire impliquées dans l'induction de l'adipogenèse, comme Krox20 ou les protéines de la famille E2F par exemple (Fajas et al., 2002).

3. Le tissu adipeux est un organe endocrine

En plus de la gestion de l'énergie, le tissu adipeux possède une fonction de sécrétion, notamment des hormones appelées adipokines mais également des lipides, appelées lipokines et des cytokines inflammatoires (**Figure 13**). Les adipokines sont capables d'agir de manière autocrine/paracrine et de manière endocrine. Les plus connues sont la leptine et l'adiponectine que nous décrirons ici.

a. La leptine

La leptine est une petite protéine de 16 kDa essentiellement sécrétée par les adipocytes blancs. Sa production est associée à la taille et au nombre d'adipocytes présents. La leptine est une protéine anorexigène, c'est-à-dire qu'elle induit la satiété. Ainsi son récepteur est principalement exprimé dans le système nerveux central, au niveau de l'hypothalamus, qui est le centre du contrôle de la prise alimentaire. L'existence de la leptine a été mise en évidence par la découverte d'un modèle de souris obèse, la souris ob/ob (Zhang et al., 1994) puis plus tard, son récepteur dans le cerveau a été trouvé. L'absence d'un récepteur à la leptine fonctionnel entraîne également une obésité sévère ainsi qu'un diabète de type 2, étudié avec le modèle animal db/db (Chen et al., 1996). La quantité de leptine dans la circulation sanguine, appelée leptinémie, est un marqueur métabolique. La leptinémie augmente avec la masse de tissu adipeux pour limiter la prise alimentaire. Néanmoins dans les cas d'obésité, la leptinémie est très élevée sans que la leptine puisse réellement être efficace. Cela signifie qu'une résistance au niveau du système nerveux s'installe (Farooqi and O'Rahilly, 2014). Les adipocytes bruns classiques sécrètent peu ou pas de leptine (Cancello et al., 1998).

b. L'adiponectine

L'adiponectine (appelée AdipoQ ou Acrp30) est une protéine exclusivement sécrétée par le tissu adipeux, essentiellement le WAT sous cutané et les adipocytes thermogéniques (Hu et al., 1996). A la différence de la leptine, la quantité d'adiponectine est corrélée négativement avec la quantité de masse grasse. On la retrouve sous forme de monomères ou de polymères, ce qui lui confère des propriétés différentes selon les organes cibles. En effet, ses récepteurs AdipoR1 et AdipoR2 sont exprimés dans différents tissus et entraînent différentes réponses. Au niveau du système nerveux central, AdipoR1 et AdipoR2 sont exprimés dans l'hypothalamus et colocalisent avec le récepteur de la leptine. L'adiponectine joue ici un rôle opposé à la leptine en stimulant l'appétit. Au niveau périphérique, AdipoR1 est exprimé dans le muscle et AdipoR2 dans le foie. La stimulation de ces récepteurs par l'adiponectine entraîne la régulation du métabolisme glucidique en favorisant le transport de glucose dans les muscles et en inhibant la néoglucogenèse dans le foie (Schraw et al., 2008). Des taux d'adiponectine circulante bas sont prédictifs du développement d'une insulino-résistance (Yadav et al., 2013).

c. Les autres sécrétions

Les adipokines

D'autres adipokines ont été décrites comme par exemple l'apeline qui agit favorablement sur le métabolisme du glucose (Castan-Laurell et al., 2011), la résistine qui est produite par le tissu adipeux infiltré de macrophages chez les individus obèses et aurait plutôt un rôle négatif (Schwartz and Lazar, 2011) ou encore l'adipsine (appelée également facteur du complément D) est une sérine protéase sécrétée qui augmente la sécrétion d'insuline en réponse au glucose et est négativement corrélée à l'obésité (Tafere et al., 2020). Le BAT secrèterait des adipokines relativement spécifique appelées "batokines" (BMP8b, FGF-21, *Neuregulin 4*, *endothelin-1* ou encore la PLTP) pouvant jouer un rôle important dans la communication avec d'autres organes et qui seront développées plus loin (Rodríguez et al., 2020).

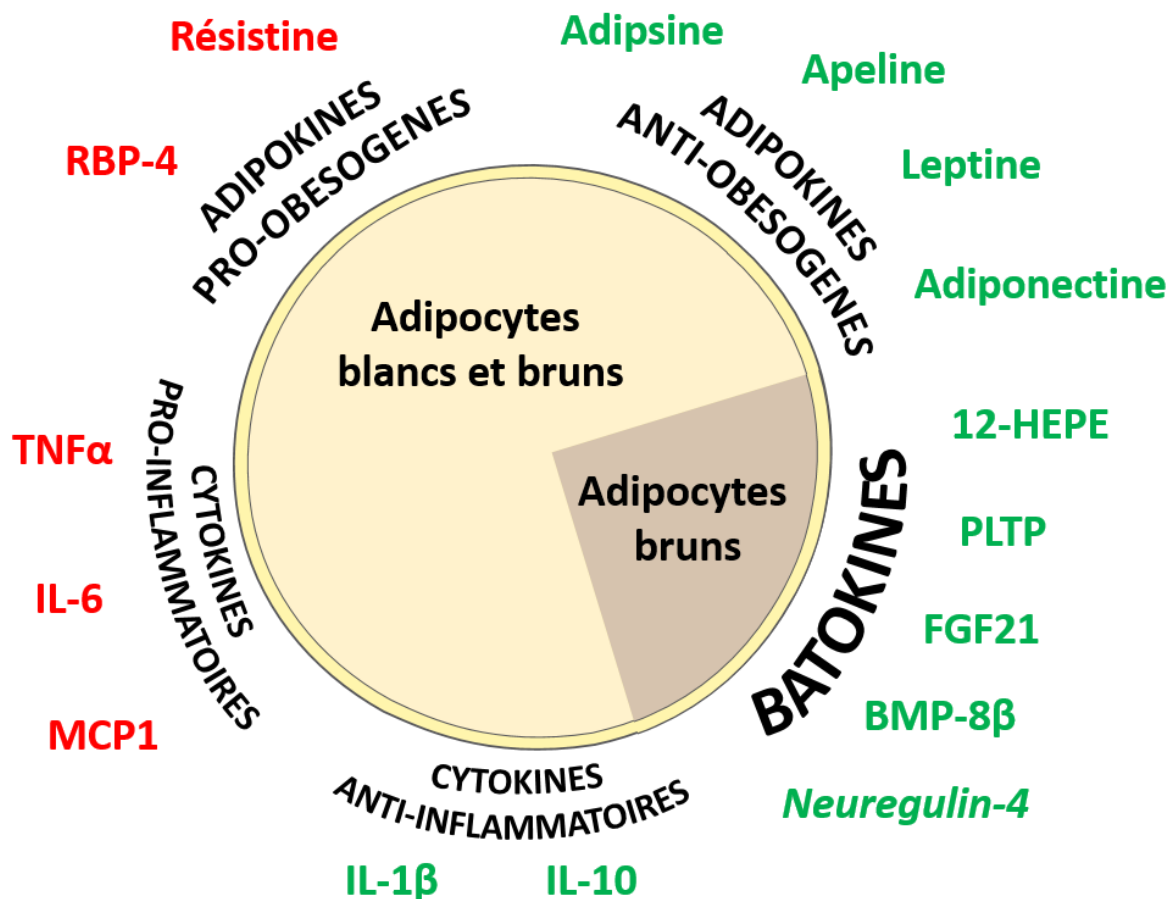


Figure 13 : Le tissu adipeux sécrète de nombreuses molécules, les adipokines, notamment des hormones qui vont favoriser l'homéostasie du glucose comme la leptine, l'adiponectine et l'apeline. Les adipokines spécifiquement sécrétées par les adipocytes bruns sont appelées les batokines et regroupent notamment le FGF-21, la BMP-8b, la neuregulin-4 ou encore le lipide 12-HEPE. D'autres vont avoir un effet négatif sur le métabolisme glucidique comme la résistine, les cytokines inflammatoires et la retino-binding protein (RBP4) qui serait pro-inflammatoire. Schéma réalisé avec *Servier Medical Art*.

Les cytokines

Bien que le principal producteur de cytokines soit le macrophage résident du tissu adipeux (Fain, 2006), ce dernier sécrète également des cytokines pro-inflammatoires comme par exemple l'interleukine 6 (IL-6), le *Tumor necrosis factor α* (TNF α) ou le chimio-attractant de monocytes (MCP1 ou CCL2) ou des cytokines anti-inflammatoires comme par exemple les interleukines 1 β (IL-1 β) et 10 (IL-10) (Blüher, 2014).

4. Le tissu adipeux brun

a. Historique

La présence d'un tissu adipeux thermogénique chez le mammifère hibernant est connu depuis 1551 avec les travaux du naturaliste Gessner (Halpern et al., 2014). L'intérêt pour ce tissu reviendra ensuite au 20^{ème} siècle où des équipes de recherche vont successivement mettre en évidence la capacité thermogénique de ce tissu permettant aux animaux de résister au froid durant l'hibernation (Smith and Hock, 1963) ainsi que sa présence chez le nouveau-né pour résister à la différence de température entre l'utérus et l'environnement extérieur (Silverman et al., 1964) (**Figure 14A**). Les capacités thermogéniques du BAT sont décrites comme diminuant après la naissance, donc l'étude du BAT chez l'humain adulte va être ralentie malgré les preuves de sa présence dans les premières années de vie chez l'enfant (Heaton, 1972) ou même chez l'adulte dans la pathologie du phéochromocytome (Ricquier et al., 1982). La recherche sur le BAT chez l'adulte va connaître un nouvel élan à partir de 2002 grâce à l'avènement de la médecine nucléaire. En effet, la technique d'imagerie médicale de PET-scan CT va être utilisée comme moyen de détection des masses cancéreuses. Le PET-scan utilise alors du glucose radio-marqué permettant de visualiser la présence de tissus métaboliquement très actifs comme c'est le cas des tumeurs. Les médecins utilisant cette méthode d'imagerie vont alors décrire des zones très actives et symétriques au niveau para-vertébral, supra claviculaire, para-aortique et inter-scapulaire, laissant alors penser à du BAT (Hany et al., 2002). De surcroit, l'activité métabolique de ces zones est régulée par le froid et les catécholamines (Nedergaard et al., 2007) et au niveau moléculaire, ce tissu exprime le marqueur unique du BAT qui est la protéine UCP1 responsable de la transformation de l'énergie chimique en énergie thermique (Zingaretti et al., 2009). Plusieurs équipes vont ensuite montrer la présence d'un BAT actif au PET-scan chez des individus exposés au froid, exprimant UCP1 et négativement corrélé à l'IMC (van Marken Lichtenbelt et al., 2009; Virtanen et al., 2009) (**Figure 14B**).

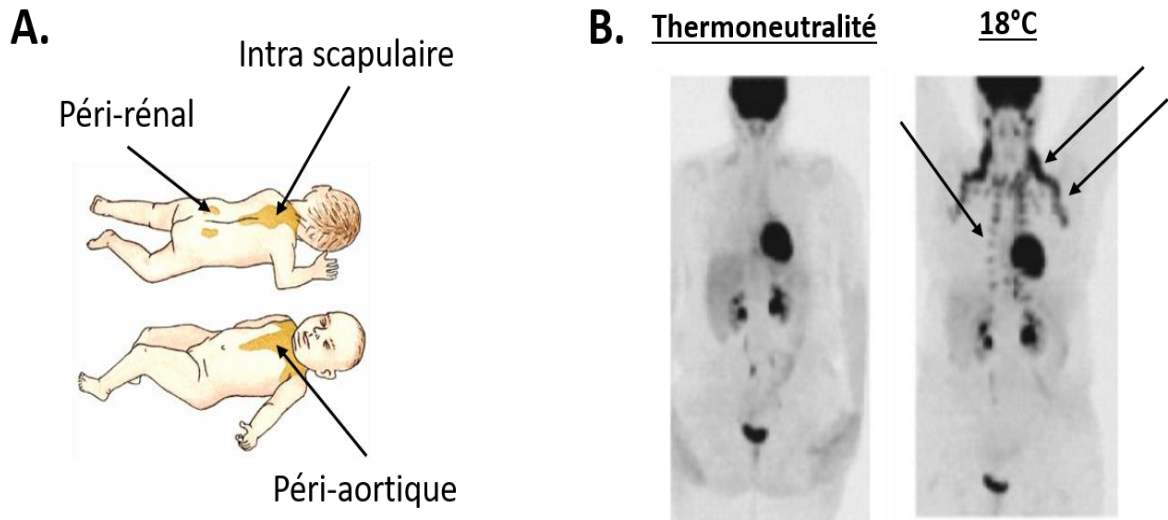


Figure 14: Historique du BAT. Le BAT a longtemps été considéré comme un tissu thermogénique uniquement présent chez le mammifère hibernant et le nourrisson (A). La médecine nucléaire a mis en évidence la présence d'un tissu thermogénique chez l'humain adulte (B). L'individu a subi une imagerie PET-scan après injection de FDG, soit après avoir été maintenu dans des conditions de température proche de la thermoneutralité (à gauche), soit à des températures relativement froides de l'ordre de 18°C, correspondant aux conditions normales d'examen (à droite). L'image laisse apparaître du tissu actif au niveau supra-claviculaire, dans le cou et en para-vertébral (flèches noires). Photos (B) d'après (Christensen et al., 2006)

b. Le tissu adipeux brun

Le tissu adipeux brun (BAT) se trouve en petite quantité comparée au tissu adipeux blanc. Par ailleurs, tous les individus adultes ne possèdent pas les mêmes quantités de tissu adipeux brun et son volume décroît avec l'âge et la prise de poids (van Marken Lichtenbelt et al., 2009). Il est localisé au niveau intra- et inter-scapulaire et cervical ainsi qu'autour du cœur et de certains vaisseaux abdominaux, particulièrement l'aorte (**Figure 15A**). Ces localisations s'expliquent par son rôle thermogénique, en cas d'exposition au froid, il permet de réchauffer les fluides, essentiellement le sang, qui vont alors maintenir la température des organes sur leur trajet (Nicholls and Locke, 1984), notamment le cerveau (Sidossis and Kajimura, 2015).

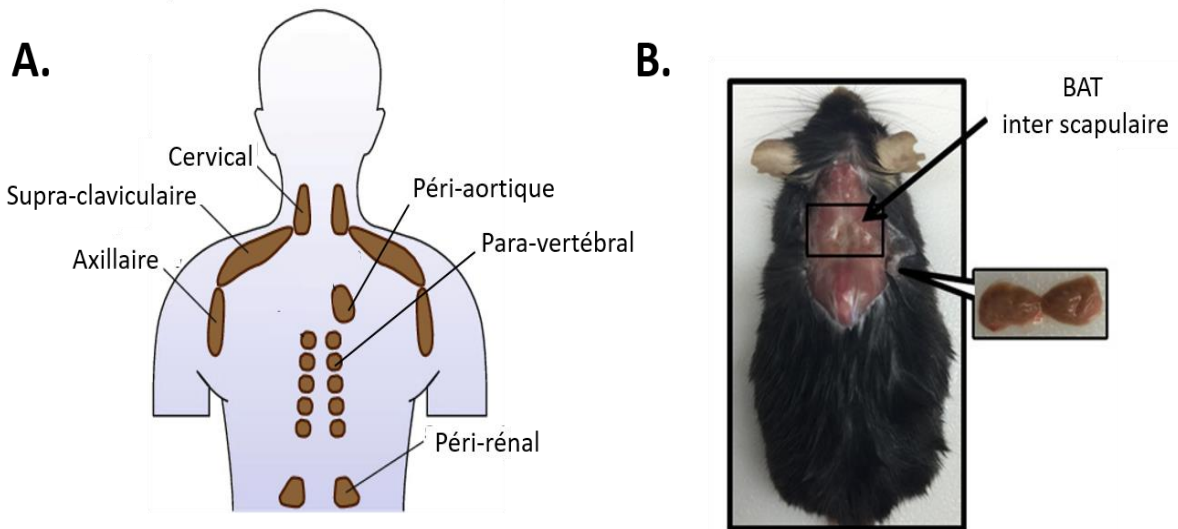


Figure 15: Les différentes localisations des dépôts de BAT chez l'Humain (A) et le BAT inter-scapulaire chez la souris (B). Illustration (A) modifiée d'après (Jung et al., 2019) Photo (B) d'après (Berryman and List, 2017).

c. L'adipocyte brun

Le BAT est constitué d'adipocytes et de pré-adipocytes ainsi que des cellules de la fraction stroma-vasculaire et c'est un tissu richement vascularisé et innervé. Comme l'adipocyte blanc, l'adipocyte brun emmagasine l'énergie sous forme de triglycérides mais au lieu de la libérer à la demande de l'organisme, il hydrolyse les triglycérides et utilise les acides gras pour produire de la chaleur. Pour cela, il possède une morphologie différente de l'adipocyte blanc. Tout d'abord, il possède plusieurs petites gouttelettes lipidiques et possède de nombreuses mitochondries riches en chaînes respiratoires et cytochrome c, ce qui est à l'origine de leur couleur brune. A l'inverse de l'adipocyte blanc, l'adipocyte brun est plus petit avec environ 20-50 μm de diamètre, et son noyau est central, ce qui lui confère une forme multiloculée polygonale (**Figure 16**) (Cinti, 2018a).

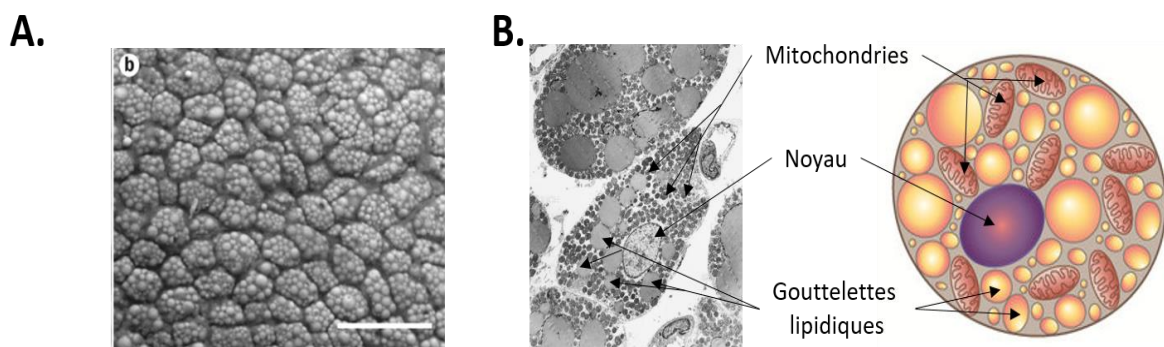


Figure 16: L'adipocyte brun. Tissu adipeux brun en microscopie électronique à balayage (A) d'après (Cinti, 2018a) et photo d'un adipocyte brun en microscopie électronique à transmission (B) et son schéma. Photo de David M. Philips, grossissement 4000x. Schéma d'après la thèse d'Alexandra Paul.

Les mitochondries des adipocytes bruns expriment une protéine unique et essentielle à la production de chaleur : *UnCoupling Protein 1* (UCP1). Cette protéine est localisée sur la membrane interne de la mitochondrie, à la suite de la chaîne respiratoire qui se termine par une ATP synthase. Dans l'adipocyte thermogénique, UCP1 crée une dérivation du circuit habituel des électrons, empêchant la synthèse d'ATP et dissipant l'énergie sous forme de chaleur.

5. L'adipogenèse brune

L'adipogenèse est un processus global très similaire à tous les tissus adipeux mais la différenciation de l'adipocyte brun nécessite des facteurs complémentaires essentiels à l'apparition du phénotype thermogénique. Le complexe de facteurs de transcription C/EBPs-PPAR γ reste central à l'adipogenèse brune même si, contrairement à l'adipogenèse blanche, C/EBP α semble moins essentiel que C/EBP β (Linhart et al., 2001).

a. L'activation β -adrénergique

La présence et l'activation des récepteurs adrénergiques β 1 par la noradrénaline est également un facteur essentiel à l'induction de l'adipogenèse brune chez les précurseurs retrouvés dans les capillaires du BAT. L'absence de récepteurs adrénergiques favorise la différenciation en adipocytes blancs (Hansen and Kristiansen, 2006).

b. PPAR γ Coactivator 1 α (PGC1 α)

L'activité thermogénique du BAT est possible grâce à l'expression de nombreux gènes essentiels à la mitochondriogenèse ainsi qu'à l'expression des protéines de la chaîne respiratoire mitochondriale. PGC1 α est un facteur clé dans la transcription des gènes essentiels à la thermogenèse induite par le froid (Harms and Seale, 2013; Puigserver et al., 1998), notamment UCP1 grâce à sa coopération avec les récepteurs nucléaires PPAR γ et TR β (Thyroid Hormone receptor). L'expression de PGC1 α est induite par SREBP-1c (Hao et al., 2010) de manière importante dans le BAT mais également dans certains adipocytes du WAT en cas d'exposition au froid ou de stimulation avec des agonistes des récepteurs β -adrénergiques. L'inactivation du gène codant pour PGC1 α chez la souris entraîne une incapacité à s'adapter au froid (Lin et al., 2004), même en présence d'UCP1 (Leone et al., 2005). De manière surprenante, les animaux invalidés pour l'expression de PGC1 α sont résistants à l'obésité et à l'intolérance au glucose induites par l'alimentation. Une hyperactivité physique due à une anomalie cérébrale (dans l'aire motrice du striatum) est l'hypothèse avancée pour expliquer ces phénomènes (Lin et al., 2004). A l'inverse, l'expression forcée de PGC1 α dans des adipocytes blancs induit l'expression d'UCP1 ainsi que celle des gènes impliqués dans la mitochondriogenèse (Tiraby et al., 2003).

c. PRDM16

Un autre facteur favorisant la différenciation en adipocyte brun est le *PR Domain Zinc finger protein 16* (PRDM16). Exprimé de manière sélective dans le BAT, il est considéré comme un « interrupteur » dans le destin cellulaire des précurseurs des adipocytes bruns. En effet, dans les progéniteurs mésenchymateux exprimant le marqueur Myf5, l'expression de PRDM16 entraîne une différenciation en adipocytes thermogéniques tandis que sa répression permet la différenciation des précurseurs en cellules musculaires, les myoblastes (Seale et al., 2008). *In vitro*, l'expression ectopique de PRDM16 dans des lignées cellulaires musculaires induit une différenciation en adipocytes exprimant les marqueurs clés adipogéniques. L'action pro-adipogénique de PRDM16 est permise par sa collaboration avec PPAR γ et PGC1 α dont il est un co-activateur transcriptionnel important (Seale et al., 2008).

Comme pour PPAR γ , l'inactivation totale de PRDM16 est létale à la naissance chez l'animal. L'inactivation partielle n'empêche pas l'adipogenèse dans le BAT mais entraîne des altérations de l'expression des gènes caractéristiques du phénotype thermogénique (Seale et al., 2007). *In vivo*, l'expression forcée de PRDM16 dans les adipocytes blancs du WAT épидидymal de souris entraîne l'expression des gènes thermogéniques comme UCP1 (Seale et al., 2007). L'altération de l'expression de PRDM16 dans des lignées et ou des cellules primaires de précurseurs bruns en utilisant des ARNs interférents abolit le programme transcriptionnel thermogénique (Seale et al., 2007)

Le rôle de PRDM16 est essentiel dans le choix de la voie de différenciation des progéniteurs adipeux, que ce soit pour former des adipocytes bruns ou des adipocytes beiges. Son rôle sera plus amplement détaillé dans un chapitre plus loin, traitant des différentes origines et différents destins des précurseurs.

6. L'adipocyte beige

L'adipocyte brun classique décrit chez la souris et chez le nouveau-né humain est essentiellement retrouvé au niveau inter-scapulaire et en région péri-rénale (**Figure 17A**). Ces dépôts de BAT sont délimités par un tissu conjonctif, les séparant du WAT (**Figure 17B**). En 1998, Guerra et al. décrit chez la souris un nouveau type d'adipocyte, apparaissant au sein du WAT et possédant les caractéristiques d'un adipocyte brun classique soit l'expression d'UCP1, une morphologie multiloculée et une capacité thermogénique (Guerra et al., 1998). L'apparition de ce nouveau type d'adipocyte a été stimulée par un traitement pharmacologique comme par un agoniste des récepteurs adrénergiques β 3.

L'analyse histologique des dépôts de BAT chez l'adulte montre plutôt un tissu où adipocytes uniloculés et multiloculés se côtoient, ce qui a conduit les scientifiques à considérer le BAT chez l'Homme comme un tissu mixte plutôt qu'un BAT classique (Cannon et al., 2020).

a. L'origine des adipocytes beiges

L'origine de ces adipocytes thermogéniques au sein du WAT est encore débattue. Certaines études semblent montrer qu'ils se forment *via* des précurseurs, d'autres suggèrent plutôt qu'ils proviennent d'une trans-différenciation des adipocytes blancs en adipocytes thermogéniques dans certaines conditions (Rosenwald and Wolfrum, 2014; Wang et al., 2013). Les études chez la souris montrent que le BAT et le WAT peuvent être considérés comme un organe unique, capable de s'adapter aux contraintes de l'environnement et de se compenser. En effet, lorsqu'un animal est exposé pendant une longue période à une température beaucoup plus basse que sa température de thermoneutralité, c'est-à-dire la température où l'organisme n'a pas besoin de produire de la chaleur supplémentaire, on observe une hyper-activation de ses dépôts de BAT classiques (inter-scapulaires et supra-claviculaires) ainsi que l'acquisition de propriétés thermogéniques par ses dépôts de WAT. Ce phénomène est appelé brunissage. A l'inverse, lorsque l'obésité est induite chez l'animal par une alimentation hypercalorique ou chez les modèles génétiquement obèses, les adipocytes bruns stockent les lipides et perdent leurs capacités thermogéniques. Ce phénomène est appelé blanchiment.

Ces adipocytes thermogéniques retrouvés dans le WAT ont été appelés adipocytes beiges ou brite (*brown-in-white*) (Petrovic et al., 2010). La terminologie beige et thermogénique sera utilisée pour le reste du document.

Les adipocytes beiges et bruns possèdent les mêmes caractéristiques : expression d'UCP1, morphologie multiloculée et capacité thermogénique.

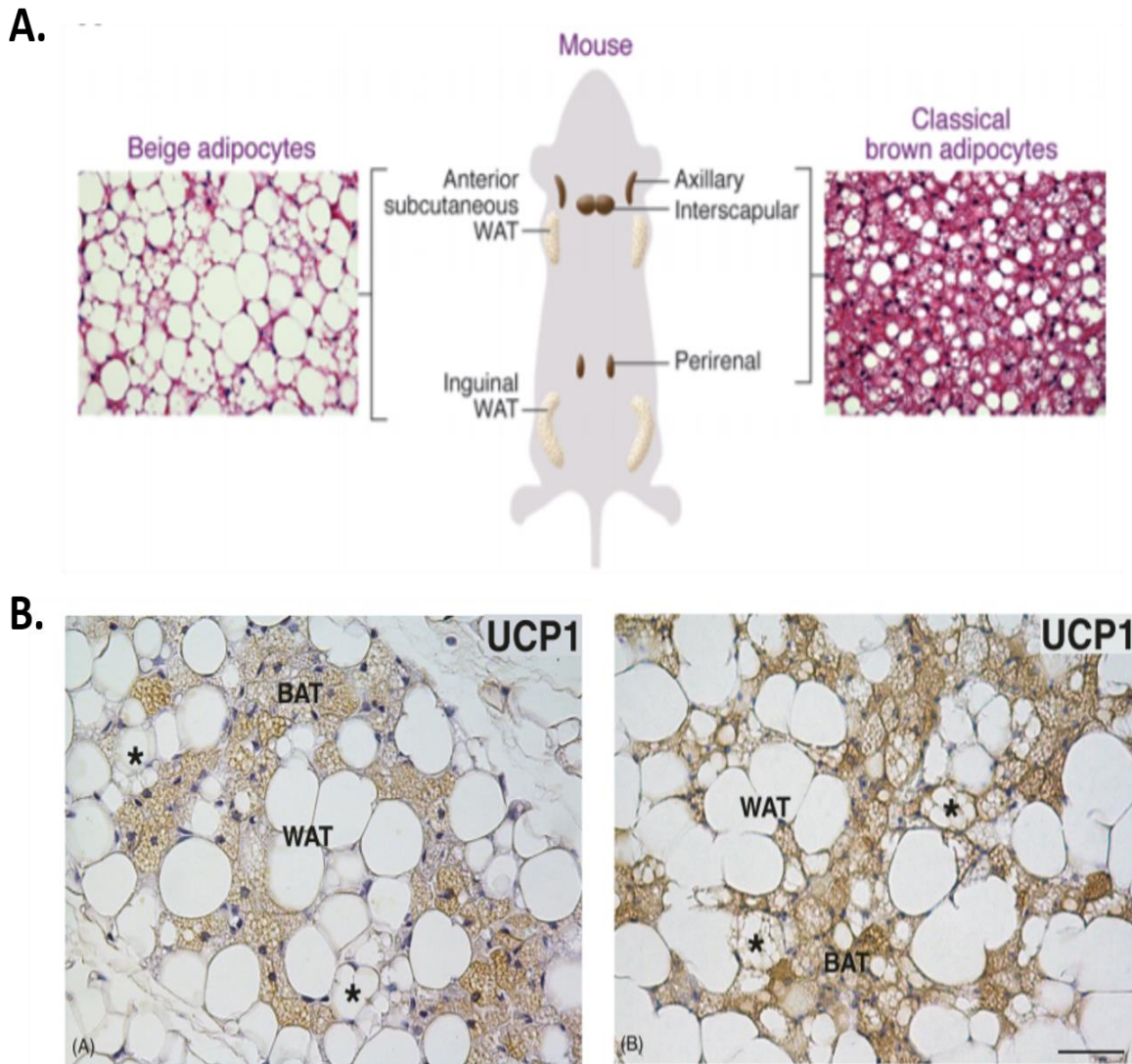


Figure 17 L'adipocyte beige. Schéma des dépôts de BAT classique et de WAT sous cutané chez la souris avec marquage hématoxyline-éosine des adipocytes (A). La différence de densité cellulaire entre le BAT et le WAT est bien visible ainsi que la présence de petits adipocytes thermogéniques dans le WAT. Les photos (B) montrent un marquage immuno-histochimique spécifique de la protéine UCP1 dans le tissu adipeux de souris (à gauche) et dans le tissu adipeux humain (à droite). Photos d'après (Cinti, 2018a)

b. Les adipocytes roses

Outre les adipocytes blancs, bruns et beiges, un autre type d'adipocyte a été décrit : il s'agit de l'adipocyte rose (*Pink adipocyte*). Décrit dans les travaux de S. Cinti (Cinti, 2018b), l'adipocyte rose apparaît lors du remodelage de la glande mammaire chez la souris lactante. Il semblerait que durant la gestation, les adipocytes blancs de la glande mammaire subissent une transdifférenciation en cellules glandulaires riches en lipides et produisant du lait.

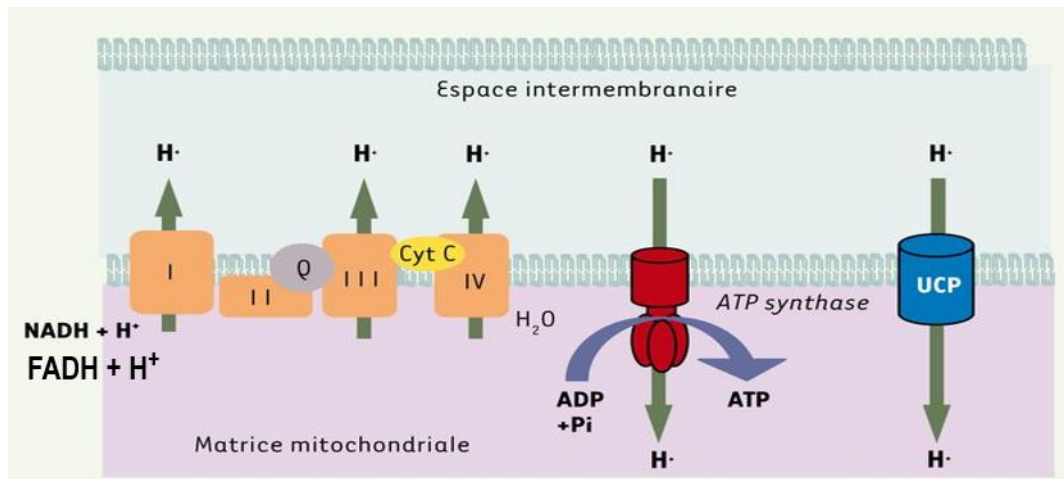
7. La thermogenèse non frissonnante

La thermogenèse est le processus chez les mammifères par lequel l'organisme dissipe de l'énergie chimique en énergie thermique, c'est-à-dire sous forme de chaleur. La thermogenèse se produit sous l'effet de la sécrétion de catécholamines, adrénaline et noradrénaline, lorsque la température environnementale diminue et que les réactions métaboliques basales ne suffisent plus à maintenir la température corporelle. A ce moment-là, les adipocytes thermogéniques (adipocytes bruns et beiges) s'activent pour produire de la chaleur, c'est la thermogenèse non frissonnante. Lorsque la température diminue, c'est tout d'abord la thermogenèse frissonnante produite par les muscles qui tente de maintenir la température corporelle. Lorsque les muscles ont épuisé leurs ressources énergétiques, la thermogenèse non frissonnante prend le relais. Le seuil auquel un processus bascule sur l'autre est différent d'un individu à l'autre, il est dépendant de l'âge, du sexe et de l'état de santé mais également de facteurs encore non identifiés. Cela rend les études chez l'Humain compliquées et nécessite un monitoring précis lors de tout protocole. De nombreuses équipes médicales étudient la possibilité d'utiliser la thermogenèse non frissonnante comme méthode pour augmenter la dépense énergétique sans que la situation soit inconfortable pour l'individu, en utilisant des vestes qui refroidissent par exemple, mais cela demande une mise au point au cas par cas (van Marken Lichtenbelt et al., 2009). La possibilité d'utiliser des méthodes pharmacologiques ou nutritionnelles serait également une opportunité et ici sera décrit le processus de thermogenèse non frissonnante ainsi que les connaissances actuelles sur son activation et son inhibition.

c. La respiration mitochondriale

Dans les adipocytes thermogéniques, la thermogenèse est possible grâce à la sécrétion de catécholamines qui va augmenter la respiration mitochondriale et la phosphorylation oxydative qui s'y déroule (**Figure 18**).

La mitochondrie est le cœur de production d'énergie de la cellule sous forme d'Adénosine TriPhosphate (ATP). La mitochondrie est constituée de deux membranes, une membrane externe perméable aux protons et une membrane interne qui contrôle le passage des protons grâce à des transporteurs. Ces transporteurs constituent la chaîne de transport d'électrons, ou chaîne respiratoire mitochondriale, qui est composée de 5 complexes : complexe I (*NADH deshydrogenase*) complexe II (*succinate deshydrogenase*), complexe III (*ubiquinol cytochrome c oxidoreductase*), complexe IV (*cytochrome oxidase*) et complexe V (*ATP synthase*).



Respiration couplée à la synthèse d'ATP

Découplage

Figure 18 Le découplage de la respiration mitochondriale par UCP1.

L'oxydation des lipides et des sucres provenant de l'alimentation permet la synthèse des deux substrats importants dans la respiration mitochondriale : NADH (*Nicotinamide Adenine Dinucleotide Hydroxide*) et FADH₂ (*Flavine-adenine Dinucleotide hydroxide*). La réduction de ces facteurs dans la chaîne respiratoire va permettre la production d'électrons qui vont être transportés dans l'espace inter-membranaire de la mitochondrie créant un gradient électrochimique. Afin de rétablir l'équilibre électrique entre les deux espaces, les électrons sont de nouveau transportés à l'intérieur de la mitochondrie par l'ATP synthase, permettant ainsi la phosphorylation de l'Adénosine Di Phosphate (ADP) en ATP et donc l'augmentation de la réserve énergétique cellulaire. C'est la phosphorylation oxydative.

En conditions basales, il existe des fuites de protons à travers la membrane interne de la mitochondrie, ce qui conduit à une dissipation de chaleur représentant environ 20% du métabolisme de base. Lorsque l'organisme nécessite une production accrue de chaleur, ce n'est pas suffisant et les adipocytes thermogéniques possèdent une protéine découplante spécifiquement dédiée à la dissipation de chaleur qui est UCP1.

d. UCP1, la protéine découplante de la respiration mitochondriale

La protéine UCP1, appelée aussi thermogénine, est la protéine permettant la dissipation de chaleur par les adipocytes thermogéniques. Elle a été décrite dans le BAT en 1982 par Ricquier et al comme étant la seule protéine du tissu dont l'activité était induite par l'exposition au froid des animaux, et qui s'inactivait lorsque la température était augmentée (Ricquier et al., 1982). Son rôle dans la thermogénèse a été confirmé en 1997 lorsque des animaux ont été invalidés pour UCP1 et qu'ils ont présenté une sensibilité accrue au froid (Enerbäck et al., 1997).

La famille des UCPs compte plusieurs protéines UCP1 à UCP5. La protéine UCP2 est exprimée de manière ubiquitaire, UCP3 est exprimée principalement dans le muscle et sont impliquées dans la prévention de l'accumulation des espèces réactives de l'oxygène (ROS) produites par la mitochondrie. Le rôle d'UCP4 et 5 n'est pas encore élucidé mais il semble qu'elles soient essentiellement présentes au niveau du système nerveux central (Bouillaud et al., 2016).

La protéine UCP1 est une protéine de 32 kDa qui est formée de 3 hélices transmembranaires contenant 2 séquences supplémentaires, la différenciant des autres membres de la famille des UCPs. En outre, le promoteur de son gène présente un complexe amplificateur (complex enhancer) (**Figure 19**) contenant les éléments de réponse aux inducteurs : un élément de réponse à la T3 (TRE), aux PPARs (PPRE), à l'acide rétinoïque (RARE) et à CREB (CRE).

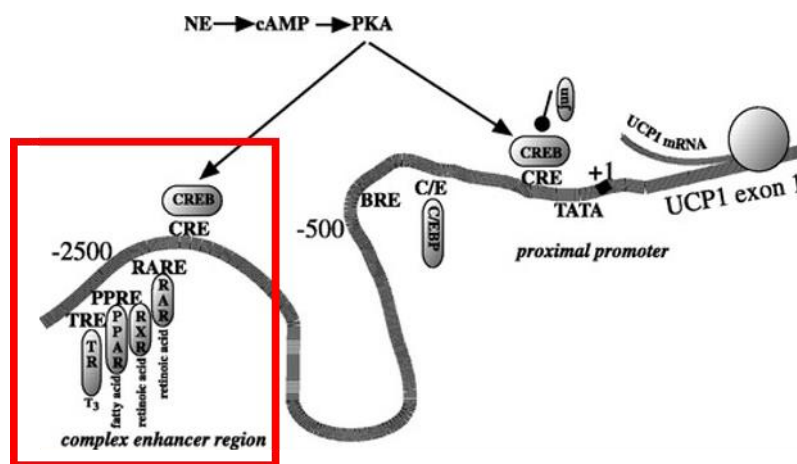


Figure 19: Schéma du promoteur du gène d'UCP1 avec le complexe amplificateur dans l'encadrement, d'après (Cannon and Nedergaard, 2004)

e. L'activation d'UCP1

L'activation d'UCP1, ou plutôt son inhibition en conditions basales, est toujours discutée autour de 2 hypothèses : i) la présence des nucléotides (ATP, ADP, GDP et GTP) serait inhibitrice (Bast-Habersbrunner and Fromme, 2020; Nicholls and Locke, 1984) et ii) l'absence de ses facteurs activateurs, notamment les AG (Cannon and Nedergaard, 2004). En effet, les AG sont des régulateurs positifs de l'activité d'UCP1 mais là encore, la manière dont l'activation a lieu reste débattue autour de 4 possibilités : i) par un effet allostérique, les AG se fixent sur UCP1 et modifient sa conformation pour permettre l'ouverture du canal aux protons, ii) les AG servent de co-facteur en se liant à UCP1 et, chargés négativement, entraînent les protons chargés positivement à traverser le canal, iii) les AG serviraient de transporteurs aux protons pour traverser de manière passive la membrane mitochondriale et passer à travers UCP1 (Divakaruni and Brand, 2011) et iv) les AG seraient en compétition avec les nucléotides sur UCP1, leur présence déplacerait ainsi la liaison et activerait UCP1 (Shabalina et al., 2004)

8. La formation et l'activation des adipocytes thermogéniques

L'origine des adipocytes thermogéniques

Comme décrit dans les paragraphes précédents, les études chez l'animal ont mis en évidence deux types distincts d'adipocytes thermogéniques : l'adipocyte brun retrouvé au sein du BAT dit classique (inter- et intra-scapulaire et cervical) et l'adipocyte beige retrouvé au sein du WAT (Kajimura et al., 2015).

Les premières analyses des lignages cellulaires ont révélé une origine commune des précurseurs des cellules musculaires et des adipocytes bruns, particulièrement par la présence du marqueur myogénique Myf5 (**Figure 20**). La répression de l'expression de Myf5, notamment par le facteur PRDM16, dans les précurseurs entraîne la différenciation des cellules en adipocytes bruns (Seale et al., 2008). Le facteur *Early B Cell Factor-2* (Ebf2) a cependant été décrit comme un marqueur plus spécifique des précurseurs d'adipocytes bruns. Connue pour être exprimé durant la différenciation et impliqué dans le maintien de l'identité thermogénique des adipocytes bruns en coopérant avec PPAR γ (Rajakumari et al., 2013), son rôle de contrôleur de la destinée des précurseurs n'était pas connu. Grâce à l'obtention d'une lignée de souris dont les cellules co-expriment Myf5 et GFP (traçage), il a été montré que l'expression de Ebf2 était très précoce, prenant place avant même le début de la différenciation (Wang et al., 2014).

Chez l'humain, les analyses histologiques sur des biopsies réalisées dans le cou mettent en évidence différentes populations d'adipocytes selon la profondeur du dépôt (Zingaretti et al., 2009). En effet, les adipocytes superficiels présentent une morphologie uniloculée et n'expriment pas UCP1 tandis que les adipocytes proches des muscles du cou sont majoritairement multiloculés et expriment UCP1. Ces observations suggèrent que les couches superficielles sont composées d'adipocytes blancs tandis que les plus profondes sont composées d'adipocytes thermogéniques, rappelant les WAT et BAT classiquement observés chez la souris (Cypess et al., 2013). Par ailleurs, les adipocytes prélevés au niveau intra scapulaire expriment Zic1, le marqueur du BAT classique retrouvé chez le rongeur (Lidell et al., 2013).

L'étude des origines des adipocytes beiges est plus complexe car les dépôts de WAT sont plus diffus dans l'organisme. Le WAT inguinal semble être le plus prompt à l'apparition d'adipocytes thermogéniques et c'est donc le plus étudié des dépôts de WAT chez la souris. Les études de lignages cellulaires mettent en évidence l'absence du marqueur Myf5 dans les adipocytes du WAT inguinal, thermogéniques ou non. Cela n'exclut cependant pas la possibilité que d'autres dépôts de WAT puissent exprimer Myf5 dans les adipocytes beiges (Sanchez-Gurmaches et al., 2016). Le

marqueur Ebf2 semble également présent dans les adipocytes exprimant UCP1 retrouvés dans le WAT inguinal alors qu'il est absent des adipocytes n'exprimant pas UCP1 (Wang et al., 2014). D'autres études mettent également en évidence des marqueurs communs entre les adipocytes beiges et les cellules musculaires lisses des vaisseaux sanguins suggérant une origine commune des précurseurs. Des analyses *in vitro* ont notamment montré que des cellules primaires de la paroi aortique chez la souris se différenciaient en adipocytes exprimant UCP1 lorsque l'expression de PRDM16 y était forcée (Long et al., 2014). La présence de cellules péri-vasculaires capables de se différencier en adipocytes thermogéniques a de nombreuses fois été signalée, les études précédentes pointant plutôt le rôle d'un autre type cellulaire, les péricytes (Tang et al., 2008).

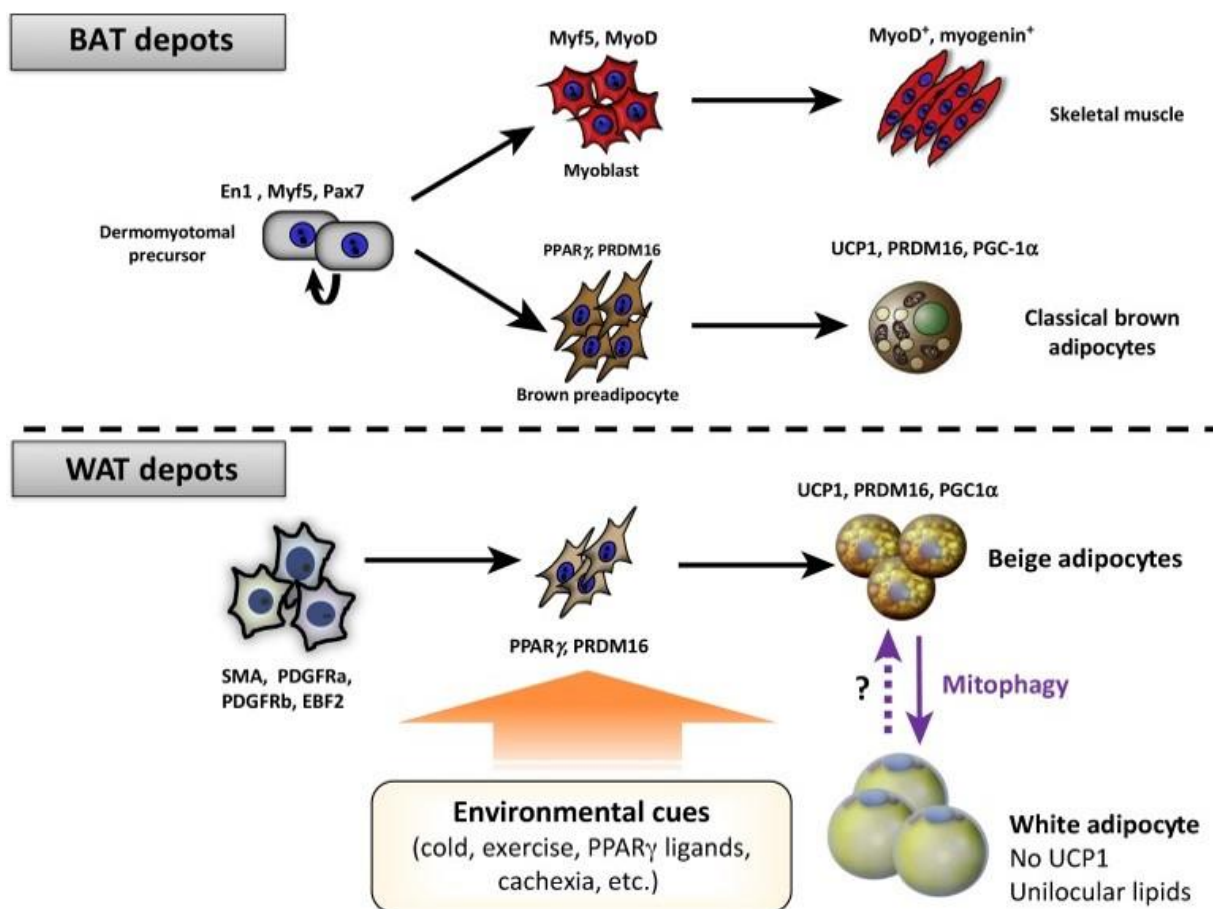


Figure 20: Les lignages cellulaires des adipocytes bruns, beiges et blancs. Les adipocytes bruns (brown) retrouvés au sein du BAT proviennent de lignées précurseurs exprimant le marqueur myogénique Myf5 tandis que les adipocytes beiges retrouvés dans le WAT expriment préférentiellement le marqueur Ebf2. L'expression de PRDM16 est commune aux adipocytes exprimant les marqueurs de la thermogénèse. PRDM16 est exprimé d'emblée dans le BAT tandis que son expression est induite par des stimuli environnementaux dans les adipocytes beiges. Chez les individus obèses, les adipocytes bruns et beiges perdent l'expression des marqueurs thermogéniques en subissant le processus de blanchiment. Schéma d'après (Ikeda et al., 2018).

Une autre question reste débattue concernant l'origine des adipocytes beiges : Comment acquièrent-ils leur phénotype thermogénique ? se forment-ils à partir de précurseurs dédiés ou sont-ils le résultat d'une transdifférenciation d'un adipocyte blanc en adipocyte beige ?

Les études les plus récentes mettent en évidence l'existence des deux processus. Sur des modèles animaux, des expériences de cartographie cellulaire montrent que l'exposition au froid induit une prolifération cellulaire dans le BAT, suggérant un recrutement de nouveaux adipocytes thermogéniques (Lee et al., 2015), mais ce phénomène n'est pas visible dans le WAT inguinal malgré la présence d'adipocytes multiloculés exprimant UCP1 (Barbatelli et al., 2010). Des études plus anciennes du laboratoire de Saverio Cinti ne montraient également pas d'augmentation de la quantité d'ADN dans le WAT, caractéristique de la prolifération cellulaire (Himms-Hagen et al., 2000). A l'inverse, d'autres expériences de cartographie cellulaire dans l'équipe de Philippe Scherer mettent en évidence qu'une exposition au froid entraîne la prolifération de nouveaux adipocytes thermogéniques au sein du WAT (Wang et al., 2013) sans implication des adipocytes matures préexistants. Ces différences entre les études peuvent s'expliquer par des limitations techniques des modèles, notamment par les techniques d'induction pharmacologique du marquage cellulaire (tamoxifène, doxycycline), mais également par les différences environnementales qui peuvent exister durant la croissance de l'animal. En effet, une hypothèse avancée est la possibilité pour les adipocytes thermogéniques du WAT de voir leurs caractéristiques phénotypiques thermogéniques masquées par les conditions environnementales, particulièrement lorsque les animaux sont maintenus à thermoneutralité et qu'il n'y a pas besoin d'effort particulier pour maintenir la température corporelle. Cette hypothèse est supportée par une étude mettant en évidence la réversibilité de l'expression des marqueurs thermogéniques, notamment UCP1, lorsqu'après avoir été exposés au froid, les animaux sont exposés de nouveau à une température proche de leur thermoneutralité (Rosenwald et al., 2013).

Ces résultats peuvent suggérer que selon les conditions environnementales, l'induction d'adipocytes thermogéniques dans le WAT peut faire appel soit à la disponibilité d'adipocytes dotés d'une activité de conversion en adipocytes beiges soit à la différenciation à partir de précurseurs, de manière dépendante de la température à laquelle les animaux ont été élevés et maintenus.

La détermination de l'origine et de l'activation des adipocytes beiges est d'autant plus importante que de nombreuses études mettent en évidence l'existence de population mixte (adipocytes blancs et thermogéniques) dans le BAT humain (Lee et al., 2014; Sharp et al., 2012).

Les voies d'activation

a. La voie β -adrénergique

Lors d'une exposition au froid, la baisse de la température est détectée par le système nerveux central qui amène le système nerveux sympathique à sécréter de la noradrénaline. Le BAT est un organe richement innervé par les fibres nerveuses sécrétant des catécholamines. La noradrénaline active ses récepteurs à la surface des adipocytes thermogéniques. De nombreuses études ont montré que la dénervation du BAT ou l'invalidation des enzymes de synthèse des catécholamines (la tyrosine-hydroxylase essentiellement) des fibres nerveuse sympathiques rend le BAT incapable de répondre aux stimuli de température (Blaszkiwicz et al., 2019).

Les récepteurs aux catécholamines existent sous plusieurs isoformes : les récepteurs $\alpha 1$ et $\alpha 2$ et les récepteurs $\beta 1$, $\beta 2$ et $\beta 3$. Les récepteurs adrénergiques sont couplés à des protéines G, les récepteurs α à des protéines Gq et Gi, inhibitrices de la signalisation tandis que les récepteurs β sont couplés à des protéines Gs, activatrices.

Chez la souris, c'est le récepteur adrénergique $\beta 3$ qui est majoritairement représenté et sollicité en cas d'exposition au froid. Il peut également être activé de manière pharmacologique par un agoniste, le CL-316,243.

L'activation des récepteurs adrénergiques $\beta 3$ entraîne une augmentation de l'AMPc et l'activation de la PKA qui va phosphoryler le facteur de transcription CREB. Le facteur CREB contrôle l'expression de gènes possédant un élément de réponse CRE sur leur promoteur, ce qui est le cas d'UCP1.

Par ailleurs, la stimulation des récepteurs β -adrénergiques va induire la lipolyse afin de produire des substrats à la thermogénèse. Les adipocytes thermogéniques utilisent également le glucose comme substrat et l'exposition au froid stimule l'expression des transporteurs de glucose à la membrane cellulaire, notamment GLUT1 (Dallner et al., 2006).

b. La voie des MAP Kinases

Une autre voie activée par la PKA est la voie des *Mitogen Activated Pathway Kinase* (MAPK) dont la MAPK p38 qui va induire phosphoryler et activer PGC1 α (Cao et al., 2004). La MAPK p38 active également le facteur de transcription 2 (*Atf-2, activated transcription factor-2*) qui induit l'expression de PGC1 α .

c. Le canal TASK1

Enfin, notre équipe a montré que le canal potassique *Twik-related acid-sensitive K⁺ channel* (TASK1) était capable de moduler l'activité des récepteurs β -adrénergiques. L'expression de

TASK1 est corrélée à l'expression d'UCP1 et les animaux invalidés pour TASK1 présentent un phénotype obèse (Pisani et al., 2016).

d. Les voies d'activation indépendantes des catécholamines

D'autres voies de régulation de la thermogenèse semblent également exister, indépendantes des voies neuronales, mais leurs réels effets sont encore discutés.

Les sécrétions du muscle

On peut distinguer des produits sécrétés par le muscle en réponse à l'exercice physique et à l'exposition au froid : l'irisine et la *meteorin-like*, deux myokines sous le contrôle de PGC1 α (Boström et al., 2012; Rao et al., 2014) et l'acide β -aminoisobutyrique (BAIBA), un acide aminé modifié, qui induit le brunissage du WAT et la β -oxydation *in vitro* et *in vivo*. En outre, une augmentation de la quantité de BAIBA plasmatique a été montrée dans une cohorte d'individus soumis à de l'exercice physique (Roberts et al., 2014).

Les batokines

On peut citer la protéine BMP8b (*Bone Morphogenic Protein*), produite par les adipocytes bruns, qui agirait de manière autocrine et paracrine pour amplifier la réponse β -adrénergique (Whittle et al., 2012).

On distingue également la *Phospholipid Transfer Protein* (PLTP) qui active la réponse thermogénique et améliore l'homéostasie du glucose (Sponton et al., 2020).

La *neuregulin 4* est une protéine sécrétée par les adipocytes bruns et par les adipocytes beige ayant acquis un phénotype thermogénique (Christian, 2014). Son rôle est essentiel dans la communication entre le système nerveux et le tissu adipeux puisqu'elle permet la croissance de l'innervation adrénergique au sein du tissu thermogénique.

Le FGF21

On peut également citer le *Fibroblast Growth Factor 21* (FGF21), sécrété par les adipocytes eux-mêmes ainsi que par le foie (Chartoumpekis et al., 2011).

Les peptides natriurétiques

Les peptides natriurétique (NP) produits par le cœur sont également capables d'activer la thermogenèse (Bordicchia et al., 2012) *via* l'activation d'une protéine kinase dépendante du GMP cyclique (PKG).

Les hormones thyroïdiennes

Les hormones thyroïdiennes sont également capables d'induire directement *via* leur récepteur nucléaire l'expression d'UCP1.

Les sécrétions digestives

Deux hormones sécrétées par l'estomac sont également impliquées dans la régulation de l'adipogenèse et de la thermogenèse : la ghréline, une hormone produite par l'estomac et dont le rôle principal est d'induire la prise alimentaire en activant une signalisation centrale dans l'hypothalamus mais qui est également impliquée dans la régulation de l'homéostasie glucidique (Sovetkina et al., 2020) ; et la sécrétine, récemment décrite comme une hormone produite par le duodénum en réponse à la prise alimentaire et qui va stimuler la thermogenèse post-prandiale en activant ses récepteurs fortement exprimés dans les adipocytes bruns (Li et al., 2018).

Les acides biliaries sécrétés lors de la prise alimentaire sont également capables de favoriser l'activité thermogénique du BAT en activant leur récepteur TGR5 (Saito et al., 2020).

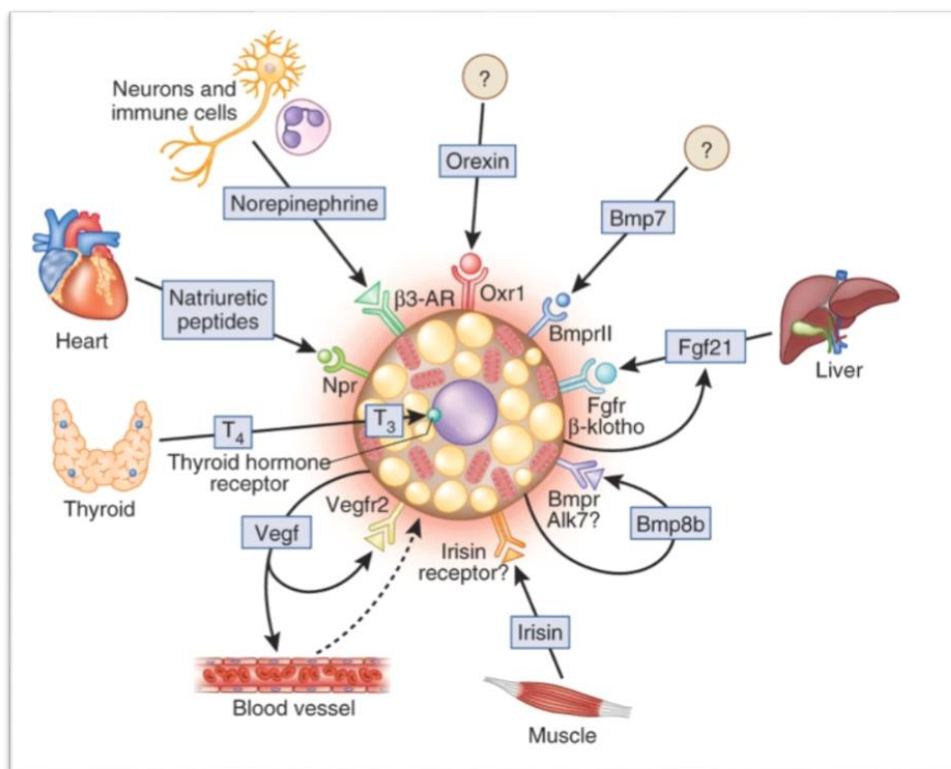


Figure 21 Schéma récapitulatif des voies d'induction de l'adipogenèse brune et de la thermogenèse. Schéma d'après (Harms and Seale, 2013)

Les neurotransmetteurs

Enfin, le neurotransmetteur orexine semble jouer un rôle important dans la thermogenèse *via* la MAPK p38 et dans le recrutement de nouveaux adipocytes thermogéniques. La sérotonine semble jouer deux rôles opposés : sa sécrétion au niveau central entraîne la thermogenèse (McGlashon et al., 2015) et favorise le recrutement tandis que sa sécrétion périphérique par des cellules de l'intestin inhibe la lipolyse et la thermogenèse et favorise la lipogenèse dans le WAT (Wyler et al., 2017).

Le microbiote intestinal

De récentes études semblent également montrer que la régulation de la thermogenèse et l'apparition de nouveaux adipocytes thermogéniques (brunissage du WAT) implique le microbiote intestinal. Outre le fait que la qualité de l'alimentation modifie le profil des bactéries du tube digestif, des expériences d'exposition au froid d'animaux ont mis en évidence une modification du microbiote intestinal. Lorsque ce microbiote est transféré chez des animaux dépourvus de germes, cela entraîne une perte de poids associée à une diminution de la taille des adipocytes viscéraux et le brunissage du WAT (Chevalier et al., 2015). Ces résultats ont été confirmés en modifiant le microbiote intestinal avec des antibiotiques (Suárez-Zamorano et al., 2015). Les mécanismes par lesquels le microbiote intestinal peut réguler le brunissage et la thermogenèse ne sont pas encore connus mais les hypothèses semblent mener au rôle des acides biliaires régulés par les bactéries et dont les récepteurs sont fortement exprimés par les adipocytes bruns (Ridlon et al., 2014). Par ailleurs, les bactéries sécrètent des acides gras appelés acides gras à chaîne courte, capables d'activer, de manière directe et indirecte, le BAT et le brunissage du WAT (Harris et al., 2020).

Les micro-ARNs

Une autre classe de molécules peut être impliquée dans la régulation de la thermogenèse et du brunissage du WAT, ce sont les microARNs (miR). Certains vont être des régulateurs positifs comme par exemple miR-196a qui va inhiber un inhibiteur de C/EBP β (Mori et al., 2012) ou miR-26 qui induit l'expression des gènes de la thermogenèse dans le modèle cellulaire hMADS (Karbiener et al., 2014). D'autres vont avoir des effets inhibiteurs sur l'activation du BAT comme miR-27 qui inhibe PPAR α , PRDM16 et CREB (Sun and Trajkovski, 2014) ou miR-125 qui inhibe la biogenèse mitochondriale (Giroud et al., 2016).

e. Les cas particuliers de thermogénèse :

La thermogénèse post-prandiale

La thermogénèse post-prandiale est considérée comme un mécanisme de thermogénèse essentielle au maintien de la balance énergétique sur le long terme. Chez la souris, la consommation d'un repas est suffisante pour induire l'activation du BAT (Glick et al., 1983) mais cela reste controversé chez l'Homme en raison de limitations techniques de mesure (Peterson et al., 2017; Vosselman et al., 2013). Une étude récente a tenté d'éclaircir l'existence ou non d'une thermogénèse post-prandiale et l'implication de la prise alimentaire en mesurant plusieurs paramètres complémentaires dont la consommation d'oxygène et le flux sanguin dans le BAT après un repas, la température et les sécrétions endocrines ainsi que la quantité d'acides gras circulants (U Din et al., 2018). Leur conclusion confirme l'activation thermogénique du BAT après la consommation d'un repas et ce, de manière indépendante des voies β -adrénergiques.

La thermogénèse indépendante d'UCP1

La thermogénèse indépendante d'UCP1 est un concept récent lorsque des études ont montré que les souris, dont l'expression d'UCP1 est invalidée, sont incapables de répondre à une exposition brutale au froid mais sont capables de s'acclimater. Ces observations laissent donc supposer qu'il existe des mécanismes de compensation en l'absence de la protéine découplante (Chang et al., 2019; Enerbäck et al., 1997). On distingue plusieurs facteurs à l'origine de la thermogénèse indépendante d'UCP1.

La créatine

Tout d'abord, la créatine, un acide organique présent dans la mitochondrie qui, sous forme phosphorylée, permet majoritairement la régénération du stock d'ATP dans le muscle lorsque la synthèse mitochondriale est insuffisante. Il a été montré que le cycle de la créatine, appelé cycle futile de la créatine, est actif dans les adipocytes thermogéniques et augmente la production de chaleur (Kazak et al., 2015) (**Figure 22A**). En effet, l'utilisation d'un analogue de la créatine qui empêche son transport, le β -guanidinopropionic acid (β -GPA), entraîne une diminution de l'activité respiratoire et donc de la dépense énergétique des adipocytes thermogéniques du WAT et du BAT chez la souris exposée au froid. La même observation est réalisée dans des adipocytes bruns humains et l'inhibition induite par le β -GPA peut être restaurée par la supplémentation en créatine.

En l'absence d'expression d'UCP1 (invalidation génétique), les animaux progressivement acclimatés au froid sont capables de maintenir leur température corporelle mais l'injection de β -GPA inhibe la thermogénèse. Par ailleurs, l'invalidation génétique spécifique du tissu adipeux de

l'enzyme de synthèse de la créatine, la *glycine amidinotransferase* (GATM), rend les animaux incapables de maintenir leur température corporelle et sensible au développement de l'obésité induite par l'alimentation (Kazak et al., 2017). Ces résultats mettent en évidence que le cycle futile de la créatine est un mécanisme participant pleinement à la thermogénèse et de manière indépendante de l'activité d'UCP1.

Le calcium

Un autre cycle futile participant à la thermogénèse non frissonnante mais indépendante d'UCP1 est le cycle du calcium (**Figure 22B**). Il a tout d'abord été décrit dans le muscle (Bal et al., 2012) puis dans les adipocytes thermogéniques du WAT (Ikeda et al., 2017). Des études montrent que le transport du calcium entre le réticulum et le cytoplasme peut engendrer une dissipation de l'énergie sous forme de chaleur. En effet, sous l'effet d'une stimulation adrénergique (exposition au froid ou agonistes des récepteurs adrénergiques), la voie de régulation du calcium impliquant la sarco / réticulum endoplasmique Ca^{2+} -ATPase (SERCA) et le récepteur à la ryanodine sont activés. La SERCA utilise l'énergie de l'hydrolyse de l'ATP pour permettre l'entrée du calcium dans le réticulum tandis que l'activation du récepteur à la ryanodine permet la sortie du calcium dans le cytoplasme, entraînant ainsi une dissipation de l'énergie sous forme de chaleur. Les études d'inactivation *in vivo* des gènes exprimant ces protéines ont mis en évidence leur importance dans la thermogénèse du WAT (Mottillo et al., 2018).

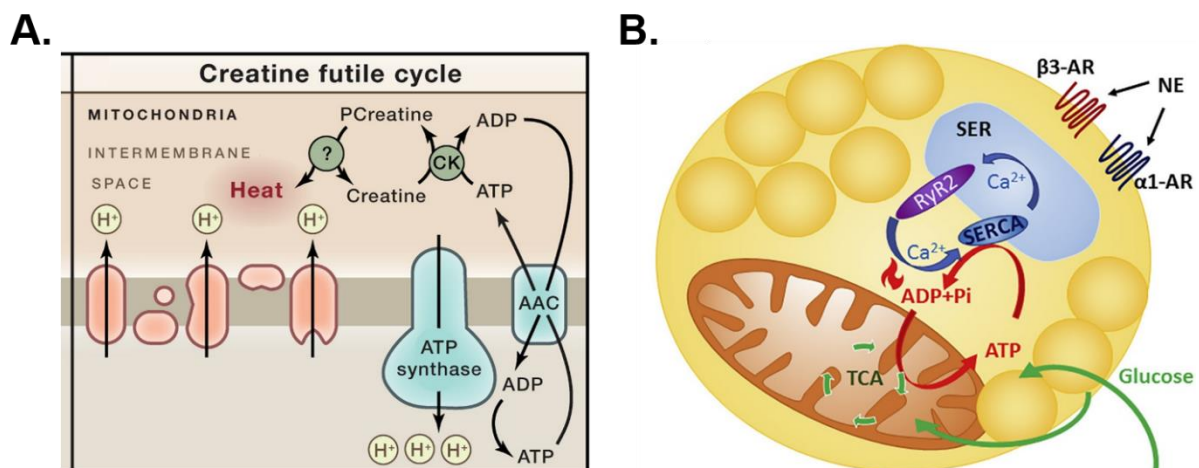


Figure 22: Les cycles futiles thermogéniques de la créatine et du calcium. Le cycle futile de la créatine (A) a lieu dans la mitochondrie et découple la synthèse d'ATP *via* l'ATP synthase, dissipant l'énergie sous forme de chaleur. Le cycle du calcium (B) a lieu entre le réticulum endoplasmique et le cytoplasme. Schémas (A) d'après (Reilly and Saltiel, 2015) et (B) d'après (Mottillo et al., 2018).

Les acides aminés N-acyles (NAAs)

Les adipocytes thermogéniques expriment l'enzyme Pm20d1 qui co-localise avec UCP1 et est également induite lors d'une exposition au froid. Pm20d1 produit des acides aminés N-acyles (*N-acyl amino acids*, NAAs) à partir d'acides aminés et d'acides gras qui semblent être capables d'interagir avec des protéines de la membrane interne de la mitochondrie en condition basale et d'activer un découplage respiratoire (Long et al., 2016).

Le turnover des lipides

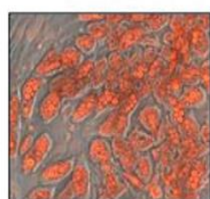
La rotation entre l'hydrolyse et la synthèse des triglycérides prenant place lors de la lipolyse induite par l'exposition au froid ou la stimulation β -adrénergique, est également impliquée dans la dépense énergétique indépendante d'UCP1 (Mottillo et al., 2014). Enfin, chez les animaux invalidés pour UCP1, il semblerait que l'enzyme *glycerol-3-phosphate dehydrogenase* soit capable de compenser et d'induire la dépense énergétique (Anunciado-Koza et al., 2008).

9. Les cellules hMADS

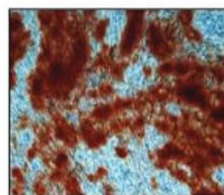
a. Description du modèle

Notre laboratoire a mis au point un modèle cellulaire à partir de biopsies pédiatriques de WAT sous-cutané : les *human Multipotent Adipose-Derived Stem cells* ou cellules hMADS (Rodriguez et al., 2004). Ce sont des cellules non transformées, possédant un caryotype normal, capables d'auto-renouvellement et sans tumorigénicité. *In vitro*, les cellules hMADS peuvent se différencier en ostéoblastes, en chondrocytes ou en adipocytes (**Figure 23**). *In vivo*, elles ont montré des capacités de régénération osseuse et musculaire (Elabd et al., 2007; Rodriguez et al., 2005). Les cellules hMADS ont été établies à partir de 12 donneurs différents mais seules 3 sont principalement utilisées et lors de mon travail de thèse, je me suis exclusivement consacrée à l'utilisation des cellules hMADS 3 provenant du WAT pré-pubien d'un donneur âgé de 4 mois.

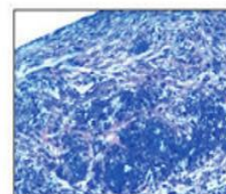
- Les cellules hMADS sont multipotentes au niveau clonal



Adipocytes



Osteoblastes



Chondroblastes

Figure 23 Photos des cellules hMADS différenciées en adipocytes, ostéoblastes et chondroblastes.

b. Différenciation des adipocytes hMADS

La prolifération des cellules hMADS se réalise en présence de sérum de veau foetal et d'un facteur mitogène FGF2 (Zaragosi et al., 2006). A la confluence, le FGF2 est enlevé pour permettre aux cellules hMADS de sortir du cycle cellulaire. Les cellules hMADS exigent un mélange d'inducteurs pour démarrer le programme de différenciation adipocytaire. La différenciation se déroule à présent dans un milieu dépourvu de sérum, pendant 18 jours et contenant en permanence de l'insuline, de la transferrine et de la T3. Durant les premiers jours de différenciation (considéré comme l'engagement), un mélange d'inducteurs est ajouté, contenant la dexaméthasone (glucocorticoïde) et de l'IBMX (inhibiteur des phosphodiesterases). Après deux jours d'induction, le mélange d'inducteurs est enlevé et un agoniste de PPAR γ est ajouté, la rosiglitazone, pendant au moins 7 jours. Cela entraîne la différenciation des cellules hMADS en adipocytes blancs exprimant les marqueurs caractéristiques adipocytaires, possédant un système de transport du glucose stimulé par l'insuline (GLUT4), capables de répondre aux agonistes des récepteurs β -adrénergiques et de sécréter la leptine et l'adiponectine (Rodriguez et al., 2004).

c. Les cellules hMADS beiges

Les cellules hMADS sont également capables de se différencier en adipocytes thermogéniques fonctionnels avec un traitement ininterrompu de rosiglitazone jusqu'au jour 18. Cela s'accompagne de l'expression des marqueurs caractéristiques des adipocytes thermogéniques comme UCP1, CPT1M, CIDEA, PGC1 α et ELOV3 et d'une augmentation de l'activité mitochondriale (Elabd et al., 2009).

Les cellules hMADS permettent également d'étudier le processus de conversion de l'adipocyte en adipocyte beige thermogénique. En effet, la reprise d'un traitement à la rosiglitazone sur l'adipocyte blanc entre les jours 14 et 18 permet de trans-différencier les adipocytes blancs en adipocytes beiges fonctionnels (Elabd et al., 2009; Pisani et al., 2011) (**Figure 24**). L'hypothèse de conversion est validée par le fait que la stimulation pendant 4 jours par la rosiglitazone exclut la possibilité d'une différenciation de nouveaux pré-adipocytes, ce sont bien les adipocytes blancs qui acquièrent le phénotype thermogénique.

D'autres inducteurs de la conversion des adipocytes hMADS blancs en beiges ont toutefois été décrits, notamment la PGI2 ou prostacycline, un métabolite oxygéné de l'acide arachidonique, est capable d'induire l'expression des marqueurs thermogéniques (Ghandour et al., 2016). Son mécanisme sera plus détaillé dans le chapitre suivant concernant les AG et leurs métabolites. Enfin, il est également possible d'induire la conversion des cellules hMADS en utilisant un agoniste pharmacologique de PPAR α , le GW7647 (Barquissau et al., 2016).

En conclusion, les cellules hMADS présentent de nombreuses caractéristiques favorables à l'étude de l'adipogenèse et à l'acquisition du phénotype thermogénique des adipocytes blancs. Ses origines humaines en font en outre, un modèle d'étude plus pertinent pour l'étude des pathologies humaines comme l'obésité.

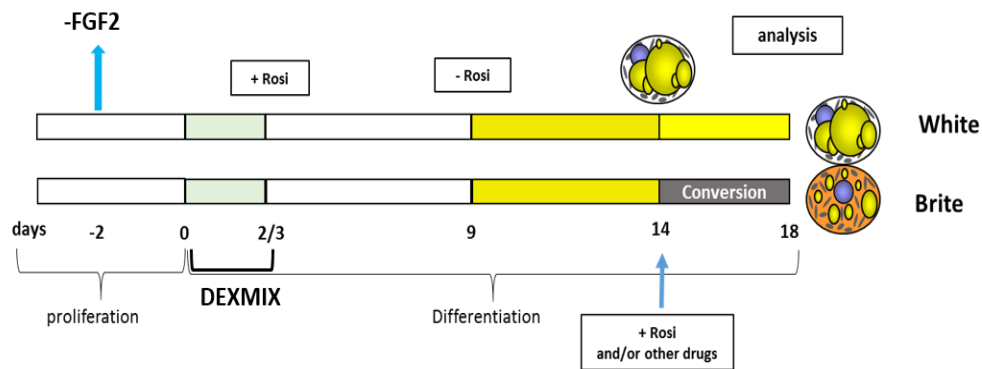


Figure 24 Protocole de différenciation des cellules hMADS en adipocytes blancs et beiges. Après une période de prolifération, l'induction de l'adipogenèse est provoquée par un mélange contenant un glucocorticoïde (dexaméthasone) et un inhibiteur des enzymes phosphodiésterases (MIX). La différenciation continue en présence de rosiglitazone (Rosi), un agoniste de PPAR γ , jusqu'au jour 9. Entre les jours 9 et 14, la différenciation en adipocytes blancs se poursuit sans rosiglitazone. Les adipocytes sont alors considérés comme des adipocytes blancs matures. Entre les jours 14 et 18, la différenciation en adipocytes blancs est maintenue (section jaune) ou la conversion en adipocytes thermogéniques (beige) est provoquée par l'ajout à nouveau de rosiglitazone ou d'autres drogues (agonistes de PPAR α et conditions expérimentales) (section grise « Conversion »).

Partie III - Les acides gras

1. Les lipides

Les lipides correspondent à une des 3 grandes familles de macronutriments sources d'énergie. On les retrouve sous plusieurs formes dont notamment les triglycérides qui servent de réserve énergétique et les phospholipides qui ont un rôle structural dans l'organisme, notamment au niveau des membranes cellulaires. On distingue aussi le cholestérol dans la famille des lipides, le cholestérol est essentiel à la synthèse des hormones stéroïdiennes et possède aussi un rôle fondamental dans la structure des membranes puisqu'il est l'élément important des plateformes métaboliques comme les radeaux lipidiques à la surface cellulaire. Les radeaux lipidiques dont le rôle est d'apporter une concentration de protéines de signalisation. Le cholestérol provient d'une synthèse de l'organisme et de l'alimentation, essentiellement les produits animaux (les végétaux apportent des phytostérols, normalement non-utilisables par l'organisme mammifère mais la possibilité d'une perturbation endocrinienne semble être à l'étude).

2. Les acides gras

Les triglycérides et les phospholipides sont constitués d'acides gras provenant soit de l'alimentation soit d'une synthèse de l'organisme. Les acides gras sont distingués par leur structure biochimique par l'absence ou la présence d'une ou plusieurs insaturations (doubles liaisons dans la chaîne carbonée) donnant lieu respectivement à un acide gras saturé, mono-insaturé ou polyinsaturé (**Figure 25**). On peut également distinguer les acides gras selon leur origine ou celle de leurs précurseurs, si elle est uniquement alimentaire, on parlera alors d'acide gras essentiel et c'est le cas des acides gras polyinsaturés linoléique (oméga6, $\omega 6$) et alpha-linolénique (oméga3, $\omega 3$). Les acides gras non essentiels sont représentés par les acides gras mono-insaturés comme l'acide oléique ou par les acides gras saturés comme l'acide palmitique. Les acides gras mono-insaturés peuvent être synthétisés à partir de l'AGS palmitique : la *delta-9-desaturase* introduit une double liaison sur l'acide palmitique ou stéarique pour former les acides palmitoléique (C16:1 n-7) et oléique (C18:1 n-9), l'acide oléique étant largement majoritaire. Une partie des acides gras mono-insaturés provient également de l'alimentation. Les AGS sont synthétisés par le foie, le cerveau et le tissu adipeux, en plus de ce qui provient de l'alimentation (Voir le rapport de l'Anses pour plus de détails sur leurs fonctions).

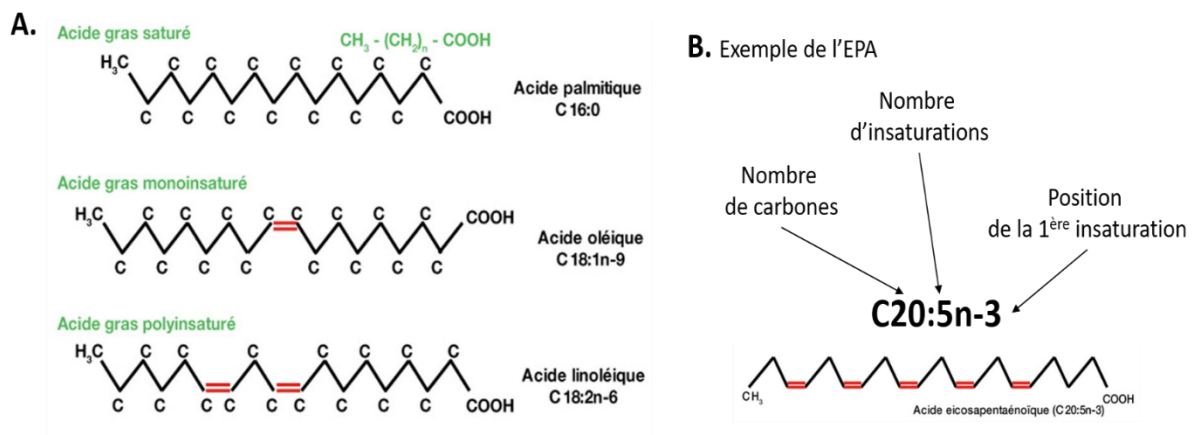


Figure 25 Les différentes sortes d'acides gras (saturé, mono-insaturé et poly-insaturé) (A) et la nomenclature commune (B)

a. Les acides gras polyinsaturés essentiels

Les dernières recommandations nutritionnelles officielles de l'ANSES (Agence Nationale de Sécurité sanitaire de l'alimentation, de l'environnement et du travail, 2011) ont pris en compte les différentes études sur les acides gras et leur impact sur la santé publique ainsi que la prévalence du surpoids et de l'obésité. « Une calorie n'est pas une calorie » et la qualité des acides gras est aussi importante que leur quantité, les acides gras $\omega 6$ et $\omega 3$ n'ayant pas les mêmes propriétés métaboliques.

Les voies de synthèse des AGPI essentiels

A partir des précurseurs essentiels LA et ALA sont respectivement synthétisés les acides gras poly insaturés (AGPI) $\omega 6$ acide arachidonique (ARA), les $\omega 3$ acides eicosapentaénoïque (EPA) et docosahexaénoïque (DHA). Les enzymes impliquées dans la synthèse et la métabolisation de ces AGPI sont communes (**Figure 26**) ce qui entraîne une compétition et rend compte de l'importance d'avoir une alimentation respectant un certain ratio entre les apports $\omega 6/\omega 3$ soit LA et ALA. Ce ratio est actuellement conseillé à 4 soit 4 LA pour 1 ALA (Simopoulos and DiNicolantonio, 2016).

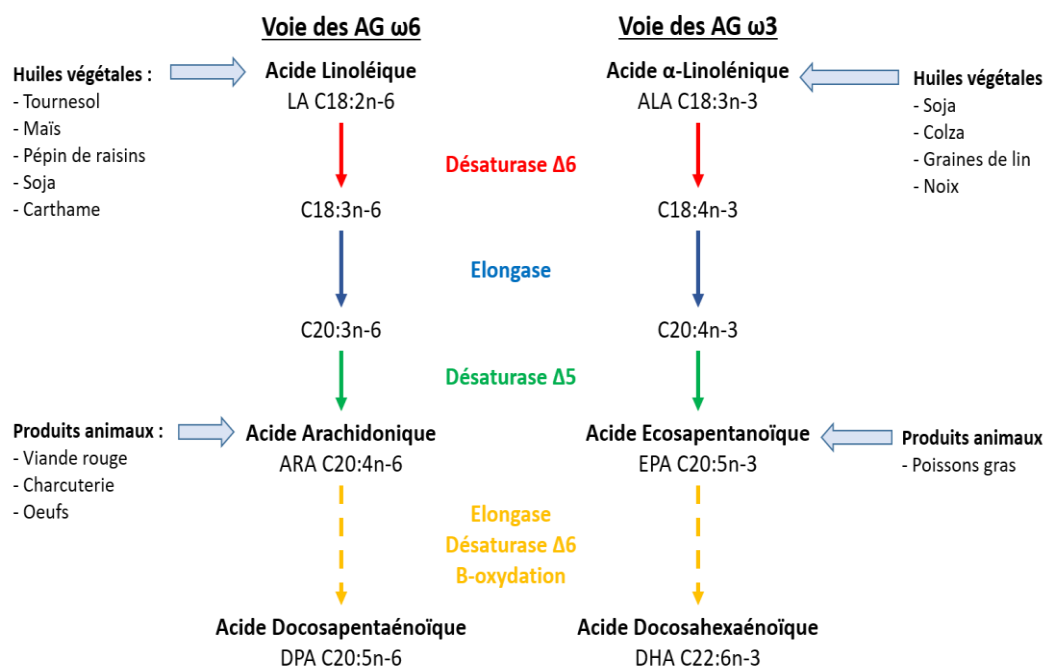


Figure 26 Les voies de synthèse des acides gras sont communes aux familles des AG $\omega 6$ et $\omega 3$

Sauf dans certains modèles animaux, notamment la souris Fat1, génétiquement modifiée pour exprimer une enzyme de *C. elegans*, qui possède la $\omega 3$ *desaturase* permettant la synthèse d'EPA et DHA à partir de sources d'AG $\omega 6$ (Kang et al., 2004; Li et al., 2014). C'est un modèle intéressant puisqu'il permet de démontrer l'importance des AG $\omega 3$ en quantité dans l'organisme. Néanmoins, en plus d'augmenter les AG $\omega 3$ il diminue les AG $\omega 6$ donc c'est double emploi et cela interroge sur ses qualités.

L'importance des AGPI essentiels dans l'alimentation

Rapporté par William Smith et Rajendrani Mukhopadhyay (Smith and Mukhopadhyay, 2012) c'est Georges et Mildred Burr en 1930 qui ont pour la première fois montré le caractère essentiel des AG dans la nutrition en étudiant les différents problèmes de santé de rats soumis à une alimentation dépourvue de lipides. Les pathologies de la peau, de l'œil et les problèmes métaboliques engendrés ont été renversés par l'ajout d'AG dans leur nutrition, notamment par du LA en petites quantités. Ce qui les amena à nommer pour la première fois AG essentiels car non synthétisés par l'organisme. Cette découverte a longtemps laissé penser que le LA était la seule source essentielle d'AG et cet AG $\omega 6$ a été supplémente jusqu' dans le milieu des années 1990 dans la nourriture, notamment le lait infantile. A partir de là, l'OMS, encouragé suite à d'autres études, l'enrichissement par les AG $\omega 6$ mais également par d'autres AG à longue chaînes, plus représentatifs du lait maternel tel que l'ARA ou encore les AG $\omega 3$. Ces études mettent en évidence

le rôle crucial des lipides dans l'alimentation et non uniquement dans le stockage d'énergie puisque la carence en AG mène à des problèmes d'ordre cutanés graves et des syndromes inflammatoires sévères.

Des études ont également montré que durant l'évolution humaine, et c'est encore le cas actuellement chez les animaux, le ratio ω_6/ω_3 consommé tournait aux alentours de 1 (1 ω_6 :1 ω_3) tandis que depuis 150 ans, le ratio tend à augmenter et est aujourd'hui aux alentours de 16 :1 dans les régimes alimentaire occidentaux hypercaloriques ou *Western diet* (Simopoulos, 2011). L'étude *INFANT Study* (Hauner et al., 2009, 2012) est la première étude prospective chez l'humain, femmes gestantes et allaitantes supplémentées en AG ω_3 et diminuées en AG ω_6 ARA. Cette étude montre que la supplémentation en AG ω_3 ainsi que la réduction en ARA n'a pas d'impact sur le poids de naissance ainsi que la prise de poids de l'enfant durant sa première année. Cette observation est contradictoire avec les études menées chez les animaux souris et rats (Massiera et al., 2003, 2010) mais pas illogique puisque le postulat principal est qu'une quantité d'AG ω_6 importante est délétère, hypothèse impossible à vérifier de manière interventionnelle chez l'humain.

b. Les AGPI essentiels et leurs dérivés sont des acteurs du métabolisme

Outre leur rôle énergétique, les AGPI ω_6 et ω_3 sont impliqués dans de nombreuses voies métaboliques. Ils sont précurseurs de métabolites essentiels dans la réponse inflammatoire de l'organisme, notamment les prostaglandines, leucotriènes et thromboxanes.

Le LA est le précurseur de l'ARA qui va être à son tour le précurseur de la majorité des médiateurs lipidiques pro-inflammatoires telles que prostaglandines et lipoxines, ce qui lui vaut d'être caractérisé d'AGPI pro-inflammatoire, contrairement à l'EPA et le DHA qui vont avoir un rôle inverse et être à l'origine des médiateurs anti-inflammatoires appelés résolvines, marésines et protectines (Serhan et al., 2014). Un rôle actif anti-inflammatoire est souvent attribué aux AG ω_3 alors qu'il semblerait que leur action soit plutôt de contrer les effets des métabolites pro-inflammatoires, en fin de réponse inflammatoire.

La régulation de la réponse inflammatoire par les médiateurs lipidiques est importante puisqu'elle conditionne la réponse aigüe à un pathogène mais peut également être impliquée dans la mise en place d'une inflammation chronique telle qu'elle est présente dans les cas d'obésité. L'inflammation chronique de bas grade caractéristique de l'obésité va entraîner notamment un dérèglement de la synthèse et de la communication métabolique de la leptine, de l'adiponectine et de l'insuline, entraînant la mise en place des comorbidités associées à l'obésité comme le diabète de type 2, les pathologies cardiovasculaires et hépatiques, des douleurs articulaires chroniques. Au-delà, cela peut même être impliqué dans l'agressivité de certains cancers puisque le système est saturé de médiateurs pro-inflammatoires.

Le LA ainsi que son métabolite ARA sont décrits comme favorisant la prise de poids menant à l'obésité, associé à une altération des sécrétions inflammatoires et adipokines menant à l'insulino-résistance et les altérations du développement adipeux (Massiera et al., 2010).

c. Les métabolites des acides gras polyinsaturés alimentaires

Les acides gras polyinsaturés $\omega 6$ et $\omega 3$ ARA et EPA sont également impliqués dans des voies de signalisation du métabolisme et de l'inflammation. En effet, ils peuvent être métabolisés en composés oxygénés par 3 grandes familles d'enzymes : les cyclooxygénases (COX), les lipo-oxygénases (LOX) et les Cytochromes P450 époxygénases (CYP450). Leurs métabolites oxygénés font partie de la famille des eicosanoïdes (20 carbones) et des docosanoïdes (22 carbones). Selon l'acide gras précurseur, les métabolites oxygénés sont appelés de série 2 (ARA), de série 3 (EPA/DHA) ou de série 1 (DGLA).

Les AGPI servant à la synthèse des métabolites oxygénés peuvent provenir des AG estérifiés en TG après lipolyse ou des AG inclus dans la membrane plasmique sous forme de phospholipides. Cette dernière voie nécessite l'intervention de la Phospholipase de type A2 pour libérer les AG de la membrane plasmique.

c.1- Les métabolites des cyclooxygénases (COX)

La famille des enzymes cyclooxygénases (COX) est la plus abondante car elle produit les principaux médiateurs inflammatoires qui sont les prostaglandines (PGs). Les COX dont l'appellation originelle est *Prostaglandin Endoperoxid H synthase* (PGHS) catalysent par une réaction de cyclooxygénation (réduction) puis de peroxydation la métabolisation de l'ARA en Prostaglandine H2 (PGH2), intermédiaire qui sera à son tour métabolisé en prostanoïdes, prostacyclines et thromboxanes (**Figure 27**). Les COX existent sous deux isoformes, COX-1 et COX-2, transcrites à partir de deux gènes distincts et régulés de manière différente. La COX-1 est exprimée de manière ubiquitaire et est la forme constitutivement active qui permet la synthèse physiologique de prostaglandines. La COX-1 est généralement localisée près de la membrane du réticulum endoplasmique. A l'inverse, la COX-2, localisée près de la membrane nucléaire, est la forme inductible dont l'expression est induite par des facteurs de croissances, l'augmentation intracellulaire d'AMPc ou encore sous l'action de facteurs inflammatoires, notamment IL-6 et NF- κ B qui possèdent des éléments de réponses sur le promoteur de la COX-2. Le promoteur de la COX-2 possède également un élément de réponse à PPAR γ et aux C/EBPs.

L'étude moléculaire et fonctionnelle des COX est d'abord importante dans la mise au point d'anti-inflammatoires non stéroïdiens mais des études ont mis en évidence leur rôle dans le métabolisme énergétique, notamment leur implication dans la formation et la fonction du tissu adipeux mais également de la mise en place de la résistance à l'insuline et de l'inflammation. En effet, la stimulation adrénérgique *in vivo* menant au recrutement d'adipocytes beiges est associée à une surexpression de la COX-2 (Madsen et al., 2010) et l'inactivation du gène inhibe l'apparition d'adipocytes thermogéniques dans le WAT sans effet sur l'activation du BAT. Par ailleurs, l'expression forcée de la COX-2 entraîne le recrutement d'adipocytes beiges (Vegiopoulos et al., 2010). Des études plus anciennes avaient d'ailleurs mis en évidence que le traitement à long terme de patients par des inhibiteurs des COX diminuait la dépense énergétique chez des patients atteints de cancer associé à un état cachexique (Davis et al., 2004; Lundholm et al., 2004). L'effet favorable de l'activité des COX semble être essentiellement lié à la production de prostaglandine I₂ (PGI₂ ou prostacycline) (Bayindir et al., 2015; Ghandour et al., 2016) dont le rôle sera décrit ci-après dans la partie traitant des prostaglandines de série 2. Toutefois, d'autres études montrent au contraire un effet délétère de l'activité des COX sur le développement de l'obésité, de la résistance à l'insuline et de l'inflammation associée. L'inhibition de l'expression de la COX-2 chez la souris diminue l'adipogenèse et l'inflammation (Ghoshal et al., 2011) et l'utilisation d'inhibiteurs spécifiques chez le rat obèse et l'humain améliore la sensibilité à l'insuline (González-Ortiz et al., 2005; Lu et al., 2018). L'implication de la COX-2 dans l'évolution de l'obésité semble être liée à la production des prostaglandines E₂ (PGE₂) et F₂ α (PGF₂ α) (Chu et al., 2010; Fain et al., 2001) qui seront également détaillées dans la partie ci-après. Une revue des manipulations moléculaires de l'expression des COX réalisées *in vivo* et *in vitro* est détaillée dans (Chan et al., 2019).

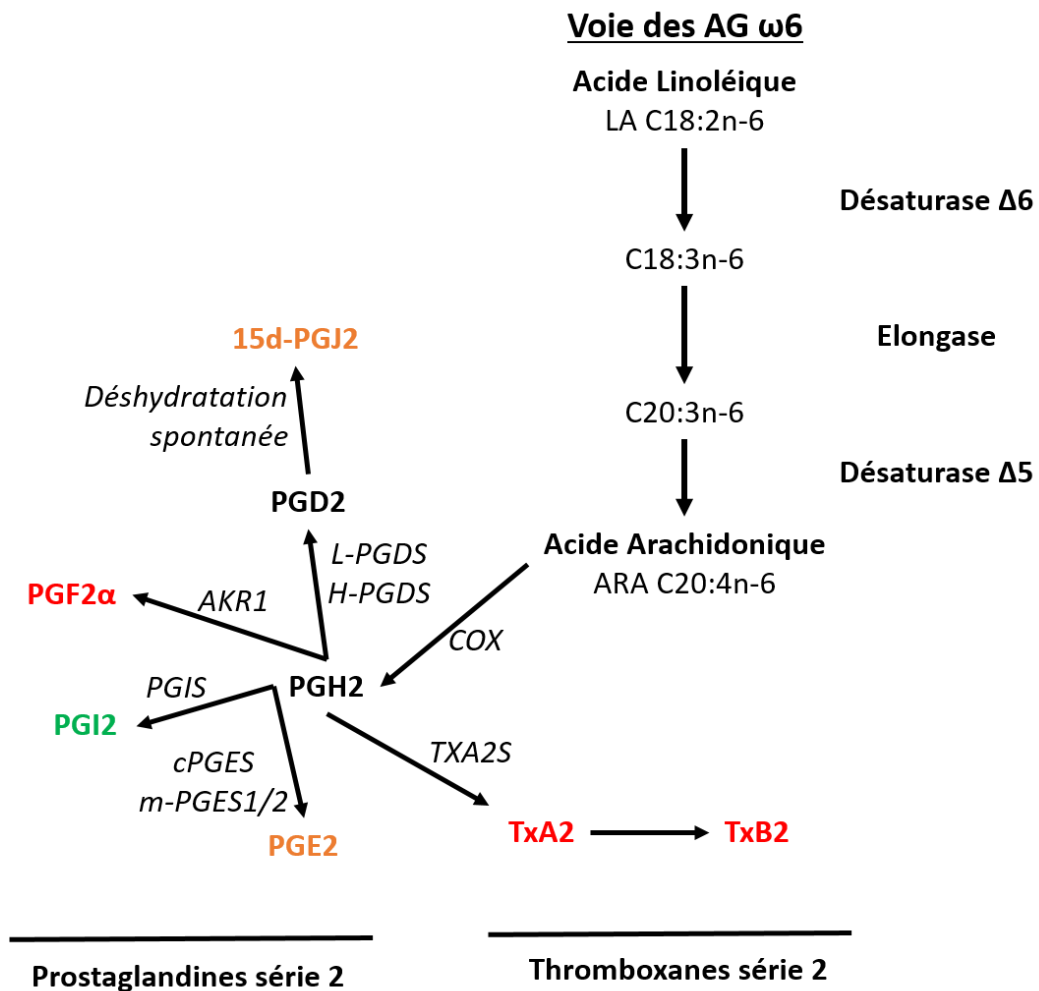


Figure 27 : les COX métabolisent l'acide arachidonique (ARA) en prostaglandines et thromboxanes de série 2. En rouge, les métabolites ayant des fonctions pro-inflammatoires connues, en vert, les métabolites ayant des fonctions anti-inflammatoires, en orange, les métabolites pouvant être pro- ou anti-inflammatoires.

L'ARA est l'AGPI majoritaire, il permet la synthèse des métabolites impliqués dans la réponse inflammatoire. L'ARA est d'abord oxydé en PGH2 par les COX puis métabolisé en thromboxane A2 par une thromboxane synthase ou en PGs par des prostaglandines synthases (PGS) : PGE2, PGF2a, PGI2 et PGD2. Les PGs agissent par le biais d'un récepteur membranaire couplé soit à une protéine G activatrice (Gs) ou inhibitrice (Gi), soit à une protéine Gq activatrice de flux calcique (**Figures 27 et 28**).

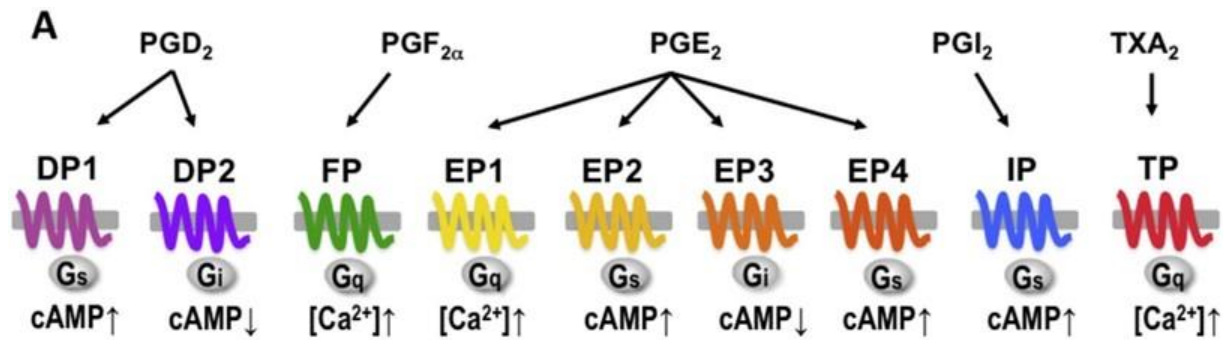


Figure 28: Les récepteurs membranaires des métabolites de série 2 produits par les cyclooxygénases. Les prostaglandines (PGs) et le thromboxane A2 (TXA2) agissent par le biais d'un récepteur membranaire couplé à une protéine G. Schéma d'après (Kawahara et al., 2015).

Les prostaglandines de série 2

La PGE₂ peut être synthétisée par 3 *Prostaglandine E Synthases* (PGES) : la PGES cytosolique et deux PGES associées aux membranes appelées les PGES microsomales (mPGES-1 et 2). La mPGES-1 semble être l'enzyme utilisée de manière préférentielle par les tissus en association avec l'activation de la COX-2 lors des réponses inflammatoires (Tanioka et al., 2000). En effet, l'expression des deux enzymes est induite par les facteurs pro-inflammatoires et la production de PGE₂ est impliquée dans la réponse inflammatoire, la fièvre, la douleur et même le cancer (Murakami and Kudo, 2004). La PGE₂ peut être tour à tour inductrice ou inhibitrice de l'adipogenèse (Barquissau et al., 2017) selon les niveaux d'expression de ses 4 récepteurs (EP1 à EP4) (**Figures 27 et 28**). Par exemple, l'action de la PGE₂ sur son récepteur EP3 semble être protecteur contre l'obésité et la résistance à l'insuline (Ceddia et al., 2016). Des études *in vivo* montrent que l'inactivation du récepteur EP3 entraîne une obésité induite par une hyperphagie (Sanchez-Alavez et al., 2007). De manière similaire, la stimulation du récepteur EP4 inhibe l'adipogenèse *in vitro* (Inazumi et al., 2011; Tsuboi et al., 2004) (**Figure 29**).

Métabolite	Enzyme/ Récepteur impliqué(e)	Modèle/ Manipulation	Effets métaboliques/ Effets cellulaires	Voie impliquée	Référence
PGE2	EP3	Invalidation globale	Hyperphagie, obésité, résistance à l'insuline	Non spécifiée	(Ceddia et al., 2016; Sanchez- Alavez et al., 2007; Xu et al., 2016)
		Invalidation globale des isoformes EP3 α et EP3 γ	Obésité, résistance à l'insuline et hyperlipidémie	AMPc/PKA	(Xu et al., 2003)
	Microsomal PGES-1	Inhibition pharmaco et siRNA	Favorise la différentiation en adipocytes blancs au lieu de beiges	PPAR γ	(García-Alonso et al., 2013)
	EP4	Agoniste/Antagoniste <i>in vitro</i>	Inhibition/Activation de la différenciation adipocytaire des 3T3- L1	AMPc	(Tsuboi et al., 2004)
	EP4	MEF de souris avec Invalidation globale	Induction de l'adipogenèse	PPAR γ 2	(Inazumi et al., 2011)
	EP2/EP4	Adipocytes primaires humains et murins	Augmentation adipogenèse	AMPc/PKA	(Vassaux et al., 1992)
	EP1	Observations <i>in vitro</i>	Inhibition adipogenèse	Calcique	(Barquissau et al., 2017)

Figure 29: Tableau des études sur le rôle de la prostaglandine E2. Tableau non exhaustif.

La PGF2 α est synthétisée par des aldo-kéto-réductases (AKR) dont il existe plusieurs isoformes, notamment AKR1B1 et AKR1C3 dans le tissu adipeux humain et Akr1b3 et 7 chez la souris. L'action de la PGF2 α est médiée par le récepteur membranaire FP couplé à une protéine Gq induisant un flux calcique (**Figures 27 et 28**). La PGF2 α possède des propriétés pro-inflammatoires (Basu, 2010). En effet, l'invalidation du gène codant pour le récepteur FP diminue les effets cardiaques induits par l'inflammation due à une injection de lipopolysaccharide (LPS) (Takayama et al., 2005) et l'utilisation d'antagonistes pharmacologiques diminue l'inflammation systémique globale engendrée par le LPS (Maehara et al., 2020). Par ailleurs, la PGF2 α inhibe la différenciation adipocytaire et l'acquisition du phénotype thermogénique (Ghandour et al., 2018). De manière surprenante cependant, l'invalidation chez la souris du gène Akr1b7 entraîne une prise de poids importante chez les animaux suggérant une rôle anti-adipogénique des enzymes de synthèse de la PGF2 α (Volat et al., 2012). Des résultats similaires ont également été obtenus *in vitro* sur des cellules 3T3-L1 dans lesquelles, l'expression de Akr1b3 a été inhibée en utilisant des petits ARN interférents (Fujimori et al., 2010) (**Figure 30**).

Métabolite	Enzyme/ Récepteur impliqué(e)	Modèle/ Manipulation	Effets métaboliques/ Effets cellulaires	Voie impliquée	Référence
PGF2 α	FP	Antagonistes pharmacologiques	Diminution de l'inflammation induite par le LPS	Non spécifiée	(Maehara et al., 2020)
		Invalidation globale chez la souris		Non spécifiée	(Takayama et al., 2005)
	Akr1b7	Invalidation globale chez la souris	Obésité, résistance à l'insuline	Non spécifiée	(Volat et al., 2012)
		Invalidation dans 3T3-L1	Augmentation de l'adipogenèse	Non spécifiée	(Tirard et al., 2007)
	Akr1b3	ARN interférents sur 3T3-L1	Augmentation de l'adipogenèse	PPAR γ	(Fujimori et al., 2010)

Figure 30: Tableau des études sur le rôle de la prostaglandine F2 α . Tableau non exhaustif.
LPS : Lipopolysaccharide

La PGI2, appelée également prostacycline, est synthétisée par la PGIS nucléaire ou de la membrane du réticulum endoplasmique. Elle agit par le biais du récepteur IP couplé à une protéine Gs et induit une augmentation intracellulaire d'AMPc (**Figures 27 et 28**). La PGI2 est également capable d'activer les récepteurs nucléaires PPARs (Brun et al., 1996), notamment PPAR β/δ , ce

qui entraîne des effets vaso-dilatateurs, anticoagulants et pro-angiogéniques (Lim and Dey, 2002). La PGI₂ est activatrice de PPAR γ et induit l'adipogénèse ainsi que la thermogénèse (Bayindir et al., 2015; Ghandour et al., 2016). L'invalidation du gène codant pour son récepteur (IP) annule les effets pro-adipogénique de la supplémentation en AG ω 6 dans l'alimentation des souris (Massiera et al., 2003). Son utilisation thérapeutique est néanmoins limitée par la demi-vie rapide de la PGI₂ qui se transforme en 6ketoPGF 1α , ce qui a conduit les études sur le développement d'analogues stables utilisables en recherche (Hildebrand, 1992; Hildebrand et al., 1990).

La PGD₂ est synthétisée par l'action de la Lipocaline-PGDS (L-PGDS) ou de l'hématopoïétique-PGDS (H-PGDS). Elle agit *via* le récepteur DP1 couplé à une Gs activant l'adénylate cyclase ou *via* le récepteur DP2 couplé à une Gi inhibant l'adénylate cyclase (**Figures 27 et 28**). C'est une prostaglandine aux propriétés pro-inflammatoires impliquée dans la réaction allergique et la douleur tactile. Le récepteur DP2 est également appelé CRTH2, un récepteur chimio-attractant pour les éosinophiles (Powell, 2003). L'activité de l'enzyme L-PGDS semble impliquée dans la production de leptine par le tissu adipeux (Yeh et al., 2019). La PGD₂ est instable et est rapidement déshydratée en 15-Deoxy-delta-12,14-prostaglandin J₂ (15delta-PGJ₂) (par l'albumine *in vitro*), aucune enzyme catalysant cette réaction n'a été mise en évidence *in vivo*. Les niveaux de 15dPGJ₂ sont donc très faibles. 15dPGJ₂ a été décrite en 1995 par deux équipes comme étant un ligand endogène de PPAR γ capable de l'activer (Forman et al., 1995; Kliewer et al., 1995) mais les faibles quantités dosées *in vivo* rendent cette hypothèse improbable (Bell-Parikh et al., 2003).

Les thromboxanes de série 2

Les thromboxanes (TX) sont synthétisées par la TX synthase (TXA₂S) à partir de PGH₂. La TXA₂S est localisée à la membrane du RE ou nucléaire et agit de concert avec la COX-1 ou la COX-2 lors d'une stimulation de la voie calcique ou des voies inflammatoires induites par le LPS respectivement (Naraba et al., 1998; Ueno et al., 2005). La forme active est TXA₂, responsable de l'agrégation plaquettaire durant la réponse inflammatoire. En milieu aqueux, TXA₂ est instable et se métabolise rapidement en TXB₂, sa forme inactive. La TXA₂ agit par un récepteur membranaire, TXA₂R ou TP, couplé à une protéine Gq induisant une voie calcique (**Figures 27 et 28**). Très étudiée dans le cadre de la réponse inflammatoire, les études montrent un rôle pro-inflammatoire de la TXA₂ dont la production est essentiellement réalisée par les plaquettes et les cellules dendritiques. La TXA₂ agit d'une manière paracrine sur les cellules endothéliales et les lymphocytes T qui expriment le récepteur TP (Kabashima et al., 2003). L'invalidation du récepteur TP chez la souris entraîne une diminution de l'inflammation systémique provoquée par une

injection de LPS, de manière similaire à l'invalidation du récepteur FP de la PGF2 α (Takayama et al., 2005) (**Figure 31**).

Métabolite	Enzyme/ Récepteur impliqué(e)	Modèle/ Manipulation	Effets métaboliques/ Effets cellulaires	Voie impliquée	Référence
TXA2	TP	Invalidation globale	Diminution de l'inflammation induite par le LPS	Non spécifiée	(Takayama et al., 2005)
		Agoniste pharmacologique <i>in vitro</i>	Différenciation de hADSCs en cellules musculaires lisses	ERK/p38 MAPK	(Yun et al., 2009)
	TXA2S	Invalidation globale	Atténuation de la fibrose du tissu adipeux et amélioration de la sensibilité à l'insuline	Non spécifiée	(Lei et al., 2015)

Figure 31 : Tableau des études sur le rôle de la thromboxane A2 (TXA2). Tableau non exhaustif.
LPS: Lipopolysaccharide, hADSC : human Adipose-derived Stem Cells

Les prostaglandines de série 3

Les COX métabolisent aussi des produits dont l'EPA est précurseur : les eicosanoïdes de série 3. On distingue notamment la PGI3 et la *15-Deoxy-delta-12,14-prostaglandin J3* parmi les prostaglandines et les TXA3 (**Figure 32**).

La PGI3 possède les mêmes effets anti-agrégation plaquettaire avec la même efficacité que la PGI2 (Kobzar et al., 2001).

La *15-Deoxy-delta-12,14-prostaglandin J3* (15delta-PGJ3) a été décrite comme stimulatrice de la sécrétion d'adiponectine (Lefils-Lacourtablaise et al., 2013).

L'activité de la COX-2 durant la réponse inflammatoire est capable d'être modifiée par l'ingestion d'aspirine (switch) et métabolise alors l'EPA en acides hydroxy-eicosapentaénoïques (HEPE) dont le 18-hepe, le précurseur des résolvines E1, E2 et E3 après action des lipoxygénases 5 et 15.

Les résolvines ont des propriétés anti-inflammatoires qui viennent contrer les effets inflammatoires des produits de l'ARA.

Les thromboxanes de série 3

La thromboxane synthase permet également la synthèse de TXA3 à partir d'EPA (**Figure 32**). Peu d'études ont été réalisées sur ce métabolite qui avait été décrit comme inactif sur l'agrégation plaquettaire (Needleman et al., 1979). Une autre étude plus récente montre que TXA3 active, comme TXA2, le récepteur TP mais avec une affinité moindre. En outre, la synthèse de PG de série 3 est majoritaire par rapport à celle des TX de série 3 (Wada et al., 2007).

Les voies de synthèse des métabolites oxygénés des AGPI sont communes à l'ARA et à l'EPA, induisant une compétition de substrats avec des affinités différentes entre l'enzyme et son ligand ainsi qu'entre les récepteurs et leurs ligands (Yang et al., 2014).

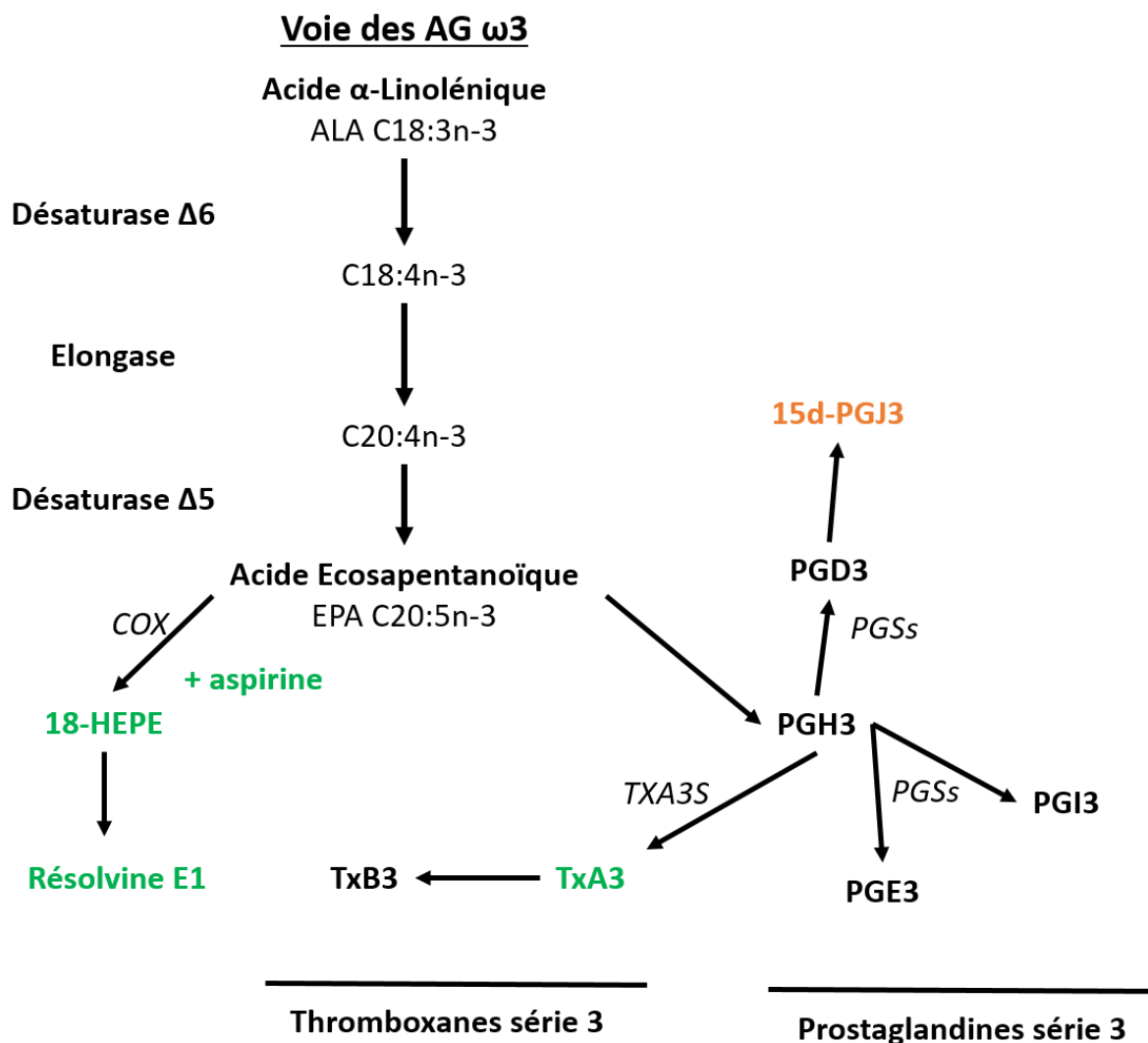


Figure 32 : Les COX métabolisent l'EPA en prostaglandines et thromboxanes de série 3. La prise d'aspirine modifie l'activité enzymatique de la COX et entraîne la production de 18-HEPE, un précurseur de la résolvine E1. En rouge, les métabolites ayant des fonctions pro-inflammatoires connues, en vert, les métabolites ayant des fonctions anti-inflammatoires.

c.2 - Les métabolites des lipoxygénases (LOX)

Les lipoxygénases (LOX) sont essentiellement à l'origine de la synthèse des leucotriènes de série 4 à partir de l'ARA et de série 5 à partir de l'EPA. Chez l'humain, on distingue 6 isoformes de LOX (7 chez la souris) selon le carbone sur lequel l'AG est oxygénée soit les LOX 5, 12, 12/15, 15, 12R et la e3 aussi appelée *epidermal LOX* (Horn et al., 2015). Les LOX 5, 12 et 15, les plus étudiées, sont représentées dans la **(Figure 33)**. Les LOX catalysent 3 réactions différentes : la di-oxygénation, l'hydroxy-peroxydation et l'époxy-oxygénation.

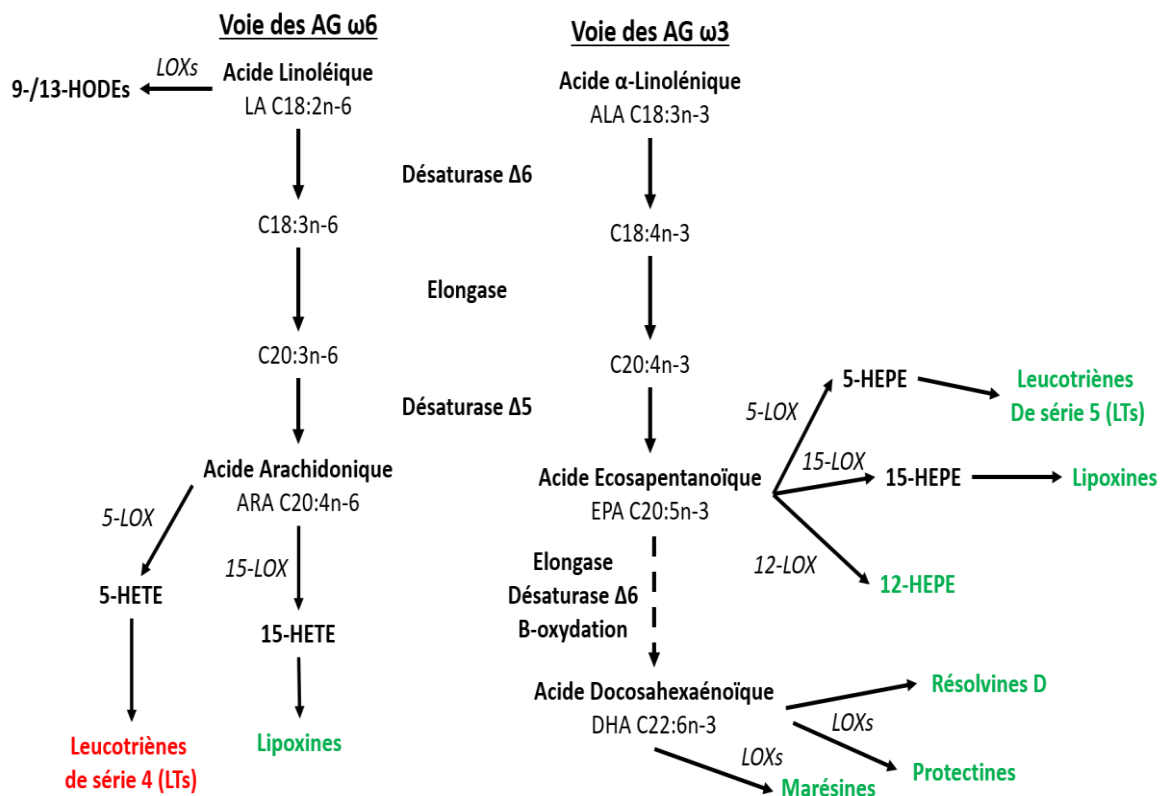


Figure 33 : Les LOX métabolisent l'ARA, l'EPA et le DHA en leucotriènes et lipoxines ainsi qu'en médiateurs de la résolution de l'inflammation appelés résolvines, marésines et protectines. En rouge, les métabolites ayant des fonctions pro-inflammatoires connues, en vert, les métabolites ayant des fonctions anti-inflammatoires.

Les produits de l'acide arachidonique (ARA) et de l'acide eicosapentaénoïque (EPA)

La métabolisation de l'ARA va former les acides hydroxyeicosatétraénoïques (HETE) dont le 5-HETE qui est le précurseur des leucotriènes (LTA4, B4, C4, D4 et E4) favorisant l'agrégation plaquettaire et la vasoconstriction (Mickael, 2014). L'EPA est lui métabolisé en acide hydroxyeicosapentaénoïques (HEPE) dont le 5-HEPE, également précurseur des leucotriènes mais leur pouvoir pro-inflammatoire est plus faible, agissant comme un faible agoniste du récepteur et

considéré comme anti-inflammatoire (Barden et al., 2018) (**Figure 33**). La 5-LOX est l'enzyme clé de la synthèse des leucotriènes qui agissent *via* les récepteurs membranaires BLT1 et BLT2. L'inactivation de l'enzyme de synthèse de la LTA4, la LTA4 hydrolase (LTA4H), protège les animaux contre l'obésité induite par une alimentation riche en graisses et augmente leur dépense énergétique sans hyperphagie (Uzawa et al., 2020). De manière surprenante, l'inactivation du récepteur BLT1 ne montre pas d'effets protecteurs contre l'obésité (Uzawa et al., 2020) mais diminue la résistance à l'insuline induite par l'obésité (Spite et al., 2011) (**Figure 34**).

Métabolite	Enzyme/ Récepteur impliqué(e)	Modèle/ Manipulation	Effets métaboliques/ Effets cellulaires	Référence
LTA4	LTA4H	Inactivation globale	Protection contre l'obésité induite par l'alimentation	(Uzawa et al., 2020)
	BLT1	Inactivation globale	Pas d'effets	
		Inactivation globale	Protection contre l'insulino- résistance induite par l'obésité	(Spite et al., 2011)
BLT1/BLT2	Antagoniste pharmacologique et siRNA dans 3T3-L1	Inhibition de la différenciation adipocytaire	(Hirata et al., 2013)	

Figure 34: Tableau des études sur le rôle de la leucotriène A4. Tableau non exhaustif.

La 15-LOX va métaboliser l'ARA et l'EPA en 15-HETE et 15-HEPE respectivement, précurseurs des lipoxines (LX) (**Figure 33**) qui sont des molécules anti-inflammatoires. Les lipoxines, et notamment LXA4, exercent leurs actions anti-inflammatoires en se liant au récepteur Formyl Peptide Receptor 2 (FPR2)/ALX et en inhibant la voie de signalisation NF- κ B (József et al., 2002; Ye et al., 2009).

L'enzyme 12-LOX produit, à partir de l'EPA, le 12-HEPE qui vient récemment d'être décrit comme une batokine sécrétée par le BAT et capable d'activer la thermogénèse *via* une action autocrine (Leiria et al., 2019).

Les produits de l'acide docosahexaénoïque (DHA)

Le DHA est le précurseur des docosanoïdes (22 carbones) à l'origine d'une classe de médiateurs lipidiques spécialisés dans la résolution de l'inflammation, appelés les résolvines de série D, les marésines et les protectines (Serhan et al., 2014) (**Figure 33, voie des AG ω 3**).

Les produits l'acide linoléique (LA)

L'acide linoléique ω 6 peut également être le précurseur direct de métabolites oxygénés par la 15-LOX : les acides 9- et 13- Hydroxyoctadécadiénoïque (HODE, 18 carbones). Ces deux métabolites ont été décrits comme des potentiels ligands de PPAR γ (Nagy et al., 1998), essentiellement synthétisés par les macrophages (Lee et al., 2016). L'entrée de ces composés dans les cellules, notamment les adipocytes, semble être possible par le transporteur et récepteur CD36 qui se lie aux oxLDL contenant les 9- et 13-HODEs (Jay et al., 2015) (**Figure 33, voie des AG ω 6**).

c.3- Les métabolites des CYP 450 époxygénases

Les produits de l'ARA : les acides epoxyeicosatriénoïques (EET)

Les CYP450 époxygénases métabolisent l'ARA en acides epoxyeicosatriénoïques (EET), une classe de médiateurs lipidiques avec des propriétés protectrices contre le stress intracellulaire. Les EET agissent essentiellement en activant des voies de protection cellulaire telle que l'Hème-Oxydase 1 (HO1). Les CYP450 existent sous plusieurs isoformes tissus-spécifiques.

Le produit de l'acide linoléique : le 12,13-dihydroxy-9Z-octadécénoic acid (12,13-diHOME)

Le LA peut également être métabolisé par les CYP450 pour produire du *12,13-dihydroxy-9Z-octadécénoic acid* (12,13-diHOME). De récentes études ont mis en évidence que ce métabolite semblait être une lipokine, sécrétée par les adipocytes bruns en cas d'exposition au froid pour potentialiser la thermogénèse en augmentant l'entrée d'AG dans la cellule (Lynes et al., 2017). Chez l'humain et chez la souris, l'exposition au froid augmente la quantité de 12,13-diHOME dans la circulation sanguine. L'injection du métabolite chez des souris augmente la thermogénèse interscapulaire et chez des animaux rendus obèses par l'alimentation, 12,13-diHOME entraîne une diminution de la quantité de triglycérides circulants. L'exercice physique semble aussi entraîner

la production de 12,13-diHOME par les adipocytes bruns pour augmenter l'entrée d'AG et leur β -oxydation dans le muscle squelettique (Stanford et al., 2018).

d. Les acides gras estérifiés sur un acide gras hydroxylé (FAHFAs)

Une autre classe de lipides présente dans le WAT a été mise en évidence en 2014 : les *fatty acids hydroxylated fatty acids* (FAHFA) (Yore et al., 2014). Il s'agit d'un acide gras « branché » sur un autre acide gras, ce qui laisse supposer une diversité importante d'espèces différentes.

Depuis leur découverte, une famille de FAHFA en particulier semble impliquée dans le métabolisme énergétique, il s'agit des PAHSAs et notamment les 5- et 9-PAHSA. Il s'agit d'un acide palmitique estérifié sur un acide stéarique hydroxylé en position 5 et 9 respectivement. Ces deux membres de la famille des PAHSAs ont été décrits comme ayant des propriétés anti-diabétiques en favorisant la tolérance au glucose. En outre, 9-PAHSA semble réguler l'activation des macrophages dans le cadre du diabète de type 1 et donc possède des propriétés anti-inflammatoires.

In vivo, la supplémentation nutritionnelle en 5- et 9-PAHSAs de souris exposées à une alimentation riche en lipides améliore leur tolérance au glucose et 9-PAHSA induit le brunissage du WAT chez des souris génétiquement obèses *ob/ob*. *In vitro*, sur le modèle 3T3-L1, 9-PAHSA semble de nouveau induire le brunissage des cellules (Wang et al., 2018).

RESULTATS :

Introduction

L'augmentation de l'obésité à l'échelle mondiale est devenue préoccupante ces dernières années et à ce jour aucun traitement pharmacologique testé n'a montré d'efficacité à long terme. Ainsi actuellement, seule la chirurgie bariatrique montre des effets positifs chez les obèses morbides et de plus en plus de patients y ont recours. Ces interventions chirurgicales sont néanmoins invasives et modifient définitivement le système digestif, le siège de sécrétions importantes dans la régulation de la prise alimentaire.

Les stratégies thérapeutiques visant à modifier le mode de vie en diminuant les apports et en augmentant la dépense énergétique semblent adaptées mais trop longues à mettre en place et à fournir des résultats et dépendent de la compliance des patients. C'est pour cela que depuis la découverte d'un tissu adipeux thermogénique fonctionnel chez l'Homme adulte (BAT), des médecins et des chercheurs ont émis l'hypothèse que l'augmentation de la dépense énergétique pouvait passer par ce tissu. En effet, le BAT consomme les lipides pour réguler la température corporelle et participe ainsi au métabolisme de base de l'organisme et à la dépense énergétique. De nombreuses études ont depuis montré que le BAT n'était pas unique puisqu'une exposition chronique au froid pouvait forcer l'apparition d'autres adipocytes thermogéniques dans le tissu adipeux blanc de stockage (WAT) et ainsi augmenter encore la dépense énergétique. L'activation du BAT et la formation d'adipocytes beiges sont évidemment régulées par l'environnement comme la température mais également par le mode de vie, c'est-à-dire l'exercice physique et la nutrition. Les apports nutritionnels, et notamment les quantités et les qualités de lipides, font l'objet d'études approfondies dans la régulation de la thermogénèse. Il a été montré que les acides gras polyinsaturés (AGPI) essentiels comme les acides gras $\omega 6$ LA et l'ARA ainsi que les acides gras $\omega 3$ ALA, EPA et DHA, sont au cœur des voies métaboliques. Ils jouent un rôle majeur dans la synthèse de précurseurs oxygénés intervenant dans la réponse inflammatoire et sont également impliqués dans la régulation de l'adipogenèse et de la thermogénèse.

Ces dernières années, plusieurs équipes de recherche, dont la nôtre, ont mis en évidence qu'une forte consommation d'acides gras $\omega 6$, notamment l'ARA, est corrélée avec l'augmentation de la masse grasse menant à l'obésité chez la souris. L'ARA est le précurseur des prostaglandines pro-inflammatoires qui vont agir directement sur le tissu adipeux pour favoriser le stockage des lipides et réguler négativement la thermogénèse. Des observations obtenues par Rayane Ghandour au laboratoire montrent que certaines prostaglandines exercent des effets favorisant la formation d'adipocytes beige (PGI₂) et d'autres plutôt inhibant cette formation (PGF₂ α). J'ai

participé à ce projet (Annexe 1) qui est à l'origine de mon projet de thèse et représente une suite logique des résultats obtenus.

Dans un premier temps, Rayane Ghandour a montré que la prostaglandine I2, ou prostacycline (cPGI2), issue de la métabolisation de l'acide arachidonique, était capable d'entraîner la conversion des adipocytes blancs en adipocytes thermogéniques *in vitro* (Ghandour et al., 2016). Le traitement des cellules hMADS différenciées en adipocytes blancs par la cPGI2 entraînait l'apparition des marqueurs de la thermogénèse dont UCP1, augmentait la mitochondriogenèse et le découplage de la respiration mitochondriale (**Figure 35**). Le traitement par cPGI2 entraînait l'expression d'UCP1 par deux voies : i) en activant son récepteur IP couplé à une protéine Gs, entraînant la phosphorylation du facteur CREB et ii) en activant PPAR γ .

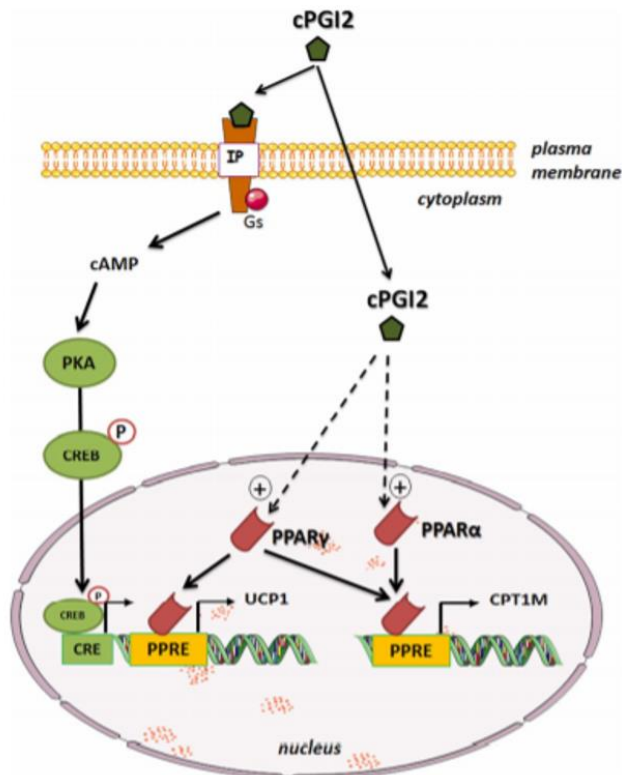


Figure 35 : Schéma récapitulatif des voies d'actions de la PGI2, d'après la thèse de Rayane Ghandour.

Dans un second temps, nous avons voulu comprendre quels étaient les mécanismes impliqués dans l'inhibition de la thermogénèse par l'AGPI $\omega 6$ ARA. Pour cela, nous avons nourris des souris avec des régimes isocaloriques enrichis en LA, le précurseur de l'ARA, ou supplémenté avec de l'ALA, le précurseur des AGPI $\omega 3$ EPA et DHA. Les résultats *in vivo* montrent que la supplémentation en $\omega 3$ augmente la réponse des tissus adipeux au CL-316,243 (agoniste $\beta 3$ -adrénergique) : i) la perte de poids des animaux est exacerbée, ce qui laisse supposer une dépense énergétique plus importante, ii) l'histologie des tissus met en évidence l'activation du BAT et le recrutement dans le WAT plus important et iii) l'expression de certains marqueurs

thermogéniques, dont UCP1, dans le BAT et le WAT est augmentée. Le dosage des métabolites oxygénés (eicosanoides) des AGPI contenus dans les tissus adipeux montre que la supplémentation en acides gras ω 3 diminue la production des prostaglandines issues de l'ARA dont notamment la PGF2 α . Ces résultats ont été confirmés *in vitro* sur le modèle cellulaire hMADS. Le traitement des adipocytes hMADS beiges par l'ARA inhibe l'expression des marqueurs thermogéniques et diminue le découplage de la respiration mitochondriale tandis que la supplémentation en EPA restaure des niveaux contrôlés. Cet effet inhibiteur semble bien être causé par la PGF2 α puisque le traitement des cellules hMADS avec un agoniste du récepteur de PGF2 α mime l'inhibition obtenue avec l'ARA (**Annexe 1**).

Mon projet de thèse s'est alors construit sur ces résultats et nous avons souhaité déterminer si d'autres métabolites des AGPI pouvaient réguler la thermogénèse et le recrutement des adipocytes beiges.

Mes travaux de thèse s'organisent en 2 parties :

- a) Dans un premier temps, la recherche de métabolites candidats
- b) Dans un second temps, nous avons mis en évidence un nouvel antagoniste du facteur de transcription majeur de l'adipogenèse et de la thermogénèse PPAR γ : l'acide carnosique

ARTICLE 1: Effects of fatty acids metabolites on adipocyte browning

Cécilia Colson¹, Pierre-Louis Batrow¹, Sebastian Dieckmann², Laurence Balas³, Claire Vigor³, Baptiste Fourmau⁴, Nadine Gautier¹, Nathalie Rochet¹, Nathalie Bernoud-Hubac⁴, Thierry Durand³, Martin Klingenspor² and Ez-Zoubir Amri^{1*}

INTRODUCTION

Afin de déterminer quels métabolites des AGPI sont impliqués dans la régulation de la formation et l'activation des adipocytes beiges, nous avons travaillé sur 3 modèles d'études :

- i) En collaboration avec le Dr. Kirsi Virtanen (Turku, Finlande), nous avons obtenu des échantillons de tissus adipeux humains sous-claviculaires prélevés, grâce à l'imagerie de PET-scan, sur des individus sains. L'imagerie a permis de cibler la zone de biopsie en suivant l'activation des tissus thermogéniques. Nous avons ainsi obtenu des échantillons de tissu adipeux actifs et donc considéré comme BAT, et des échantillons de tissus adipeux adjacent, non actifs et donc considérés comme WAT.
- ii) Des prélèvements de WAT sous cutanés et de BAT inter-scapulaire chez la souris suite à des interventions nutritionnelles avec une modulation des ratios $\omega 6/\omega 3$ et un challenge terminal avec du CL-316,243.
- iii) Notre modèle cellulaire hMADS différencié en adipocyte blanc et beige.

Pour les 3 modèles d'études, nous avons réalisé un profil des oxylipines présentes dans les tissus et le transcriptome des échantillons de tissus humains a également été séquencé. Cela nous a permis de confirmer que nous avons bien une différence d'expression d'UCP1 dans les tissus adipeux humains considérés comme BAT et ceux considérés comme WAT (**Figure 36**). Dans les deux autres modèles (BAT et WAT de souris, hMADS *in vitro*), l'expression des marqueurs est analysée par PCR quantitative.

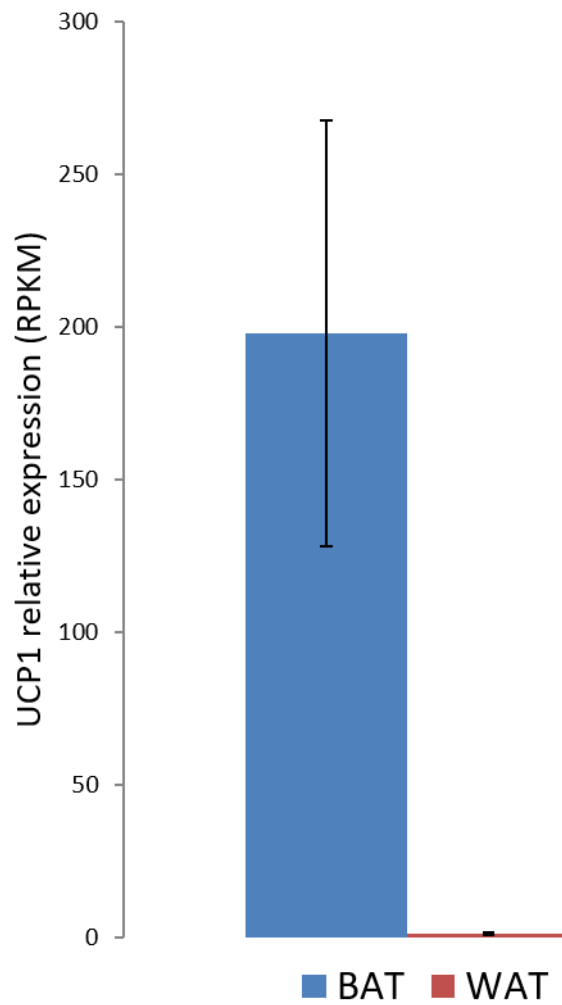


Figure 36 : Quantification des transcrits d'UCP1 par séquençage des ARNm des biopsies humaines prélevée au niveau sous-claviculaire de 15 patients soit 30 biopsies appariées (BAT et WAT)

Les profils obtenus pour les biopsies humaines ont mis en évidence des oxylipines candidates, corrélées positivement avec l'expression d'UCP1 (**Figure 37**).

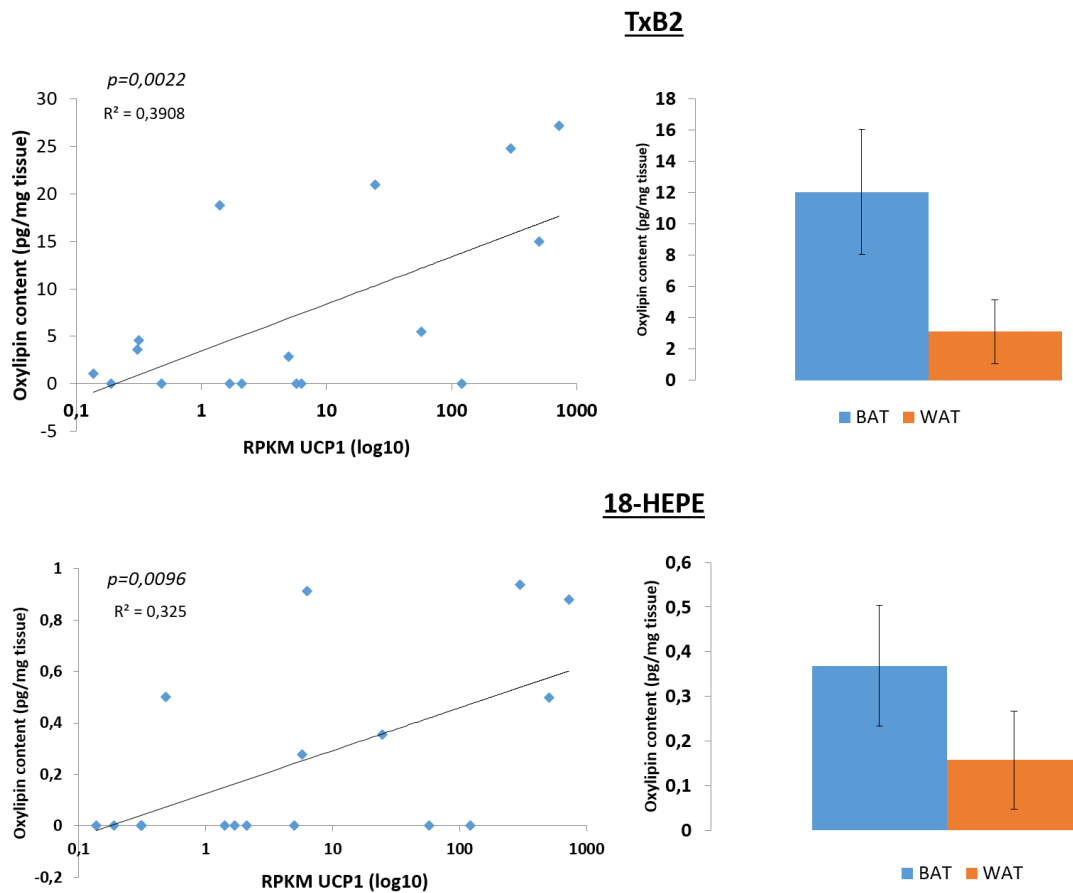


Figure 37: Corrélatifs et dosages du TxB2 et du 18-HEPE dans les biopsies humaines.

Nous avons également recherché dans la littérature des métabolites d'acides gras décrits pour être impliqués dans la régulation de la thermogénèse et le recrutement des adipocytes thermogéniques. De récentes études ont mis en évidence deux oxylipines qui favorisent la thermogénèse : 12-HEPE et 12,13-diHOME (Leiria et al., 2019; Lynes et al., 2017).

Effects of fatty acid metabolites on adipocytes browning

Cécilia Colson¹, Pierre-Louis Batrow¹, Sebastian Dieckmann², Laurence Balas³, Claire Vigor³, Baptiste Fourmau⁴, Nadine Gautier¹, Nathalie Rochet¹, Nathalie Bernoud-Hubac⁴, Thierry Durand³, Martin Klingenspor² and Ez-Zoubir Amri^{1*}

¹Université Côte d'Azur, CNRS, Inserm, iBV, Nice, France

²Chair for Molecular Nutritional Medicine, Technical University of Munich, TUM School of Life Sciences, Freising, Germany.

³Institut des Biomolécules Max Mousseron, UMR 5247, CNRS, Université Montpellier, ENSCM, Faculté de Pharmacie, Montpellier, France

⁴Univ-Lyon, Inserm UMR 1060, Inra UMR 1397, IMBL, INSA-Lyon, 69100, Villeurbanne, France

*Correspondence: Dr. Ez-Zoubir Amri, iBV, Institut de Biologie Valrose, Université Côte d'Azur, CNRS UMR 7277, Inserm U1091, Tour Pasteur; Faculté de Médecine; 28, avenue de Valombrose, 06107 Nice, France. Tel: +33 493 37 70 82; Fax: +33 493 81 70 58; E-mail: amri@unice.fr

Key words: browning; PGI₂; TXA₂; FAHFA; 15dPGJ₂; 15dPGJ₃

Abstract

Obesity is a complex disease highly related to diet and lifestyle and is associated with low amount of thermogenic adipocytes. Therapeutics that regulate brown adipocyte recruitment and activity represent interesting strategies to fight overweight and associated comorbidities. Lipokines have been shown to regulate thermogenesis through the increase of energy expenditure and systemic metabolism. The purpose of this work was to analyze the role of fatty acid metabolites in the control of brown/brite adipocyte formation. We used a human cell model (hMADS cells) which differentiates into white adipocytes and converts into brite adipocytes upon rosiglitazone or PGI₂ treatment. Gene expression was quantified using RT-qPCR and protein levels were assessed by western blotting. We show here that lipokines such as FAHFA, 12,13 diHOME, 12-HEPE and 15dPGJ_{2/3} were not able to induce browning of white hMADS adipocytes. Thromboxane A₂ (TXA₂), but not its inactive metabolite TXB₂, inhibited the rosiglitazone-induced browning of hMADS adipocytes. PGI₂ and TXA₂, originating from the metabolisation of a common precursor PGH₂ and have opposite effects vasodilator and vasoconstrictor, respectively. Our data pinpoint TXA₂ as a lipokine inhibiting brown adipocyte formation that might be antagonized by PGI₂. Our data open new horizons in the development of potential therapies based on the control of thromboxane A₂/prostacyclin balance to combat obesity and associated metabolic disorders.

Introduction

The increasing prevalence of overweight and obesity has reached “epidemic” proportions with over than 2 billion people overweight (BMI > 25 kg/m²) and at least 650 million of them clinically obese (BMI > 30 kg/m²) (1). Obesity represents a risk factor for hypertension, type 2 diabetes, and cardiovascular diseases. This increase in body weight, accompanied by an increase in the mass of adipose tissue, results from an imbalance between energy intake and energy expenditure. So far pharmacological remedies or lifestyle interventions normalizing this imbalance with significant long-term success are not efficient and for morbidly obese patients no effective treatment other than bariatric surgery is available.

The adipose organ, composed of white (WAT) and brown adipose tissue (BAT), plays a central role in the control of energy homeostasis (2-4). In contrast to WAT, BAT is specialized in adaptive thermogenesis in which the uncoupling protein 1 (UCP1) plays a key role (5,6). It is well established that active brown and brite or beige adipocytes are present in healthy adults and that these adipocytes hold great promise as novel antiobesity strategy in humans (7-9,10,11). Cold exposure is the most potent physiologic stimulus of BAT thermogenesis that results in increased energy expenditure in mice and humans. Several compounds have been reported to promote brite adipogenesis or white to brite conversion (12-15).

A number of lipids are known to play key roles in cell signaling, homeostasis and disease. They are substrates and products of enzymes constituting metabolic pathways. Lipids derive directly from diet or de novo synthesis from precursors and their metabolism is under the control of environmental and genetic variations. Reports suggested that lipids, termed as lipokines, besides serving as fuel source for heat generation might play a crucial role in controlling the differentiation and activation of thermogenic adipocytes (13,14,16). Interestingly, 12,13-diHOME and 12-HEPE have recently been described to be strongly correlated with BAT activity (17-19). Prostaglandins and prostacyclins, as well as the recently discovered family of fatty acid hydroxylated fatty acid (FAHFA), represent also potential effectors of the white into brown adipose tissue conversion and are of particular interest since their bioavailability can be modulated by nutrition (20,21).

In the present study we analyzed the involvement of fatty acid metabolites, oxylipins, towards brite adipocyte formation and function. We have used a unique human cell model, namely the human Multipotent Adipose-Derived Stem (22,23) cells, which can undergo adipocyte differentiation process and convert into functional brite thermogenic adipocytes upon stimulation with rosiglitazone, the potent PPAR agonist (25, 26, 28, 31). We tested various

compounds either selected from the literature (FAHFA, prostaglandins and metabolites) or identified in our previous correlative study (24). We found that none of the compounds tested was efficient in the control of brite adipocyte formation, except thromboxane A2 which exhibited an inhibitory effect on white adipocyte browning.

Material and methods

Reagents

Cell culture media, insulin and trypsin buffers were purchased from Invitrogen, fetal bovine serum from Eurobio, hFGF2 from Peprotech and other reagents from Sigma-Aldrich Chimie or Cayman. 15d-PGJ3 was synthesized from PGD3 as previously described (25). FAHFAs were synthesized by L. Balas as previously described (26). All compounds were checked by MS/MS before their use in culture.

Cell culture

The establishment and characterization of hMADS cells (human Multipotent Adipose-Derived Stem) have previously been described (22,27). Cells were seeded at a density of 5000 cells/cm² in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS, 15 mM Hepes, 2.5 ng/ml hFGF2, 60 mg/ml penicillin, and 50 mg/ml streptomycin. hFGF2 was removed when cells reached confluence. Cells were induced to differentiate, in a serum free medium, at day 2 post confluence (designated as day 0) in DMEM/Ham's F12 (1:1) media supplemented with 10 µg/ml transferrin, 10 nM insulin, 0.2 nM triiodothyronine, 1 µM dexamethasone and 500 µM isobutyl-methylxanthine for 4 days. Cells were treated between day 2 and 9 with 100 nM rosiglitazone (a PPAR γ agonist), to enable white adipocyte differentiation. At day 14, conversion of white to brite adipocytes was induced by rosiglitazone or the indicated compound for 4 days. Media were changed every other day and cells used at the indicated days.

Isoprostanoid quantification was carried out as described previously (28).

Adiponectin levels

hMADS cells were differentiated into white adipocytes and induced to convert into brite adipocytes in the absence or the presence of 15Delta-PGJ3. Media were collected 6 hours after the last medium change, centrifuged and frozen at -80°. Adiponectin levels were measured in media using an ELISA kit as per the manufacturer's instructions (Invitrogen).

Isolation and analysis of RNA

These procedures followed MIQE standard recommendations and were conducted as previously described (29). The oligonucleotide sequences, designed using Primer Express software, are shown in Supplementary Table 1. Quantitative PCR (qPCR) were performed using SYBR qPCR premix Ex TaqII from Takara (Ozyme, France) and assays were run on a StepOne Plus ABI real-time PCR machine (Perkin Elmer Life and Analytical Sciences, Boston). The

expression of selected genes was normalized to that of 36B4 housekeeping gene and then quantified using the comparative- Δ Ct method.

Western blot analysis

Proteins were extracted from cells or tissues as previously described (30). Equal amounts of cellular proteins, 30 to 50 μ g, were separated by electrophoresis using gradient gels (4-15%) and blotted onto PVDF membranes. Following blocking, membranes were incubated Primary antibody incubation was performed overnight at 4°C (anti-UCP1, Abcam #ab10983, dilution 1:1000; anti-TBP, CST #D5C9H, dilution 1:1000). Primary antibodies were detected with HRP-conjugated anti-rabbit or anti-mouse immunoglobulins (Promega). Detection was performed using Immobilon Western Chemiluminescent HRP Substrate (Merk-Millipore). Chemiluminescence obtained after adding Pierce ECL western blotting substrate (Thermo Scientific) was detected using an Amersham Imager 600 and quantified with Image Lab 5.0 software (Bio-Rad, France).

Statistical analyses

Data are expressed as mean values \pm SEM and were analyzed using InStat software (GraphPad Prism version 8.3.0 for Windows, GraphPad Software, San Diego, California USA). Data were analyzed by Student's t-test or one-way ANOVA followed by a Student-Newman-Keuls post-test. Differences were considered statistically significant when $p < 0.05$.

Results

FAHFA did not affect the browning process of white adipocytes

Lipidomic analysis of adipose tissues revealed the existence of a novel class of branched fatty acid esters of hydroxy fatty acids (FAHFA) that are endowed with anti-inflammatory and anti-diabetic properties (20,31). We aimed to test the effects of the most characterized FAHFAs on the process of white adipocyte browning. We investigated the effect of 10 different FAHFA compounds, chemically synthesized, using the hMADS cell model.

Cells were induced to differentiate into white adipocytes for 14 days and then induced to convert into brite adipocytes for 4 days in the presence of different FAHFAs or rosiglitazone as control. Gene expression analysis showed no effect of palmitic acid hydroxystearic acid (5-PAHSA) on mRNA levels of the key thermogenic marker UCP1, whereas rosiglitazone induced its expression as expected (Figure 1, left panel). FABP4 mRNA expression was not affected by 5-PAHSA treatment in the absence (white) or the presence (brite) of rosiglitazone (Figure 1, right panel). We observed that 5-PAHSA treatment did not affect rosiglitazone-induced brite adipocyte formation, indicating that this FAHFA did not potentiate or interfere with PPAR γ activation (Figure 1). Similar observations were obtained with other FAHFAs (7-PAHSA, 5-PAHPA, 5-OAHPA, 9-OAHPA). Our data are in contrast with a recent study showing that 9-PAHSA promotes browning of 3T3-L1 adipocytes and white adipose tissue of Wild-type and ob/ob mice (32). This discrepancy might be due to human versus murine system.

Absence of effects of prostaglandins and their derivatives on browning of white adipocytes

We and others showed that prostacyclin 2 (PGI₂), through the use of a stable compound cPGI₂, affected brown adipocyte formation and function (33-35). Herein, we aimed to test whether polyunsaturated fatty acids omega-6 and omega-3-derived prostanoids (PGI₂ and PGI₃) and metabolites ((15-deoxy-delta 12,14-PGJ₂ (15dPGJ₂) and 15-deoxy-delta 12,14-PGJ₃ (15dPGJ₃)) affected the browning process of white adipocytes.

White hMADS adipocytes obtained after 14 days of culture as described above, were treated with PGI₃, 15dPGJ₂, 15dPGJ₃ from day 14 to day 18. Treatment with carbaprostacyclin (cPGI₂, a stable analogue of PGI₂) or with 100 nM rosiglitazone were used as positive controls. UCP1 mRNA levels were analyzed at day 18 as a marker of brite adipocyte conversion. Figure 2A shows that UCP1 gene expression was increased in cPGI₂-treated cells compared to untreated cells in a dose-dependent manner though less efficiently than 100 nM rosiglitazone in agreement with previous reports (33). PGI₃ treatment has no effect on UCP1 gene expression. cPGI₂ and rosiglitazone, but not PGI₃, also enhanced the expression of FABP4 as

expected (Figure 2, middle panel). Finally, PLIN1 a standard marker of adipogenesis was not affected by any of these compounds (Figure 2, right panel).

Then, we tested whether 15dPGJ2 and 15dPGJ3, previously reported to be potential PPAR γ ligands, were able to induce brite adipocyte formation. Figure 3A shows that both compounds were not able to induce UCP1 gene expression compared to the huge effect of rosiglitazone. 15dPGJ2 and 15dPGJ3 enhanced FABP4 gene expression to a lesser extent than rosiglitazone (Figure 3B). As expected adiponectin (adipoQ) gene expression was increased by rosiglitazone and slightly by 15dPGJ2 but not by 15dPGJ3 (Figure 3D), whereas PLIN gene expression was not affected (Figure 3C). In parallel we analyzed whether adiponectin secretion was affected. Figure 4 shows brite adipocytes secreted higher adiponectin amount compared with white adipocytes and that neither 15dPGJ2 nor 15dPGJ3 altered adiponectin secretion.

Effects of some fatty acid metabolites: oxylipins

In a previous work, we quantified a panel of 36 fatty acid metabolites in brown and white adipose tissues of mice, in human brown and white adipose tissue biopsies and in hMADS adipocytes in order to correlate their levels to UCP1 mRNA expression ((24) and data not shown). We aim to analyze the effects of some of these metabolites as well as two compounds recently identified to play a role in thermogenesis (17-19).

We asked whether these fatty acid metabolites can affect the differentiation of brite adipocytes. For this purpose, as described above, hMADS cells were first induced to differentiate into white adipocytes for 14 days before testing for the next 4 days the potential of these metabolites either to induce the conversion into brite adipocytes or to modulate the rosiglitazone-induced browning.

12,13 di-HOME (Figure 5A) and 12-HEPE (Figure 5B) did not induce UCP1 gene expression (left panels) and did not interfere with rosiglitazone-induced effect (right panel). Both compounds did not affect FABP4 and PLIN1 mRNA levels (Figure A and B).

We obtained similar observations with 18-HEPE, which had no effect on any marker analyzed on white and brite adipocytes (Figure 6).

Finally, we tested the effects of thromboxane A2 (TXA2) and its metabolite thromboxane B2 (TXB2). Since TXA2 is rapidly degraded into TXB2 we used its stable analogue the carbocyclic thromboxane A2 (CTA2). As shown in Figure 7A, CTA2 did not induce the expression of UCP1 in white adipocytes. However, it significantly inhibited the rosiglitazone-induced UCP1 gene expression. CPT1M, FABP4 and PLIN1 mRNA levels were not affected by CTA2 treatment neither in white nor in brite adipocytes. TXB2, which represents the inactive product

of TXA2 did not affect UCP1 gene expression in white and brite adipocytes. Consistently to gene expression data, UCP1 protein level decreased upon CTA2 treatment (Supplementary Figure 1A) in brite hMADS adipocytes with an optimal inhibitory effect at 1 μ M of CTA2, whereas no effect was observed with TXB2 treatment (Supplementary Figure 1A).

Discussion

The obesity has become pandemic and the need to restore the energy balance has led to development of new therapeutic strategies. Formation and activation of brown adipose tissue represent a potential target that is being highly studied. Lipokines, potential therapeutic targets, have been shown to regulate thermogenesis through increased energy expenditure and improved systemic metabolism (16,36). Metabolites of ω 6 and ω 3 polyunsaturated fatty acids represent signaling molecules involved in the control of adipogenesis, energy balance and for some of them in the activation and recruitment of brown adipocytes in mice and humans (21). In a previous work, we analyzed the association between UCP1 mRNA expression and a panel of fatty acid metabolites, where few compounds correlated negatively or positively (24). Furthermore, isoprostanooids are a group of non-enzymatic oxygenated metabolites of polyunsaturated fatty acids that represent important lipid mediators in biological processes, such as tissue repair, blood clotting, blood vessel permeability, inflammation and immunity regulation. In the present work we quantified cellular and secreted isoprostanooids in white and brite hMADS adipocytes and found no differential expression in the levels of these isoprostanooids (Supplementary Figure 2).

In this study, using a human cell model, we analyzed the effects of various fatty acid metabolites on the formation of brown/brite adipocytes reported in our previous work and from the literature (19,20,24,37). Fatty acid esters of hydroxylated fatty acids have been discovered as novel class of endogenous mammalian lipids having anti-inflammatory and antidiabetic effects (38-40). 5-PAHSA treatment of hMADS adipocytes did not induce any conversion of white to brite adipocytes or inhibit rosiglitazone-induced UCP1 mRNA expression. Our observations contrast with previous data showing that 9-PAHSA promotes browning of white 3T3-L1 adipocytes and in vivo using wild type and ob/ob mice (32). Since FAHFAs act through GPR120, this discrepancy might be explained by a difference between human and mouse model, albeit hMADS cells express this receptor (data not shown).

Prostaglandins play important role in the control of adipogenesis and thermogenesis (16,41). Our data show that PGI3, PGD3 and PGJ3 (data not shown), derived from the ω 3 polyunsaturated fatty acid, were inefficient to induce UCP1 gene expression in contrast to prostacyclin (PGI2) derived also from the ω 3 polyunsaturated fatty acid.

We further analyzed the effects of metabolites that were reported to endogenous natural ligand of PPAR γ and master transcriptional regulator of adipogenesis (42,43). The PGJ2 and PGJ3 metabolites, 15-deoxy-delta 12,14-PGJ2 and 15-deoxy-delta 12,14-PGJ3, respectively, were inefficient to induce any browning of white hMADS adipocytes. 15-deoxy-delta 12,14-PGJ3

did not induce adiponectin secretion in contrast to what was reported in a mouse cell model (25). Further studies are necessary to clarify the role of 15-deoxy-delta 12,14-PGJ3 in adipogenesis as very few experiments are reported. In the absence of effects of these prostaglandins and their derivatives on UCP1 expression, it remains important to analyze other targets such as lipolysis, oxygen consumption, glucose transport ...

Among other oxylipins, 12,13-diHOME, 12-HEPE and 18-HEPE failed to induce browning process of white adipocytes. However, thromboxane A2 (TXA2) inhibited the rosiglitazone-induced expression of UCP1 mRNA and protein. We found thromboxane B2 (TXB2) levels associated with UCP1 expression in our previous study ((24)and unpublished data). TXA2 is a chemically unstable lipid mediator involved in several pathophysiologic processes. In human platelets, TXA2 is the major arachidonic acid derivative via the cyclooxygenase (COX1) pathway. Assessment of TXA2 biosynthesis can be performed through measurement of serum TXB2 (44). The use of CTA2, stable analogue of TXA2, allowed us to show that TXA2 play an anti-browning effect, probably favoring whitening of brown adipocytes. TXA2 exerted their effects through thromboxane A2 receptor (TBXAR2) which is expressed in hMADS adipocytes. Thromboxane A2 is an arachidonate metabolite that is a potent stimulator of platelet aggregation and a constrictor of vascular and respiratory smooth muscles and considered as a mediator in diseases such as myocardial infarction, stroke, and bronchial asthma (45). It has been reported that TXA2 modulate peripheral tissue insulin sensitivity and adipose tissue fibrosis (46). Furthermore, it has been shown that TXB2 (reflecting TXA2) circulating levels were higher in obese compared to lean patients (47). COX catalyze the first step in the conversion of arachidonic acid into prostanoids which includes prostacyclin (PGI2) and TXA2 that have opposite effects vasodilator and vasoconstrictor, respectively (48). Given that PGI2 is favoring adipogenesis and thermogenesis, TXA2 plays the opposite effect resembling to the antagonistic effect on platelet aggregation. PGI2 and TXA2 derive from the same precursor, arachidonic acid, through the action of cyclooxygenase (COX1 and 2) giving rise a common substrate (PGH2). PGH2 is then metabolized by specific enzymes prostaglandin I synthase (PGIS) or thromboxane synthase to produce PGI2 or TXA2 respectively. It is tempting to speculate that inhibiting the production of TXA2 locally or development of specific TXA2 receptor antagonist in association with increasing PGI2 levels will favor white and brown adipocyte formation and function. More detailed studies, in vitro and in vivo, are necessary to decipher the mechanisms behind these effects. Our data open new horizons in the development of potential therapies based on the control of thromboxane A2/prostacyclin balance to combat obesity and associated metabolic disorders.

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Author contributions:

Conceived and designed the experiments: CC, EZA. Performed the experiments:

Analyzed the data:

Wrote the manuscript: EZA, CC. All authors approved the final version of the manuscript.

Conflicts of interest:

The authors declare no conflict of interest

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Figure legends

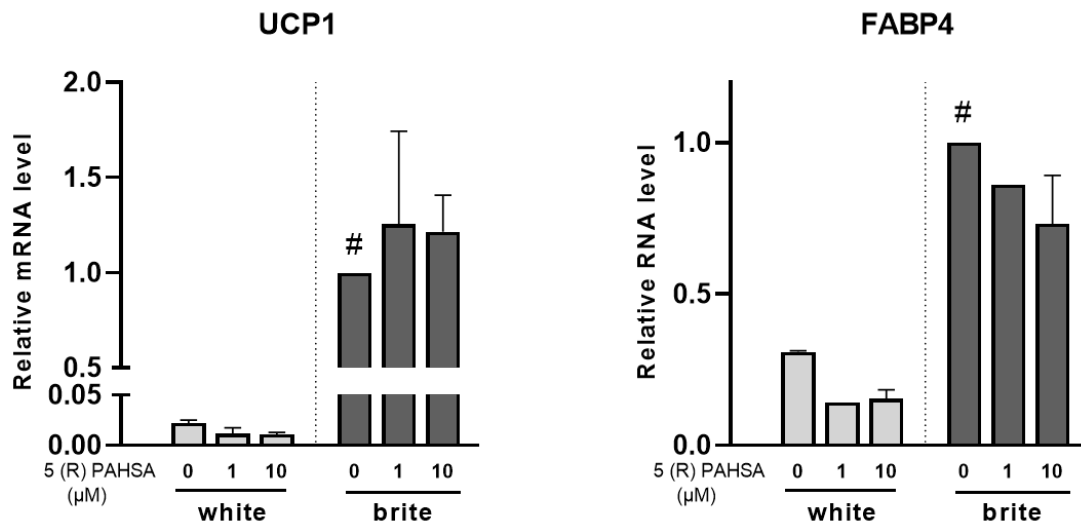


Figure 1. Effects of FAHFA on UCP1 mRNA expression.

White hMADS adipocytes were converted (from day 14 to day 18) into brite adipocytes using the PPAR γ agonist rosiglitazone in the absence (-) or the presence (+) of 1 or 10 μ M 5-PAHSA. mRNA levels of UCP1 and FABP4 markers were measured. Histograms display mean \pm SEM of three independent experiments; Paired student, $p < 0.05$ considered as significant: #, white vs brite adipocyte.

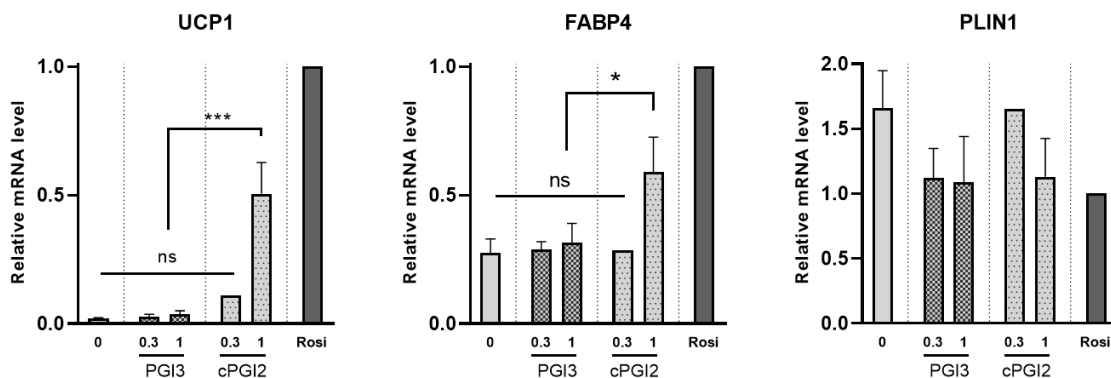


Figure 2. Prostaglandin I3 effects on UCP1 gene expression.

White hMADS adipocytes were induced to convert (from day 14 to day 18) into brite adipocytes in the presence of various amounts of PGI3 using cPGI2 and the PPAR γ agonist rosiglitazone (Rosi) as positive controls. mRNA levels of UCP1, FABP4 and PLIN1 markers were measured. Histograms display mean \pm SEM of three independent experiments; Paired student, $p < 0.05$ considered as significant: *, ***, treated vs untreated or PGI3 treated.

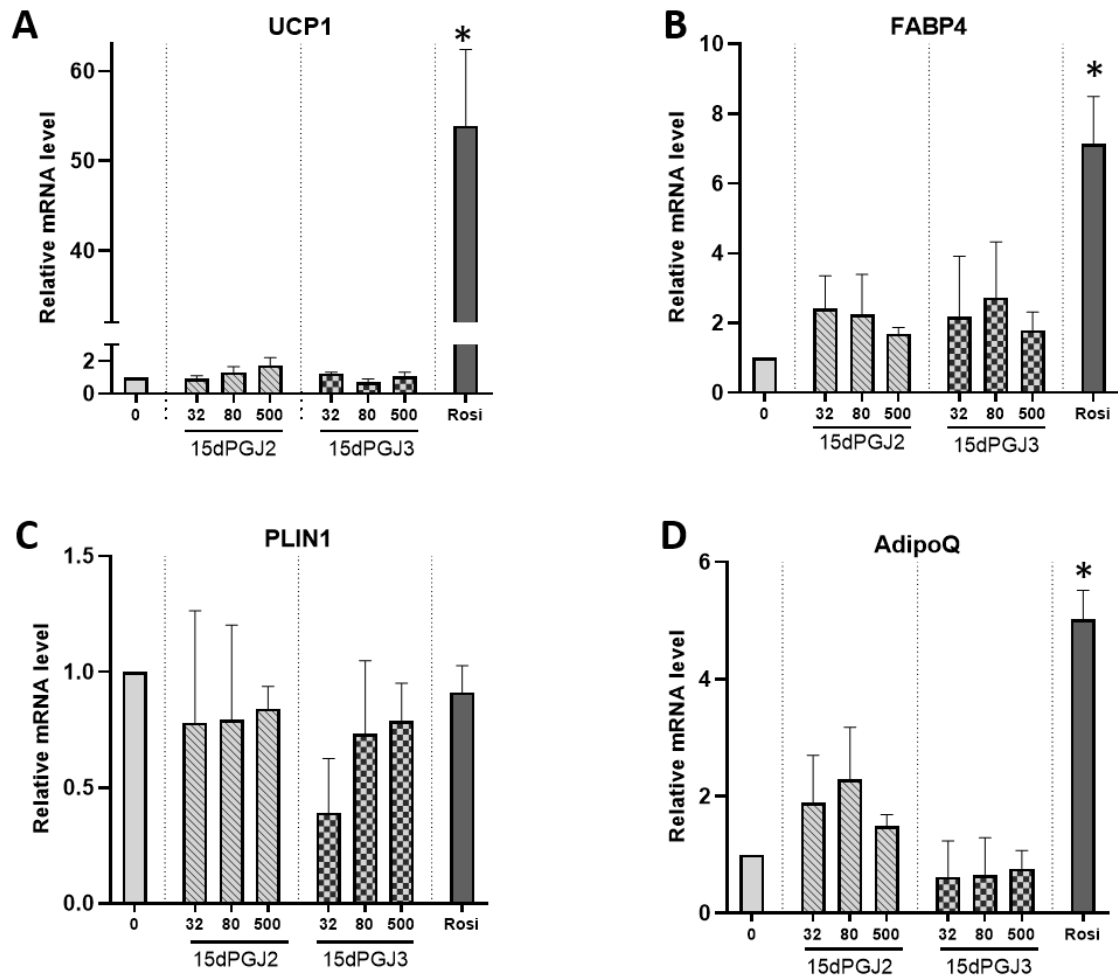


Figure 3. Potential PPAR γ activators: 15-deoxy-delta 12,14-PGJ2, 15-deoxy-delta 12,14-PGJ3

White hMADS adipocytes were induced to convert (from day 14 to day 18) into brite adipocytes in the presence of various amounts of 15-deoxy-delta 12,14-PGJ2, 15-deoxy-delta 12,14-PGJ3 using rosiglitazone as positive control. mRNA levels of UCP1 (A), FABP4 (B), PLIN1 (C) and adiponectin (AdipoQ) (D) markers were measured. Histograms display mean \pm SEM of three independent experiments; Paired student, $p < 0.05$ considered as significant: *, white vs brite adipocyte.

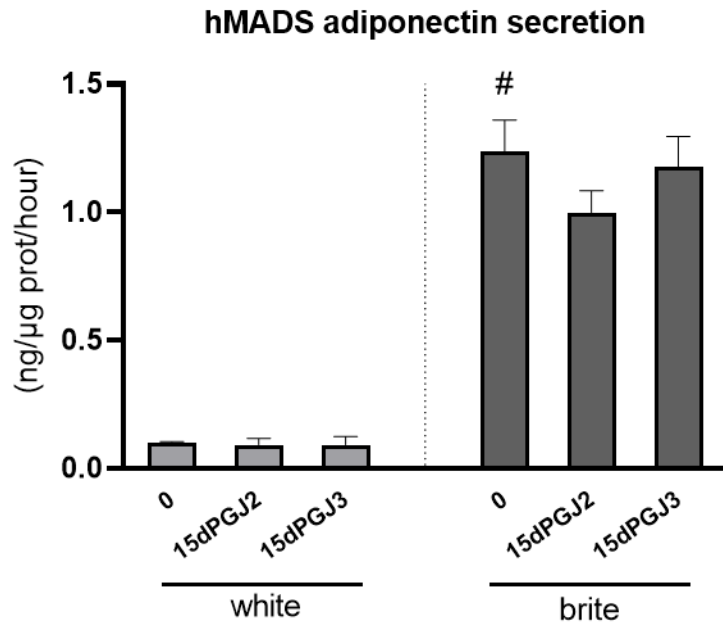


Figure 4. Adiponectin secretion

White hMADS adipocytes were induced to convert (from day 14 to day 18) into brite adipocytes in the presence of 0.5 μ M 15-deoxy-delta 12,14-PGJ2 or 15-deoxy-delta 12,14-PGJ3 in the absence or the presence of 100 nM rosiglitazone. Adiponectin levels were measured in the media six hours after the last medium change. Histograms display mean \pm SEM of three independent experiments; Paired student, $p < 0.05$ considered as significant: #, white vs brite adipocyte.

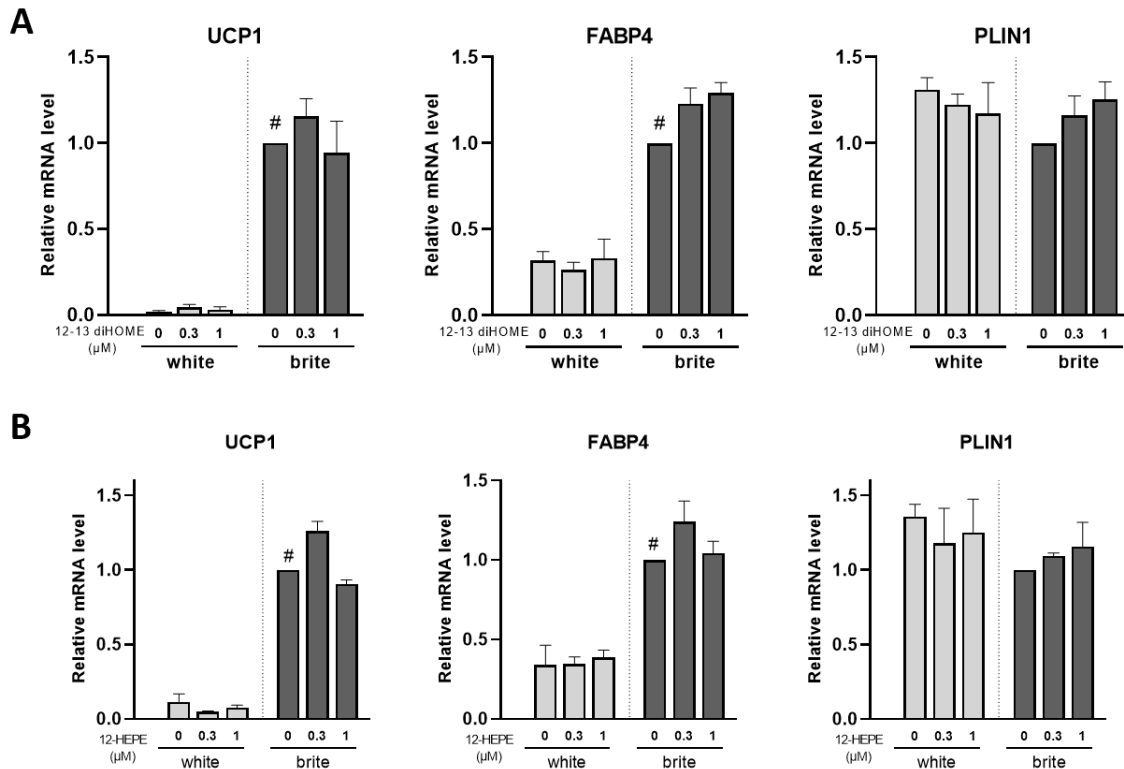


Figure 5. Effects of 12,13 diHOME and 12-HEPE on UCP1 mRNA expression

White hMADS adipocytes were induced to convert (from day 14 to day 18) into brite adipocytes in the presence of various amounts of 12,13 diHOME (A) or 12-HEPE (B) in the absence or the presence of 100 nM rosiglitazone. UCP1, FABP4 and PLIN1 mRNA levels were measured. Histograms display mean \pm SEM of three independent experiments; Paired student, $p < 0.05$ considered as significant: #, white vs brite adipocyte.

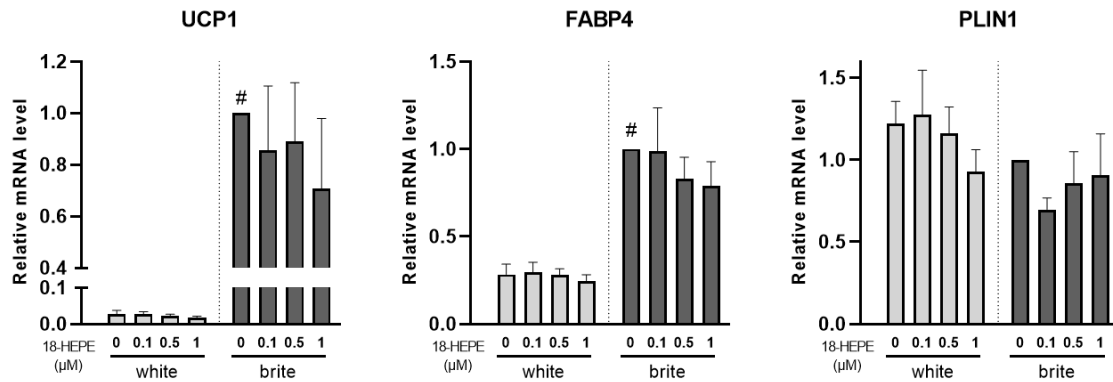


Figure 6. Effects of 18-HEPE on UCP1 mRNA expression

White hMADS adipocytes were induced to convert (from day 14 to day 18) into brite adipocytes in the presence of various amounts of 18-HEPE in the absence or the presence of 100 nM rosiglitazone. UCP1, FABP4 and PLIN1 mRNA levels were measured. Histograms display mean \pm SEM of three independent experiments; Paired student, $p < 0.05$ considered as significant: #, white vs brite adipocyte.

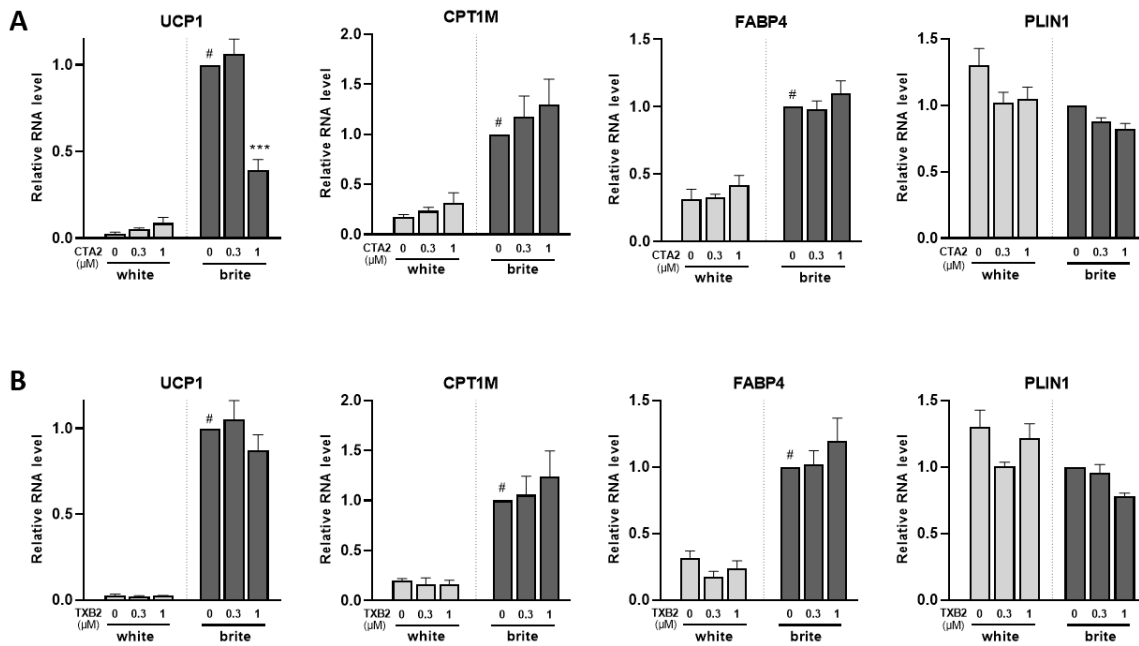
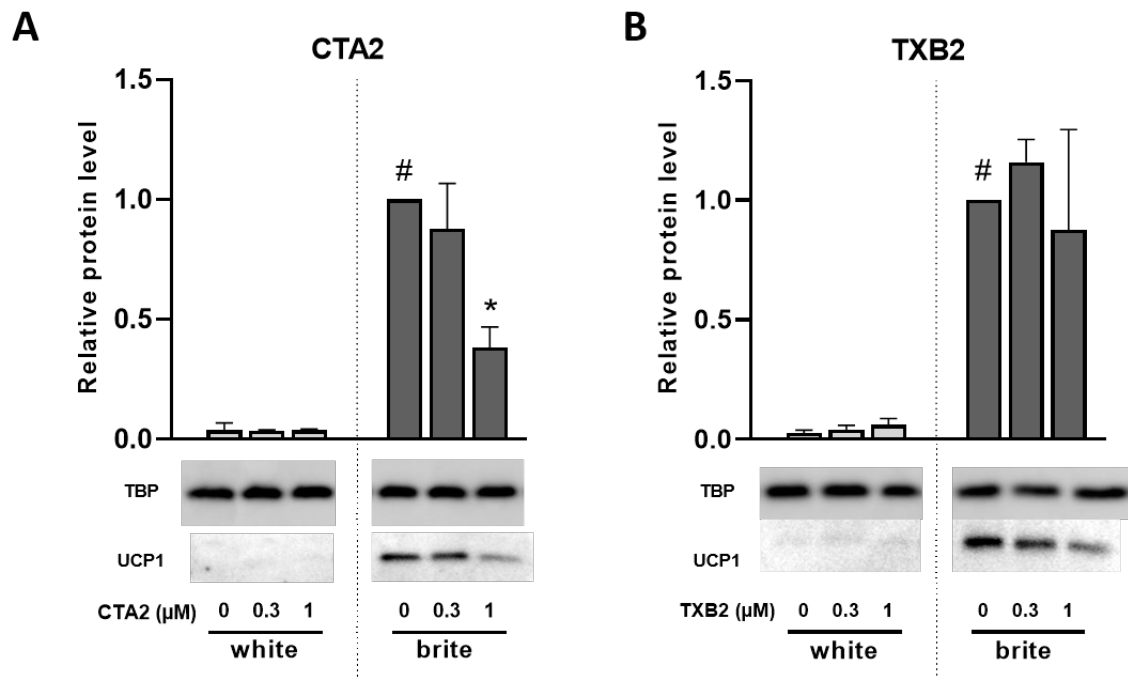


Figure 7. Effects of CTA2 and TXB2 on conversion of white in brite hMADS adipocytes

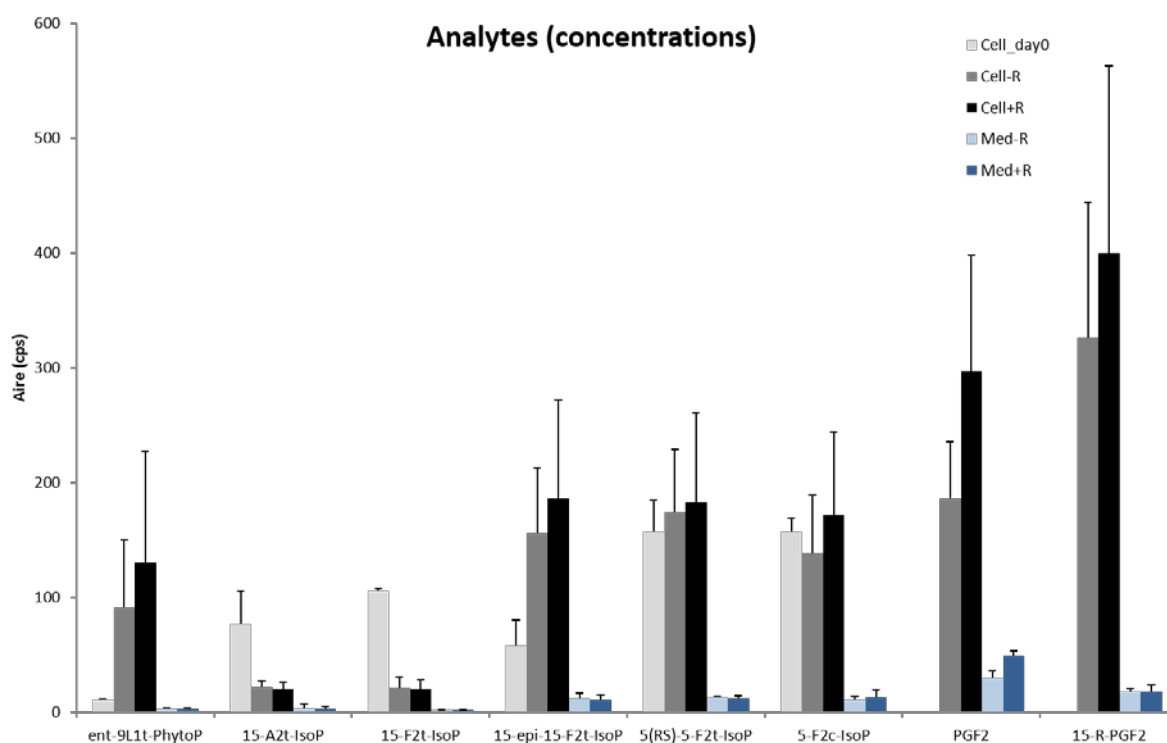
White hMADS adipocytes were induced to convert (from day 14 to day 18) into brite adipocytes in the presence of various amounts of CTA2 (A) or TXB2 (B) in the absence or the presence of 100 nM rosiglitazone. UCP1, CPT1M, FABP4 and PLIN1 mRNA levels were measured. Histograms display mean \pm SEM of three independent experiments; Paired student, $p < 0.05$ considered as significant: #, white vs brite adipocyte, ***, treated vs untreated.

Supplementary material



Supplementary Figure 1. Effects of CTA2 and TXB2 on UCP1 protein expression

White hMADS adipocytes were induced to convert (from day 14 to day 18) into brite adipocytes in the presence of various amounts of CTA2 (A) or TXB2 (B) in the absence or the presence of 100 nM rosiglitazone. 40 μg total protein extracts were analyzed by Western blot. Histograms display mean \pm SEM of two independent experiments; Paired student, $p < 0.05$ considered as significant: #, white vs brite adipocyte, *, treated vs untreated.



Supplementary Figure 2. Isoprostanes quantification in cell extracts and medium of white and brite adipocytes.

White hMADS adipocytes were induced to convert (from day 14 to day 18) into brite adipocytes. Cell extracts and media collected 6h after the last medium change were analyzed for isoprostanes levels. Histograms display mean \pm SEM of three independent experiments.

Supplementary Table 1. Sequence of primers used for gene expression analysis.

name	Reverse primer	Forward primer
PPAR γ 2	ATCAGTGAAGGAATCGCTTTCTG	CAAACCCCTATTCCATGCTGTT
UCP1	CCAGGATCCAAGTCGCAAGA	GTGTGCCCAACTGTGCAATG
FABP4	CAACGTCCCTTGGCTTATGCT	TGTGCAGAAATGGGATGGAAA
CPT1-M	GAGCAGCACCCCAATCAC	AACTCCATAGCCATCATCTGCT
PLN1	GATGGGAACGCTGATGCTGTT	ACCCCCCTGAAAAGATTGCTT
36B4	TGCATCAGTACCCCATTCATCAT	AGGCAGATGGATCAGCCAAGA

DISCUSSION DU PAPIER

Durant ce travail, j'ai identifié plusieurs oxylipines candidates dans la régulation de la thermogénèse et la formation de l'adipocyte beige.

Dans un premier temps, j'ai testé les effets d'une nouvelle classe de lipides décrite comme favorable à la formation des adipocytes thermogéniques *in vitro* et *in vivo*, les acides gras hydroxylés sur un acide gras (Fatty acid hydroxylated fatty acids, FAHFAs). La famille des PAHSA et notamment les 5- et 9-PAHSAs ont été décrits comme améliorant l'homéostasie glucidique et induisant le brunissage dans le tissu adipeux blanc (Wang et al., 2018). Contrairement à ce qui a été décrit, le traitement des adipocytes hMADS blancs et beige n'a pas induit de modification dans l'expression d'UCP1. Néanmoins, nous n'avons pas étudié le métabolisme glucidique et il se peut que 5- et 9-PAHSAs influencent cet aspect dans les adipocytes hMADS. D'autre part, la littérature décrit jusqu'ici des effets sur des cultures d'adipocytes murins (3T3-L1) ou des souris, l'absence d'effets que nous observons pourrait être causée par des différences avec le modèle humain, hMADS.

Ensuite, de récentes études de la même équipe ont mis en évidence deux métabolites lipidiques, 12,13-diHOME et 12-HEPE, produits et sécrétés par le muscle et le BAT respectivement. Les deux oxylipines semblent produites lors d'une exposition au froid ou lors d'exercice physique et agirait de manière endocrine et paracrine pour augmenter l'entrée de glucose dans les adipocytes bruns et favoriser la formation et l'activité des adipocytes thermogéniques (Leiria et al., 2019; Lynes et al., 2017; Stanford et al., 2018). Les deux composés ont été testés sur des adipocytes hMADS blancs et beiges et aucun des deux n'induit l'expression d'UCP1 ou potentialise les effets induits par la rosiglitazone.

Nous avons ensuite souhaité tester une classe de métabolites sur lesquels notre laboratoire avait déjà travaillé, les prostaglandines et les prostacyclines. Parmi eux, le laboratoire avait montré que la prostacycline 2 (PGI₂) était capable d'induire le brunissage dans les adipocytes hMADS (Ghandour et al., 2016) *via* une voie directe par son récepteur IP et une voie indirecte activant PPAR γ et ainsi l'expression d'UCP1. La PGI₂ provient de la métabolisation de l'acide arachidonique ω 6 par les COX et intervient dans la réponse inflammatoire, notamment par ses propriétés d'anti-agrégation plaquettaire et vaso-dilatatrices. Son analogue de la série 3, PGI₃, provenant de la métabolisation de l'EPA ω 3, est peu étudié mais est décrit comme ayant des propriétés anti-inflammatoires équivalentes à la PGI₂ (Kobzar et al., 2001). Nous avons donc souhaité tester son potentiel sur la conversion des adipocytes hMADS blancs en beiges. Les résultats confirment que la PGI₂ induit le brunissage des adipocytes blancs, avec une induction

d'UCP1 cependant plus faible que la rosiglitazone. Néanmoins, la PGI3 n'est pas capable d'induire l'expression d'UCP1 et ne modifie pas l'expression des marqueurs adipogéniques comme FABP4 ou PLIN1.

Ensuite parmi les prostaglandines, les 15dPGJ2 et 15dPGJ3 sont des produits de la métabolisation par les COX de l'ARA et de l'EPA respectivement. La 15delta-PGJ2 est décrite comme un agoniste de PPAR γ , capable d'induire la différenciation adipogénique (Forman et al., 1995; Kliewer et al., 1995). Son analogue de la série 3 a récemment été montré comme favorisant la sécrétion d'adiponectine (Lefils-Lacourtablaise et al., 2013), une adipokine produite par le tissu adipeux ayant des propriétés insulino-sensibles. Nos résultats ne montrent aucun effet des deux prostaglandines sur la conversion des adipocytes hMADS blancs en beige puisqu'elles ne sont pas capables d'induire l'expression d'UCP1 malgré une légère augmentation de l'expression de FABP4. Ces résultats ne sont donc pas compatibles avec la littérature décrivant la 15dPGJ2 comme un ligand potentiel de PPAR γ , dont les protéines UCP1 et FABP4 sont des cibles. Une étude plus récente suggère d'ailleurs que la production physiologique de 15dGPJ2 est insuffisante pour que les concentrations soient capables d'activer PPAR γ et avance l'hypothèse que cette prostaglandine ne soit qu'un produit de dégradation sans activité métabolique (Powell, 2003). Néanmoins la 15dPGJ2 semble augmenter légèrement l'expression de l'adiponectine et nous avons souhaité connaître ses effets sur la sécrétion de l'adipokine. Le dosage de la sécrétion d'adiponectine ne montre aucun effet des deux prostaglandines, contrairement à ce qui avait été décrit pour la 15dPGJ3. Encore une fois, la différence de modèle entre la souris et l'humain peut être à l'origine des contradictions observées. Seul un fort différentiel de sécrétion d'adiponectine entre l'adipocyte blanc et beige est visible.

Un critère essentiel à prendre en considération et qui pourrait expliquer que nos résultats soient contradictoires avec ce qui est parfois décrit dans la littérature, c'est la potentielle instabilité des métabolites d'acides gras. En effet, comme cela a été montré avec la PGI2, il est possible que la demi-vie des métabolites d'acides gras soit très courte en milieu aqueux, expliquant l'absence d'effets visibles dans notre étude.

Les différences observées entre les modèles murins et humains peuvent également être expliquées par des profils lipidiques distincts entre les modèles. En effet, lors de notre collaboration avec l'équipe du Dr. Martin Klingenspor à l'Université Technique de Munich (TUM), nous avons analysé les profils lipidiques obtenus dans les biopsies humaines de tissus adipeux et dans les échantillons de tissus adipeux de souris maintenues à thermoneutralité (30°C) ou exposées au froid (5°C) (Dieckmann et al., 2020) (**Annexe 2**). Ces résultats ont mis en évidence des distributions très différentes des métabolites d'acides gras entre les tissus humains et murins,

notamment avec une quantité plus importante de métabolites issus de l'ARA chez l'humain que chez la souris. Par ailleurs, il est ressorti de cette étude que les oxylipines peuvent être considérées comme caractéristiques d'un tissu adipeux blanc ou thermogénique et permettre de distinguer le WAT du BAT chez la souris uniquement. Chez l'Homme, les profils d'oxylipines du BAT sont équivalents aux profils d'oxylipines observés dans les adipocytes beiges de la souris (WAT sous cutané après exposition des animaux à 5°C).

Enfin, à partir de corrélations positives entre l'expression du facteur clé de la thermogénèse, UCP1, et la quantité de certaines oxylipines, j'ai supposé que les métabolites 18-HEPE et TxB2 pouvait réguler favorablement la formation de l'adipocyte thermogénique. 18-HEPE, issu de la métabolisation de l'EPA par les COX est généralement étudié pour son rôle de précurseurs d'une classe de métabolites anti-inflammatoires, les résolvines de la série E (Serhan et al., 2014). Le traitement des adipocytes hMADS blancs et beige par le 18-HEPE n'a montré aucun effet positif ni négatif sur l'expression d'UCP1 ou sur l'expression de certains marqueurs de l'adipogénèse, FABP4 et PLIN1. Un BAT activé est généralement associé à un état anti-inflammatoire et il est possible que la présence du 18-HEPE soit due à l'existence d'une réserve de précurseurs lipidiques afin de répondre à une inflammation.

La TxB2 est le produit inactif de la métabolisation de la TxA2, impliquée dans la réponse inflammatoire *via* ses propriétés sur l'agrégation plaquettaire et la vasoconstriction. La TxA2 est métabolisée à partir de l'ARA ω_6 par les COX puis par la Thromboxane Synthase et est le pendant de la PGI2 dans la réponse inflammatoire. En effet, elles possèdent des effets opposés et c'est la balance entre ces deux métabolites qui régule la réponse inflammatoire vasculaire (Needleman et al., 1979). La TxA2 est instable et c'est son produit TxB2 qui est utilisé comme marqueur plasmatique. Nous avons testé les effets de ces deux composés sur les adipocytes hMADS blancs et beige en utilisant un analogue stable de la TxA2, CTA2. Les résultats montrent un fort effet inhibiteur de la CTA2 sur l'expression de l'ARNm et de la protéine d'UCP1 sans que les autres marqueurs de la thermogénèse (CPT1M) et de l'adipogénèse (FABP4, PLIN1) ne soient impactés. En revanche, aucun effet de TxB2 n'est observé, ce qui est cohérent avec son statut de produit de métabolisation inactif.

La TxA2 est donc un inhibiteur du brunissage et nous pouvons émettre l'hypothèse qu'elle agit en regard de la PGI2 qui elle, induit la formation de l'adipocyte thermogénique selon le statut inflammatoire de l'individu. En effet, l'obésité se caractérise par une inflammation chronique de bas grade, ce qui entraîne la production de TxA2 et inhibe le brunissage chez les individus obèses. Nous pouvons également supposer que l'obésité induit une dérégulation de la production de PGI2,

peut être au profit d'autres prostaglandines issues de l'acide arachidonique ou une diminution de l'expression des récepteurs de la PGI₂ au niveau des adipocytes, favorisant ainsi la réponse à la TxA₂. En conclusion, la voie de synthèse de la TxA₂ peut être envisagée comme une cible dans la lutte contre l'obésité.

ARTICLE 2: The *Rosmarinus* bioactive compound carnosic acid is a novel PPAR antagonist that inhibits the browning of white adipocytes

Cécilia Colson¹, Pierre-Louis Batrow¹, Nadine Gautier¹, Nathalie Rochet¹, Gérard Ailhaud¹, Franck Peiretti² and Ez-Zoubir Amri^{1*}

INTRODUCTION

Les facteurs de transcription PPARs sont impliqués dans de multiples processus physiologiques et plus particulièrement le métabolisme lipidique. Dans cette famille, le récepteur nucléaire PPAR γ est l'acteur majeur de l'adipogenèse et de la formation des adipocytes thermogéniques.

Durant mon projet de thèse, nous avons cherché à identifier des molécules capables de réguler l'activité de PPAR γ et l'étude de la littérature nous a mené vers une molécule utilisée en médecine traditionnelle. Extrait du romarin et de la sauge, le composé bioactif appelé acide carnosique est décrit à la fois comme un inhibiteur de certaines enzymes lipoxygénases métabolisant des oxylipines de la famille des HETEs et des HODEs (Poeckel et al., 2008) et comme un inhibiteur de l'adipogenèse ciblant l'activité de C/EBP β (Gaya et al., 2013; Ibarra et al., 2011). L'acide carnosique possède des propriétés anti-inflammatoires et anti-diabétiques, utilisées sous forme de décoction, infusion et application topique (Birtić et al., 2015; Li et al., 2019).

Les effets potentiels de l'acide carnosique sur un modèle adipocytaire humain et notamment sur l'apparition du phénotype thermogénique n'étaient cependant pas décrits.

Article

The *Rosmarinus* Bioactive Compound Carnosic Acid Is a Novel PPAR Antagonist That Inhibits the Browning of White Adipocytes

Cécilia Colson ¹, Pierre-Louis Batrow ¹, Nadine Gautier ¹, Nathalie Rochet ¹, Gérard Ailhaud ¹, Franck Peiretti ² and Ez-Zoubir Amri ^{1,*} 

¹ Université Côte d'Azur, CNRS, Inserm, iBV, 06103 Nice, France; Cecilia.COLSON@univ-cotedazur.fr (C.C.); Pierre-Louis.Batrow@unice.fr (P.-L.B.); Nadine.Gautier@univ-cotedazur.fr (N.G.); rochet@unice.fr (N.R.); ailhaud@unice.fr (G.A.)

² Aix Marseille Université, INSERM, INRAE, C2VN, 13007 Marseille, France; Franck.PEIRETTI@univ-amu.fr

* Correspondence: amri@unice.fr; Tel.: +33-493-37-70-82; Fax: +33-493-81-70-58

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Abstract: Thermogenic brown and brite adipocytes convert chemical energy from nutrients into heat. Therapeutics that regulate brown adipocyte recruitment and activity represent interesting strategies to control fat mass such as in obesity or cachexia. The peroxisome proliferator-activated receptor (PPAR) family plays key roles in the maintenance of adipose tissue and in the regulation of thermogenic activity. Activation of these receptors induce browning of white adipocyte. The purpose of this work was to characterize the role of carnosic acid (CA), a compound used in traditional medicine, in the control of brown/brite adipocyte formation and function. We used human multipotent adipose-derived stem (hMADS) cells differentiated into white or brite adipocytes. The expression of key marker genes was determined using RT-qPCR and western blotting. We show here that CA inhibits the browning of white adipocytes and favors decreased gene expression of thermogenic markers. CA treatment does not affect β -adrenergic response. Importantly, the effects of CA are fully reversible. We used transactivation assays to show that CA has a PPAR α/γ antagonistic action. Our data pinpoint CA as a drug able to control PPAR activity through an antagonistic effect. These observations shed some light on the development of natural PPAR antagonists and their potential effects on thermogenic response.

Keywords: carnosic acid; PPA; adipocyte; conversion; antagonist; browning

1. Introduction

Mammalian adipose organs can be divided into two distinct types of adipose tissues: white and brown. White adipose tissue (WAT) is specialized in the storage and release of fatty acids [1], which are required as an energy source for heart and muscles. In contrast, brown adipose tissue (BAT) dissipates energy in the form of heat by uncoupling the mitochondrial respiratory chain from ATP synthesis [2,3]. Adipose tissue is the largest endocrine organ and links metabolism and immunity [4]. It is a major actor in the regulation of energetic metabolism and represents a potential therapeutic target to combat fat mass disorders such as obesity and hypermetabolism in critical illness.

Obesity has become a worldwide socioeconomic burden and constitutes a substantial risk factor for hypertension, type 2 diabetes, cardiovascular diseases, and certain cancers [5,6]. Despite enormous efforts, there is no efficient treatment for patients with obesity, except bariatric surgery for morbidly obese patients. Various lifestyle interventions and proposed pharmacological remedies have not yet been shown to induce sustained body weight reduction with long-term benefits to the health and well-being of patients with obesity [7].

Critical illnesses lead to more than 50% of deaths in the first week and may be due to sepsis, burns, or cancer-induced cachexia, which induce a negative energy balance with hyper-activation of BAT and high recruitment of new thermogenic adipocytes located within WAT, known as beige or brite adipocytes (brown-in-white) [8]. High increases in catecholamine production, such as epinephrine and norepinephrine, and pro-inflammatory factors are observed in critical illness, but pharmacologic treatments are associated with secondary effects such as gastrointestinal and cardiovascular failures. Obesity appears to be protective against death induced by critical illness, and this process is called the “obesity paradox”.

Adipocytes with thermogenic characteristics located within WAT are known as brite (brown-in-white) or beige adipocytes and are found as islets that can be induced in response to cold, β -adrenergic, or peroxisome proliferator-activated receptor (PPAR) agonists and to other stimuli [9–11]. The number of brown and brite adipocytes declines with age and with the development of obesity [12,13]. Identification of cellular and molecular mechanisms involved in the formation and activation of brite adipocytes may lead to the development of novel therapeutic tools. In this respect, several compounds, some of which are natural compounds, have already been reported to play key roles [11,14]. PPARs play critical roles in white and brown adipogenesis and function and, thus, represent key targets [15–17]. To identify potential compounds that are able to control brown fat formation and/or activation, we focused on the active principles that are found in plants used in traditional medicine of which the leaves are traditionally consumed as tea or for topical treatments [14,18]. We focused our attention on carnosic acid (CA), a major active component from rosemary (*Rosmarinus officinalis*), a plant well known for its medicinal properties [19,20]. CA exhibits multiple bioactive properties including antioxidant, anti-inflammatory, and anticancer activities [21,22]. It also affects adipose tissue development and hepatic steatosis and improves cholesterolemia and glycemia [21,23]. Rosemary extracts containing mainly CA are used in Europe and Asia as an antioxidant in food additives [24]. CA was found to inhibit adipogenesis in rodent cell models such as 3T3-L1 by inhibiting clonal mitotic expansion and gene expression of master genes [25,26]. CA also impacts adipocyte differentiation and glucose transport [26,27]; however, the effects of CA on the formation and function of brown/brite adipocyte are presently unknown.

Herein, we report the role of CA in the browning of white adipocytes and in the inhibition of the expression of thermogenic markers of brite adipocytes in human and murine cell models.

2. Materials and Methods

2.1. Reagents

Cell culture media, insulin, and trypsin buffers were purchased from Invitrogen (Cergy Pontoise, France). Fetal bovine serum was from Eurobio (Les Ulis, France). hFGF2 was from Peprotech (Neuilly sur Seine, France), and other reagents were from Sigma-Aldrich Chimie (Saint-Quentin Fallavier, France) or Cayman (Montigny-le-Bretonneux, France).

2.2. Cell Culture and Stromal Vascular Fraction Preparation

2.2.1. hMADS Cells Culture

The establishment and characterization of human multipotent adipose-derived stem (hMADS) cells have previously been described [28,29]. Briefly, these cells, which were isolated from white adipose tissue removed from surgical scraps of infants undergoing surgery, did not enter senescence while exhibiting a diploid karyotype, were non-transformed though they expressed significant telomerase activity, did not show any chromosomal abnormalities after 140 population doublings (PDs), and maintained their differentiation properties after 160–200 PDs. hMADS cells were able to withstand freeze/thaw procedures, and their differentiation could be directed under different culture conditions into various lineages [29]. The cell populations that have been studied were isolated from the pubic

region fat pad of a 4-month-old male donor (hMADS3). Cells were seeded at a density of 5000 cells/cm² in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 15 mM Hepes, 2.5 ng/mL hFGF2, 60 mg/mL penicillin, and 50 mg/mL streptomycin. hFGF2 was removed when cells reached confluence. Differentiation was induced in cells in a serum-free medium at day 2 post-confluence (designated as day 0) in DMEM/Ham's F12 (1:1) media supplemented with 10 µg/mL transferrin, 10 nM insulin, 0.2 nM triiodothyronine, 1 µM dexamethasone, and 500 µM isobutyl-methylxanthine for 4 days. Cells were treated between days 2 and 9 with 100 nM rosiglitazone (a PPAR γ agonist) to enable white adipocyte differentiation. At day 14, conversion of white to brite adipocytes was induced by a second rosiglitazone treatment for 4 days. Alternatively, for some experiments, cells were treated with GW7647, a PPAR α agonist. Media were changed every other day, and cells were used at the indicated days.

2.2.2. Mouse Adipose Tissue SVF Preparation and Culture

Twelve-week-old C57Bl/6J mice were euthanized by cervical dislocation to isolate stromal vascular fractions (SVF), as described previously [30–32]. Briefly, interscapular BAT depots were sampled, washed in PBS, and minced. Adipose tissue samples were digested for 45 min at 37 °C in DMEM containing 2 mg/mL collagenase A (Roche Diagnostics, Meylan, France) and 20 mg/mL bovine serum albumin (Sigma-Aldrich Chimie, Saint-Quentin Fallavier, France). Lysate was successively filtered through 250-, 100-, and 27-µm nylon sheets and centrifuged for 5 min at 500× g. The pellet containing stromal vascular fraction (SVF) cells was subjected to a red blood cell lysis procedure. SVF cells were plated and maintained in DMEM containing 10% FBS until confluence. Differentiation was induced in the same medium supplemented with 1 µM dexamethasone, 0.5 mM isobutylmethylxanthine, and 170 nM insulin for two days. Cells were then maintained for 7–10 days in DMEM containing 10% FCS in the presence of 10 nM insulin and 2 nM triiodothyronine for white and 1 µM Rosiglitazone for brown adipogenesis. Media were changed every other day.

2.3. Isolation and Analysis of RNA

These procedures followed Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) standard recommendations and were conducted as described previously [33]. The oligonucleotide sequences, designed using Primer Express software, are shown in Supplementary Table S1. Quantitative PCR (qPCR) were performed using SYBR qPCR premix Ex TaqII from Takara (Ozyme, Montigny-le-Bretonneux, France), and assays were run on a StepOne Plus ABI real-time PCR machine (PerkinElmer Life and Analytical Sciences, Boston, USA). The expression of selected genes was normalized to that of the 36B4 housekeeping gene and then quantified using the comparative- Δ Ct method.

2.4. Lipolysis Assays

Lipolysis was assessed by measuring glycerol release [34] from differentiated cells as previously described [28,35]. Differentiated adipocytes were insulin-deprived for 30 min; then, fresh medium was added. The cells were immediately subjected to 1 µM isoproterenol, a pan β -adrenergic receptor agonist for 90 min. The sampled medium was used to measure glycerol release with a free glycerol reagent (Sigma-Aldrich Chimie, Saint-Quentin Fallavier, France), according to the manufacturer's instructions. The results were normalized to the protein amount.

2.5. Western Blot Analysis

Proteins were extracted from cells or tissues as previously described [36]. Equal amounts of cellular proteins, 30 to 50 µg, were separated by electrophoresis using gradient gels (4–15%) and blotted onto PVDF membranes. Following blocking, membranes were incubated. Primary antibody incubation was performed overnight at 4 °C (anti-UCP1, Abcam #ab10983, dilution 1:1000; anti PPAR γ , CST #2430, dilution 1:1000; and anti-TBP, CST #D5C9H, dilution 1:1000). Primary antibodies were

detected with HRP-conjugated anti-rabbit or anti-mouse immunoglobulins (Promega, Charbonnières Les Bains, France). Detection was performed using Immobilon Western Chemiluminescent HRP Substrate (Merck-Millipore, Molsheim, France). Chemiluminescence obtained after adding Pierce ECL western blotting substrate (Thermo Scientific, Asnières sur Seine) was detected using an Amersham Imager 600 and quantified with Image Lab 5.0 software (Bio-Rad, Marnes-la-Coquette, France).

2.6. Cell-Based PPAR Transactivation Assay

HEK293 cells were transfected with Peroxisome Proliferator Response Element (PPRE)-driven Firefly luciferase (addgene 1015) and SV40-driven Renilla luciferase coding vectors together with PPAR γ or PPAR α expression vector. PPAR γ -LBD-Gal4 or PPAR α -LBD-Gal4 expression vector (given by Dr. Teruo Kawada, Kyoto University, Japan) was transfected along with the SV40-driven Renilla luciferase expression vector in HEK293 cells stably expressing the Gal4 response element-driven firefly luciferase reporter (pGL4.35(luc2P/9XGAL4UAS/Hygro) vector from Promega, Madison, WI, USA). Thirty-six hours after transfection, cells were exposed to the tested compounds for an additional 16 h; then, firefly and Renilla luciferase activities were measured in the cell lysates using the reagents Genofax A and C (Yelen) in an EnSight multimode reader (Perkin Elmer, Courtaboeuf, France). PPAR transactivation activity of the compounds is calculated as ratio of firefly to Renilla luciferase activity.

2.7. Statistical Analyses

Data are expressed as mean values \pm SEM and were analyzed using InStat software (GraphPad Prism version 8.3.0 for Windows, GraphPad Software, San Diego, CA, USA). Data were analyzed by Student's *t*-test or one-way or two-way ANOVA followed by a post hoc multi-comparison test (Tukey or Bonferroni). Differences were considered statistically significant when $p < 0.05$ in Student's *t*-test. When ANOVA was performed, adjusted *p*-values < 0.05 were considered to assess statistical differences.

3. Results

3.1. Carnosic Acid Inhibits the Browning Process of White Adipocytes

In the first series of experiments, we investigated the effect of CA on adipogenesis using a well-characterized human cell model (hMADS: human Multipotent Adipose-Derived Stem) [28]. Treatment of hMADS cells with CA at the beginning of the differentiation process led to complete loss of adipogenesis (data not shown), in agreement with previous reported studies using mouse cell models [25,26].

In a second series of experiments, we tested if CA affected the formation and function of brite adipocytes. For this purpose, once differentiated into white adipocytes, hMADS cells, upon stimulation with PPAR agonists, can undergo the browning/browning process and can convert into functional brite thermogenic adipocytes [33,37]. Differentiation was induced in hMADS cells into white adipocytes for 14 days, and then conversion into brite adipocytes was induced for 4 days using rosiglitazone, a potent PPAR γ agonist, in the absence or the presence of CA. CA is widely used in vitro from 1 to 10 μ M [38], and we observed an optimal inhibitory effect on gene expression at 10 μ M in our cell model using a range of 0–20 μ M (Supplementary Figures S1 and S3). Up to 10 μ M CA treatment, no effect on cell morphology and lipid accumulation was observed, discarding any cytotoxic effect (Figure 1A), whereas the 20 μ M CA treatment was associated with cytotoxicity (data not shown). Gene expression analysis showed a pronounced decrease of mRNA levels of key thermogenic markers such as UCP1 and CPT1M in brite hMADS adipocytes treated with CA, whereas perilipin 5 (PLIN5) and PGC1 α were only slightly affected (Figure 1B). UCP1 protein levels decreased upon CA treatment, which is consistent with gene expression data (Figure 1C) in brite hMADS adipocytes with an optimal inhibitory effect at 10 μ M of CA.

The mRNA levels of adipocyte marker FABP4 were also significantly reduced (Figure 1D). We observed that the inhibitory effect of CA was more potent on brite adipocytes than on white

adipocytes, indicating that PPAR γ may play a key role. In line with this assumption, PPAR γ 2 mRNA levels were significantly reduced in the presence of CA whereas PPAR α mRNA levels increased slightly but not significantly (Figure 1D). However, under these conditions, PPAR γ protein levels were not affected (Figure 1C).

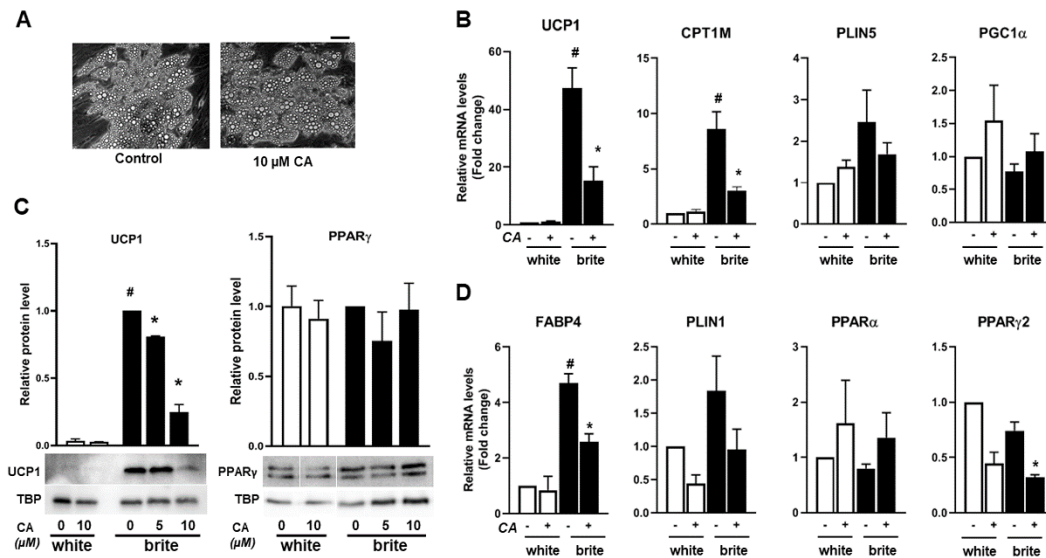


Figure 1. Carnosic acid (CA) inhibits the browning process of human white adipocytes. White human multipotent adipose-derived stem (hMADS) adipocytes were converted (from day 14 to day 18) into brite adipocytes using the peroxisome proliferator-activated receptor γ (PPAR γ) agonist rosiglitazone in the absence (–) or the presence (+) of 10 μ M CA (CA): (A) photomicrographs of differentiated hMADS cells treated or not with 10 μ M CA, bar: 20 μ m; mRNA levels of thermogenic (B) and adipogenic (D) markers; and (C) western-blot analysis using 40 (left panel) or 50 (right panel) μ g total protein extracts. Histograms display mean \pm SEM of three independent experiments; paired student, $p < 0.05$ was considered significant: #, white vs. brite adipocyte; *, untreated vs. CA treated condition.

We had shown previously that activation of PPAR α induces browning of white adipocytes [16]. In a similar manner to rosiglitazone-induced browning, GW7647 (a PPAR α agonist) also induces gene expression of key thermogenic markers such as UCP1 and CPT1M and an adipogenic marker, FABP4, which was inhibited in the presence of CA (Supplementary Figure S1C,F).

CA's effects on thermogenesis are not unique to human cells as we observed a similar inhibition in mouse primary adipocytes. Differentiation into adipocytes in stroma-vascular cells from brown adipose tissue of mice was induced in the absence or presence of rosiglitazone for 7 days, and cells were treated for the last 4 days with 10 μ M CA. CA treatment did not modify UCP1 expression when cells were differentiated in the absence of rosiglitazone (Supplementary Figure S2). However, as expected, when UCP1 mRNA levels were induced in the presence of rosiglitazone, this induction was inhibited under 10 μ M CA treatment (Supplementary Figure S2). Similar observations were obtained when using stroma-vascular cells derived from subcutaneous adipose tissue.

In conclusion, these results show that CA inhibits the expression of the UCP1 gene in human and mouse brown adipocytes.

3.2. Carnosic Acid Inhibits Thermogenic Marker Gene Expression of Human Brite Adipocytes

To investigate whether CA modulates the browning process, white and brite hMADS adipocytes (obtained following rosiglitazone treatment from days 14 to 18) were treated or not with 10 μ M of CA at day 18 for 4 days. Cells were harvested and analyzed at day 22. CA treatment did not affect cell morphology and lipid accumulation, which rules out any cytotoxic effect (data not shown). The mRNA abundance of key thermogenic markers UCP1 and CPT1M (Figure 2A and Supplementary Figure S3B,E)

and adipogenic markers FABP4 and PPAR γ 2 (Figure 2B) decreased in the presence of 10 μ M CA, more in white adipocytes than in brite adipocytes. PLIN1 and PPAR γ 2 mRNA levels displayed a significant decrease (Figure 2B) in white and brite hMADS adipocytes upon CA treatment. PGC1 α mRNA levels were not significantly affected by CA treatment (Figure 2A). Consistently, CA treatment induced a decrease of UCP1 at the protein level in brite hMADS adipocytes (Figure 2C).

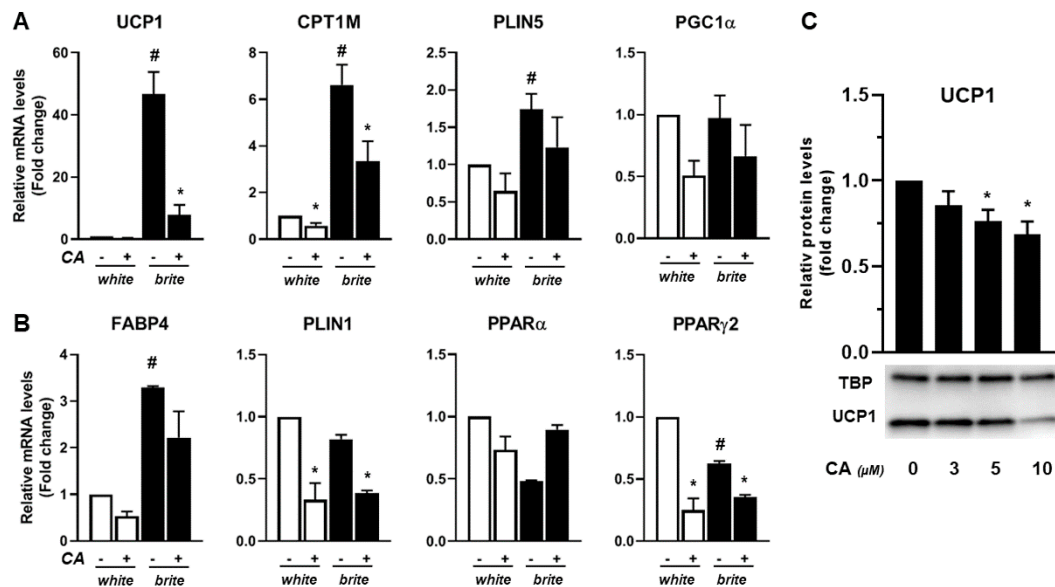


Figure 2. CA inhibits thermogenic marker gene expression of human brite adipocytes. White or brite hMADS adipocytes (at day 18) were maintained in the absence (–) or the presence (+) of 10 μ M CA between days 18 to 22, and mRNA levels of thermogenic (A) and adipogenic (B) markers were analyzed. Forty micrograms of total protein extracts were analyzed by Western blot (C) representative of three experiments. Histograms display mean \pm SEM of three independent experiments; paired student, $p < 0.05$ was considered significant: #, white vs. brite adipocyte; *, untreated vs. CA treated condition.

In a similar way, CA inhibited the expression of the thermogenic marker of brown adipocytes obtained upon GW7647 treatment through downregulation of thermogenic and adipogenic markers, with an efficient dose at 10 μ M CA (Supplementary Figure S3C,F).

Altogether, our results show that CA inhibits the browning process of white adipocytes by preventing (Figure 1) or inhibiting (Figure 2) the expression of key thermogenic markers.

3.3. Carnosic Acid Is a Potential Competitor of Rosiglitazone

Our aim was to test whether there was competition between rosiglitazone and CA. Thus, we evaluated the efficiency of the CA inhibitory effect. We treated white hMADS adipocytes during the browning process with various amounts of rosiglitazone in the absence or the presence of 5 and 10 μ M of CA. White hMADS adipocytes were converted into brite adipocytes by rosiglitazone in a similar dose-dependent manner. UCP1 gene expression was maximal at 30 nM rosiglitazone, whereas a slight inhibition was observed at 10 μ M rosiglitazone (Figure 3A). The effects observed are in agreement with affinity of rosiglitazone for PPAR γ , with a Kd in the range of 10–50 nM [39]. In the presence of CA, the effect of rosiglitazone was slightly decreased at 5 μ M CA and almost completely inhibited at 10 μ M CA, i.e., more than 70% inhibition (Figure 3A). Similar observations, albeit to a lesser extent, were obtained with other thermogenic markers such as CPT1M (Figure 3B) and the adipogenic marker FABP4 (Figure 3C), whereas the expression of PLIN1, which is not a PPAR γ target, was not affected (Figure 3D). Of note, in the absence of rosiglitazone, CA showed no effect on UCP1 expression (see Figures 1 and 2 and data not shown).

Altogether, these observations show that CA inhibits the effects of rosiglitazone and thus might compete with rosiglitazone on PPAR γ . Our data strongly suggest that CA behaves as an antagonist of rosiglitazone on PPAR γ .

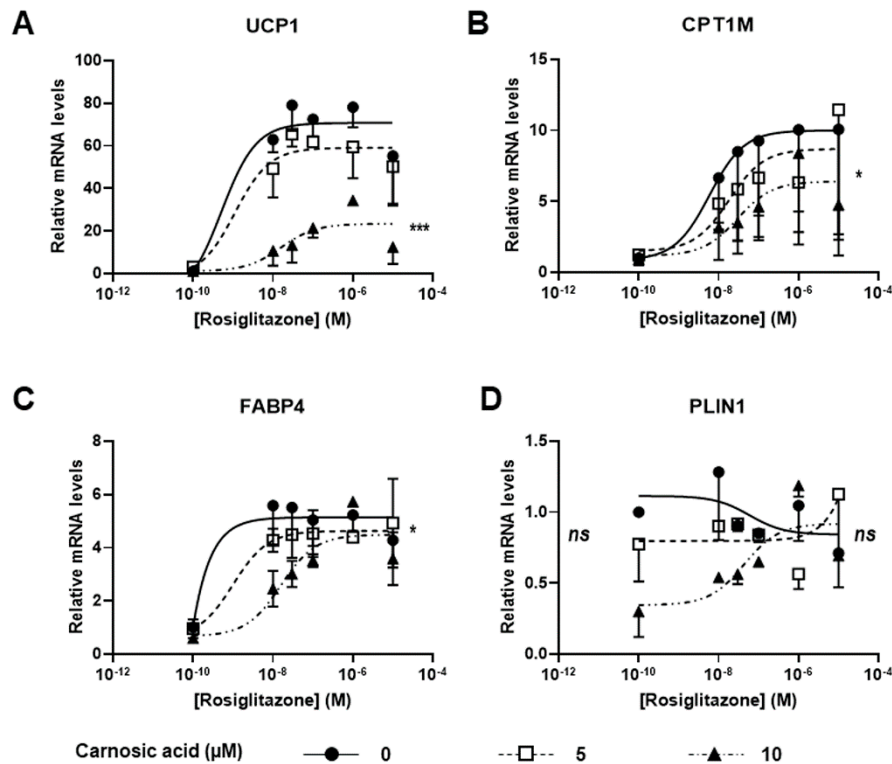


Figure 3. Efficiency of CA against rosiglitazone: conversion into brite adipocytes of white hMADS adipocytes was induced (between days 14 and 18) in the presence of varying amounts of rosiglitazone (10 nM–10 μM) in the absence (●) or the presence of 5 (□) or 10 (▲) μM CA. mRNA levels of thermogenic (UCP1 and CPT1M (A,B)) and adipogenic (FABP4, PLIN1, (C,D)) markers were analyzed. Each point on the curves represents mean ± SEM of two independent experiments (two replicates for each experiment); one-way ANOVA followed by Tukey post hoc test, the untreated vs. CA 10 μM-treated condition: *, adjusted $p < 0.05$; ***, adjusted $p < 0.01$.

3.4. Carnosic Acid Antagonizes Rosiglitazone-Induced Activation of PPAR γ

To better understand the molecular mechanisms of CA inhibitory effects, we analyzed the effect on PPAR γ transcriptional and transactivation activities in a cell system. For this purpose, in an initial series of experiments, a transcriptional activation assay (PPRE-based luciferase) was carried out to investigate the effect of CA on the direct transcriptional activity of full length PPAR γ . PPAR γ activation was measured by luciferase activity, reflecting the transcriptional activation of the PPRE driving luciferase in the presence of varying amounts of rosiglitazone and in the absence or the presence of 40 μM CA. As expected, rosiglitazone induced luciferase activity, and CA strongly inhibited the rosiglitazone-induced response (Figure 4A).

In the second series of experiments, we used a transactivation analysis based on the PPAR γ -LBD-GAL4 chimera assay [40]. In this assay, the PPAR γ ligand-binding domain was fused to the GAL4 DNA-binding domain and the firefly luciferase reporter gene was under the control of GAL4-binding elements (Figure 4B). In this system, CA did not affect the transactivation response of the luciferase reporter. Rosiglitazone treatment (50 nM) led to a strong transactivation of PPAR γ , whereas CA treatment decreased the rosiglitazone-induced transactivation in a dose-dependent manner (Figure 4B).

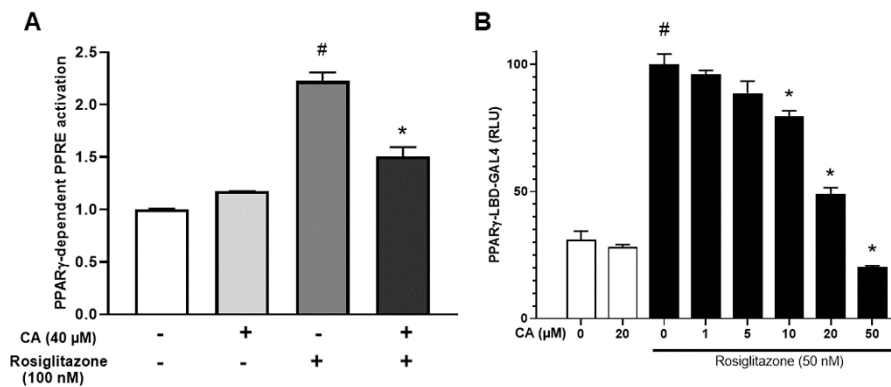


Figure 4. CA antagonizes rosiglitazone-induced activation of PPAR γ . **(A)** HEK293 cells were transfected with PPRE-driven firefly luciferase and SV40-driven Renilla luciferase encoding vectors together with the PPAR γ expression vector. Cells were then incubated in the presence of 100 nM rosiglitazone with or without 40 μ M CA. PPRE promoter activity was calculated as the ratio of firefly/Renilla luciferase and expressed as fold over the control situation. **(B)** Concentration-dependent PPAR γ transactivation activities of rosiglitazone were measured in the presence of varying amounts of CA using a PPAR γ -LBD-GAL4 chimera assay. Values are expressed as % of the maximal response measured with rosiglitazone (50 nM). Data are displayed as mean \pm SEM of two to three independent experiments (three replicates for each experiments). Student test, $p < 0.05$ was considered significant: #, rosiglitazone conditions vs. control; *, CA conditions vs. untreated.

Our data show that CA targets the ligand-binding domain of PPAR γ and affects the transcriptional activity by reducing the maximal response without affecting the half maximal effective concentration (EC₅₀).

PPAR α activation by GW7647 was also decreased in the presence of CA (Supplementary Figure S4). These results strongly suggest that CA inhibits PPAR ligand activation, potentially in a noncompetitive manner, and its effect seems to be stronger on PPAR γ than on PPAR α .

3.5. The Carnosic Acid Inhibitory Effect Is Normalized upon its Removal

We next checked whether the CA inhibitory effects were reversible. To answer this question, white hMADS adipocytes were treated with the PPAR γ agonist rosiglitazone in the absence or the presence of CA for 72 h. CA was then removed or not for the last 24 h. As expected, rosiglitazone induced an increase in the expression of thermogenic markers UCP1 and CPT1M during the 3 days of treatment, that was inhibited in the presence of 10 μ M CA (Figure 5A). Removal of CA for the last 24 h allowed full recovery of UCP1 and CPT1M mRNA levels (Figure 5A). Similar observations were obtained with the adipogenic markers FABP4 and PLIN1 (Figure 5B). Thus, it appears that the CA inhibitory effects on the browning process are reversible.

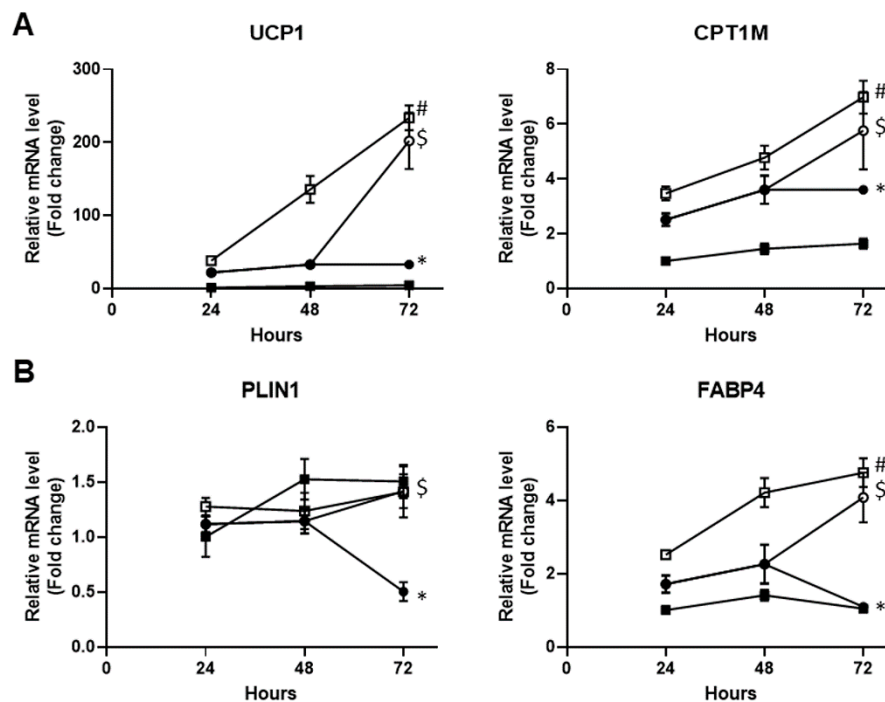


Figure 5. The CA inhibitory effect is normalized upon its removal. Conversion of white hMADS adipocytes (from day 14 to day 17) into brite adipocytes was induced with 100 nM rosiglitazone in the absence (□) or the presence (●) of 10 μ M CA for 72 h. CA was removed for the last 24 h (○). Untreated white hMADS adipocytes (■) served as the control. mRNA levels of thermogenic (UCP1 and CPT1M, **A**) and adipogenic (FABP4 and PLIN1, **B**) markers were analyzed. Data are displayed as mean \pm SEM of two independent experiments (two replicates for each experiments). Two-way ANOVA followed by multi-comparisons and Bonferroni correction, adjusted $p < 0.05$ was considered significant: #, white brite adipocyte; *, untreated vs. CA treated condition, §: significant for treated 72 h vs. 48 h.

3.6. Effects of Carnosic Acid on Lipolysis

Lipolysis is one of the major adipocyte functions which enables the release of stored energy. Under β -adrenergic stimulation, the adipocyte hydrolyzes stored triglycerides into glycerol and free fatty acids. Importantly, given that thermogenic adipocytes use high amounts of fatty acids to produce heat, we tested whether CA affected lipolysis. For this purpose, brite hMADS adipocytes were treated or not with 10 μ M CA for 4 days and stimulated by isoproterenol, a pan β -adrenergic receptor agonist in order to induce lipolysis.

Our results showed a decrease in glycerol release when brite hMADS adipocytes were treated with CA (Figure 6). Nevertheless, the same reduction (around 2-fold) was observed under basal and isoproterenol-stimulated conditions, suggesting that CA decreased global lipolytic capabilities with no specific effect on β -adrenergic stimulation.

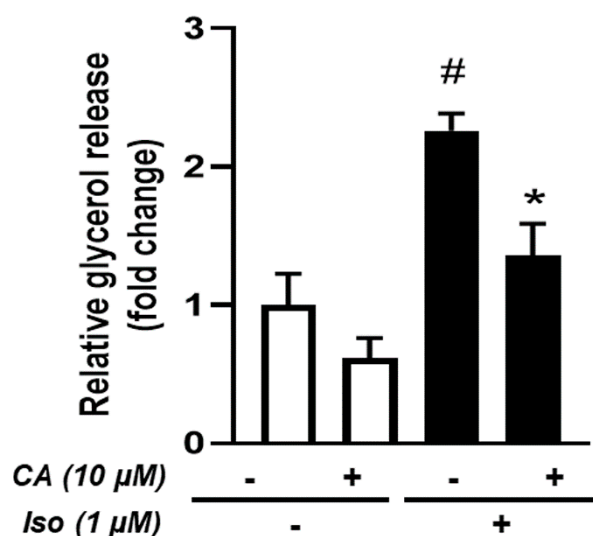


Figure 6. Effects of CA on lipolysis: conversion of hMADS adipocytes into brite adipocytes was induced in the absence (–) or the presence (+) of 10 μM CA for 4 days. Basal and isoproterenol-induced lipolysis were measured during the last hours. Data are displayed as mean ± SEM of three independent experiments; paired student, $p < 0.05$ was considered significant: #, no isoproterenol challenge (iso –) vs. isoproterenol treatment (iso +); *, untreated vs. CA-treated condition.

4. Discussion

The control of brown/brite adipocyte formation and function represents a promising therapeutic target to combat development and redistribution of fat mass and metabolic disorders. Several studies have shown that herbal medicine can play a role in the control of adipogenesis and metabolic diseases. Targets of herbal compounds that affect insulin signaling include the insulin receptor pathway and PPARs, among others [14,41,42]. Herein, we showed that CA, a major compound from rosemary, plays a PPAR α/γ antagonist role inhibiting the browning process of white adipocytes and inhibiting thermogenic marker expression in brite adipocytes. PPARs represent a transcription factor family playing key roles in the regulation of important metabolic pathways and cellular functions in the pathophysiology of diabetes and obesity. So far, three different PPAR isoforms, designated α , β/δ , and γ , have been identified [43]. PPAR γ is a master gene that drives adipogenesis, lipid metabolism, and browning process of white adipocytes [44,45]. The endogenous agonists of PPARs are a class of fatty acids and their derivatives that originate from the diet, de novo lipogenesis, and lipolysis [43]. Natural endogenous ligands have not yet been identified, but chemical ligands/activators such as thiazolidinediones (for example, rosiglitazone) are potent activators of PPAR γ and have been used in a clinical setting for the treatment of insulin resistance [46]. However, these benefits are associated with secondary effects, such as fluid retention, body weight gain, bone loss, myocardial ischemia, diarrhea, and heart failure [46]. We had previously reported that human mature white adipocytes can acquire the brown fat cell properties upon PPAR γ or PPAR α activation [16,32]. The brown identity of a fat cell relies on its capacity to sustain thermogenesis, for example, possessing sufficiently high mitochondrial oxidative capacities associated with activation of the bona fide uncoupling protein, UCP1. Indeed, CA treatment of white hMADS adipocytes prevents the rosiglitazone-induced browning as measured by UCP1 mRNA and protein expression as well as another thermogenic marker, CPT1M. Moreover, CA treatment of brite hMADS adipocytes led to a loss of the UCP1 expression that is characteristic of the whitening process. The effects of CA are reversible, demonstrating that the inhibitory effects of CA did not result in disappearance of the thermogenic expression profile. CA antagonizes the effects of rosiglitazone in a dose-dependent manner leading to the reduction of maximal response at 10 μM and small effects on the half maximal doses. Depending on the gene, the effects are either very pronounced (UCP1), moderate (FABP4), or finally weak effects on adipogenic markers that are not

PPAR targets (PLIN1). Similar effects of CA were observed when using mouse primary cultures of adipocyte precursors with lower inhibition, which might be due to the presence of fetal calf serum and the existence of potential proteins trapping CA.

Using transactivation assays, our data show that CA antagonizes rosiglitazone at the PPAR γ ligand-binding domain. Regarding PPAR α , the effects are less efficient with 15% inhibition compared to 50% for PPAR γ . As these experiments were carried out in the presence of fetal calf serum, one cannot rule out the presence of a potential PPAR α agonist leading to a high background response. Indeed, in the presence of CA, the background response was lower and the maximal response to PPAR α (GW7647) was slightly inhibited (Supplementary Figure S4). Altogether, our observations indicate that CA is a noncompetitive PPAR antagonist with a higher affinity for PPAR γ than for PPAR α .

Several studies have shown that CA exerts an inhibitory effect on adipocyte differentiation [25,26,47] and might be a potent therapeutic agent of overweight/obesity and associated diseases [23,27,48,49]. It has been shown that the inhibitory effects of CA on adipogenesis can be mediated by inhibition of PPAR γ and C/EBPs, affecting also the LIP/LAP ratio [25], although the main function of CA appears to be related to antioxidative features as a quencher of reactive oxygen species [50]. Other functions have been reported to act through the EGFR/MAPK pathway, Akt pathway, or induction of glutathione S-transferase [51–53] as well as inhibition of lipoxygenases that affect potential PPAR activators/ligands [54]. To our knowledge, our data represent the first demonstration that CA plays an antagonistic role against ligand binding on PPAR γ and, therefore, may represent a potential new class of natural compounds with known pharmacological properties in the treatment metabolic disorders. Altogether, CA seems to control both adipogenesis and thermogenesis, and for the latter, it might be through tight control of thermogenic markers including the most important, UCP1. Under conditions where browning of white adipocytes is exacerbated, such as in critical illness after a severe burn injury or cachexia [8,55–57], CA treatment may represent a potential therapeutic option. Animal models and patients with severe burns develop hypermetabolism with massive browning of white adipose tissue, hepatic steatosis, and cachexia, which are harmful and have limited therapeutic treatments. In a similar manner, cancer-associated cachexia, a wasting syndrome, is associated with increased browning of white adipose tissue, which leads to higher thermogenesis with increased lipid mobilization and energy expenditure, further worsening the clinical situation and risk of death. Inhibition of white adipose tissue browning in burn and cancer patients represents a promising approach that can be potentially achieved with CA treatment alone or in combination with other approaches. In line with this, it has been suggested that CA is efficient against obesity-associated cancers, in particular against colon cancer [22]. Furthermore, it is known that PPAR activation by thiazolidinediones is associated with bone fracture [58], and we could speculate that CA treatment has the potential to restore bone mass, given that betulinic acid, a PPAR γ antagonist, is known to favor osteogenesis at the expense of adipogenesis [59].

CA is a phenolic diterpene, and given its lipidic nature, one can hypothesize that CA is able to interfere with PPAR ligands in the ligand-binding domain in a reversible manner to regulate receptor activity. In the absence of rosiglitazone, CA was able to inhibit adipogenic marker expression, suggesting that CA might antagonize endogenously synthesized ligands/activators. We cannot rule out the possibility that CA might exert some effects on the recruitment of cofactors and/or affect PPAR modifications such as phosphorylation. Further studies are necessary to shed some light on the mechanisms of interaction of CA and PPARs. The development of a new generation of natural PPAR antagonists represents a promising approach to address selectivity and therapeutic concerns.

5. Conclusions

In this work, we showed for the first time that CA had a PPAR α/γ antagonistic action in a human cell model. CA inhibited the browning process of white adipocytes and decreased the expression of thermogenic key markers, suggesting that CA is able to induce whitening in thermogenic adipocytes. Genes that are under the control of PPAR, harboring a PPRE in their promoter, were affected upon

activation of PPAR α/γ . CA did not affect the β -adrenergic response; however, it inhibited the lipolytic ability associated with brite adipocytes. Importantly, the effects of CA were fully reversible. More detailed studies are necessary to decipher the mechanisms behind these effects. Our data open new horizons in the development of natural PPAR antagonists with potential effects on metabolic disorders.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2073-4409/9/11/2433/s1>, supplementary Figures S1–S4, supplementary Table S1. Figure S1: CA inhibits the browning process of human white adipocytes; Figure S2: CA inhibits the browning process of mice primary adipocytes; Figure S3: CA inhibits thermogenic marker gene expression of human brite adipocytes; Figure S4: CA antagonizes rosiglitazone-induced activation of PPAR α ; Table S1: Sequence of primers used for gene expression analysis.

Author Contributions: Conceived and designed the experiments: C.C. and E.-Z.A.; performed the experiments: C.C., P.-L.B., N.G., N.R., F.P., and E.-Z.A.; analyzed the data: C.C., P.-L.B., G.A., F.P., and E.-Z.A.; and wrote the manuscript: C.C. and E.-Z.A. All authors have read and agreed to the published version of the manuscript.

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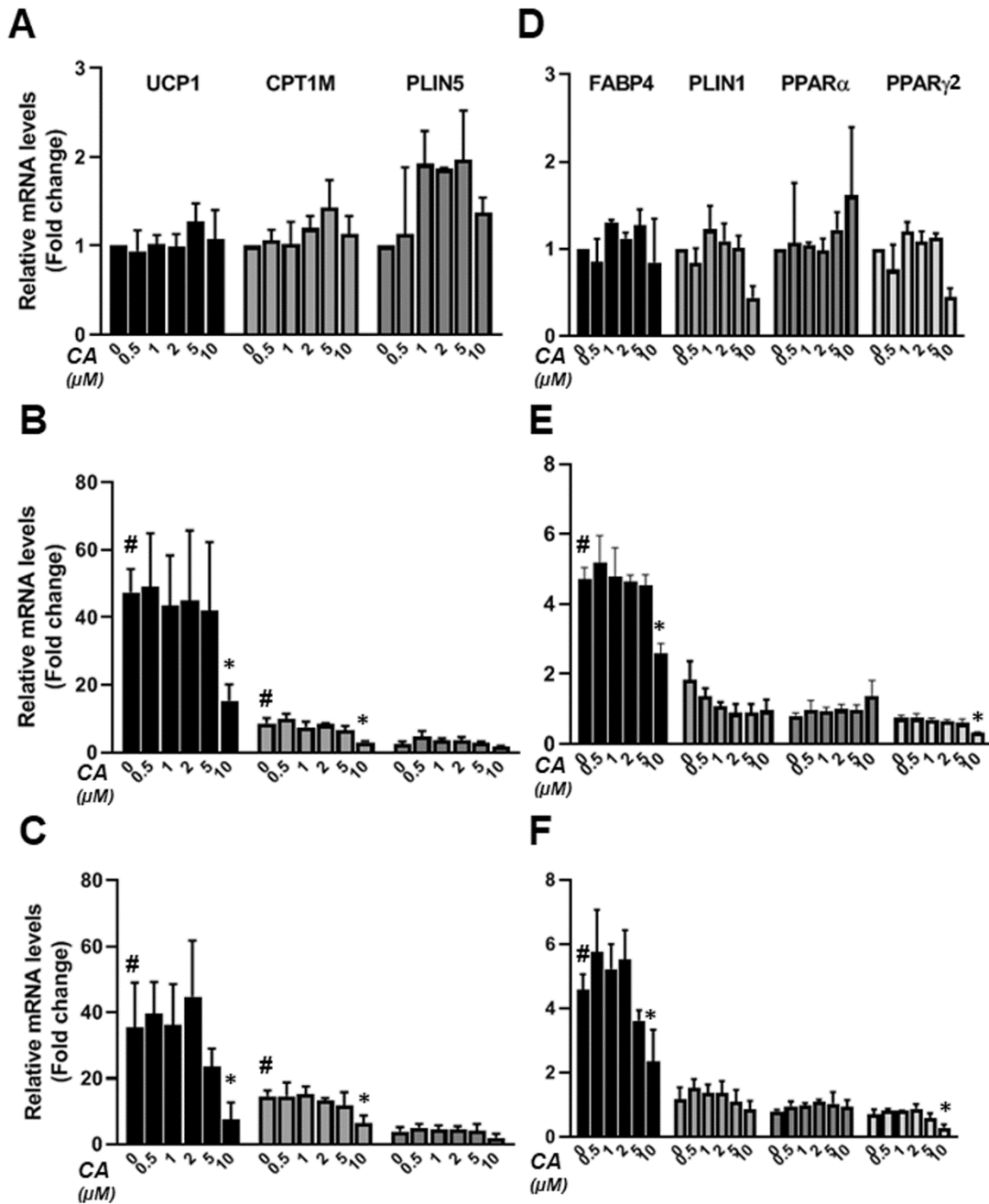
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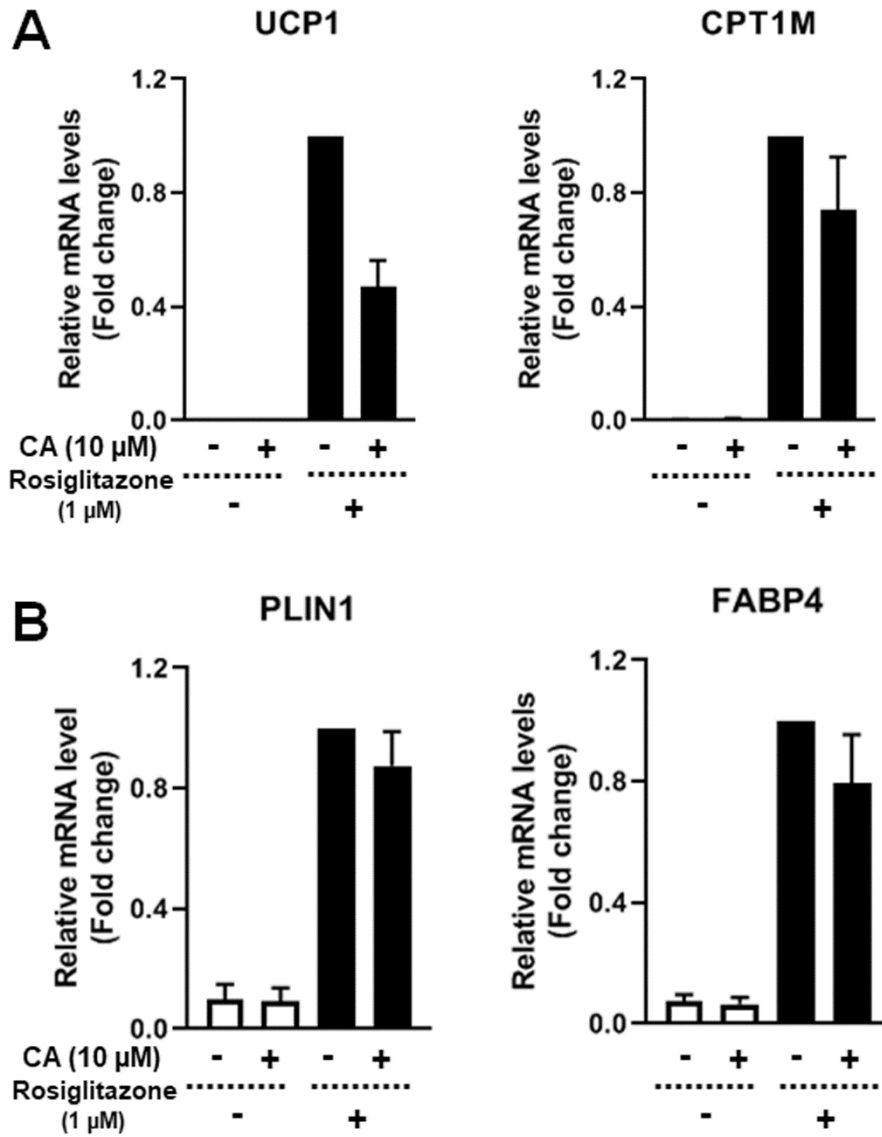


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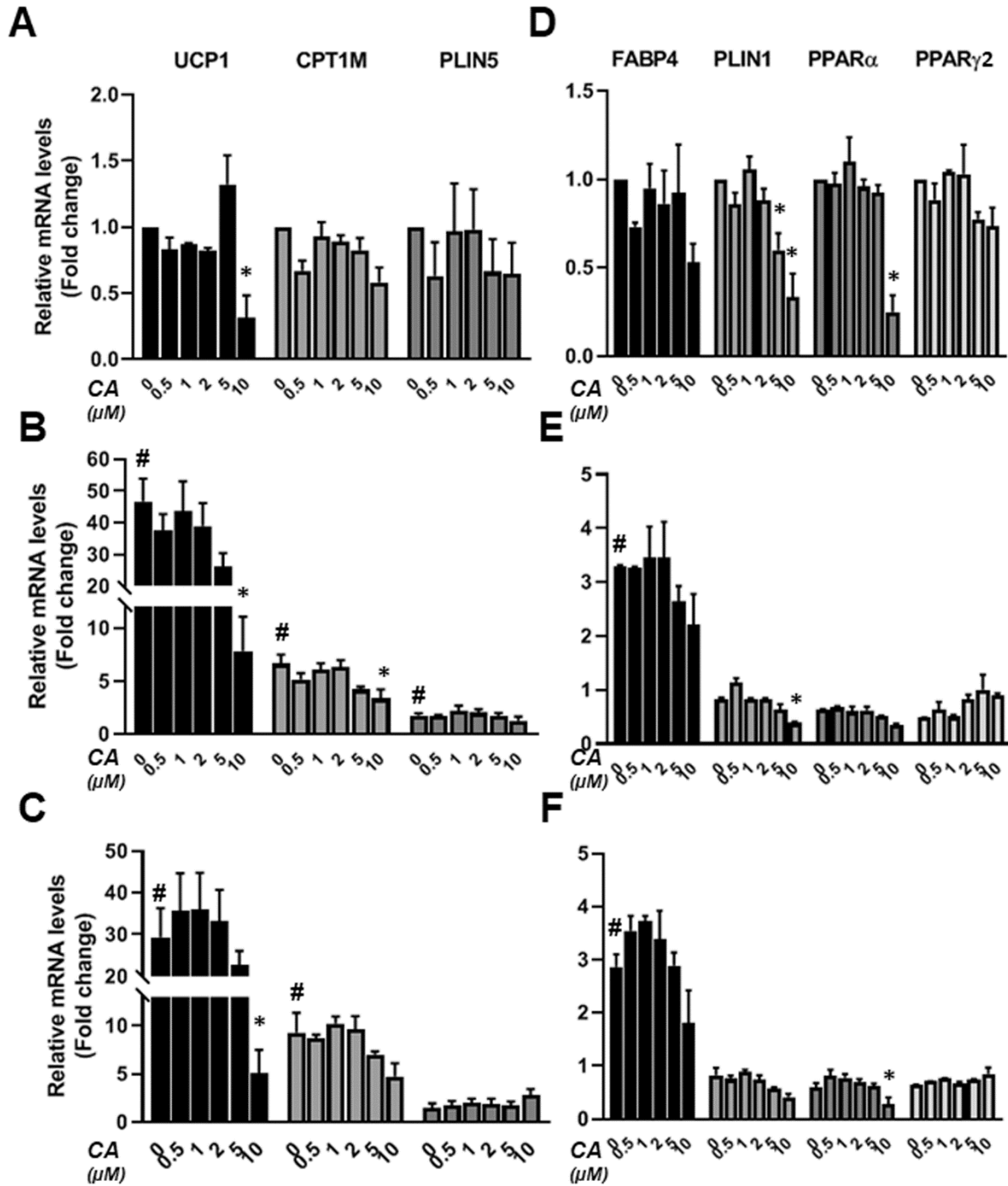
Supplementary material



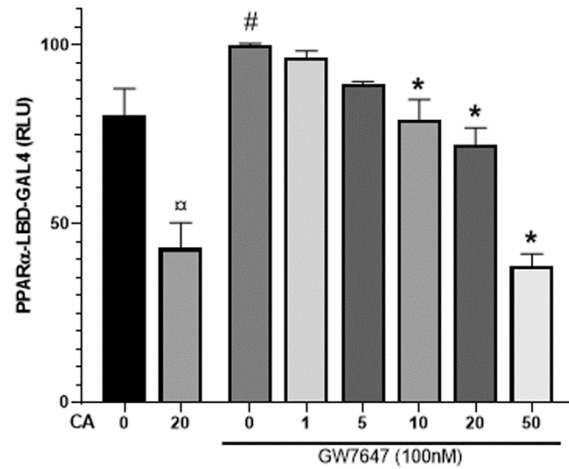
Supplementary Figure S1: CA inhibits the browning process of human white adipocytes. White hMADS adipocytes (A, D) were converted (days 14 to 18) into brite adipocytes with 100 nM rosiglitazone (B, E) or 300 nM GW7647 (C, F) in the presence of increasing CA concentrations. mRNA levels of thermogenic (A-C) and adipogenic (D-F) markers were analyzed. Paired student, $p < 0.05$ considered as significant: #, white vs brite adipocyte; *, untreated vs CA treated condition.



Supplementary Figure S2: CA inhibits the browning process of mice primary adipocytes. SVF cells derived from mice BAT were induced to differentiate into white or brown (rosiglitazone-treatment) adipocytes in the absence (-) or presence (+) of 10 μ M of CA for the last 4 days and mRNA levels of thermogenic (A) and adipogenic (B) markers were analyzed. Histograms display mean \pm SEM of three independent experiments.



Supplementary Figure S3: CA inhibits thermogenic marker gene expression of human brite adipocytes. hMADS adipocytes were differentiated into white adipocytes (A, D), or brite adipocytes using rosiglitazone (B, E) or GW7647 (C, F) in the presence of increasing CA concentrations and mRNA levels of thermogenic (A-C) and adipogenic (D-F) markers were analyzed. Paired student, $p < 0.05$ considered as significant: #, white vs brite adipocyte; *, untreated vs CA treated condition.



Supplementary Figure S4: CA antagonizes rosiglitazone-induced activation of PPAR α . Concentration-dependent PPAR α transactivation activities of GW7647 were measured in the presence of varying amounts of CA using a PPAR α -LBD-GAL4 chimera assay. Values are expressed as % of the maximal response measured with GW7647 (100 nM). Data are displayed as mean \pm SEM of two to three independent experiments (three replicates for each experiments). Paired student, $p < 0.05$ considered as significant: #, GW7647 treated *vs* untreated; * and α , CA treated *vs* untreated.

Supplementary Table S1. Sequence of primers used for gene expression analysis.

Human oligonucleotide sequences		
name	Reverse primer	Forward primer
PPAR γ 2	ATCAGTGAAGGAATCGCTTTCTG	CAAACCCCTATTCCATGCTGTT
PPAR α	TCCAAAACGAATCGCGTTGT	GGCGAACGATTCGACTCAAG
PGC1 α	CTGTGTCACCACCCAAATCCTTAT	TGTGTCGAGAAAAGGACCTTGA
UCP1	CCAGGATCCAAGTCGCAAGA	GTGTGCCCAACTGTGCAATG
FABP4	CAACGTCCCTTGGCTTATGCT	TGTGCAGAAATGGGATGGAAA
CPT1-M	GAGCAGCACCCCAATCAC	AACTCCATAGCCATCATCTGCT
PLN1	GATGGGAACGCTGATGCTGTT	ACCCCCTGAAAAGATTGCTT
PLN5	CTACGAGCACTCTGTGGGGA	GGTCTATCAGCTCCAGCGTCT
36B4	TGCATCAGTACCCCATCTATCAT	AGGCAGATGGATCAGCCAAGA

Mice oligonucleotide sequences		
name	Reverse primer	Forward primer
36B4	TCC AGG CTT TGG GCA TCA	CTTTATCAGCTGCACATCACTCAGA
Cpt1m	GGCTCCAGGGTTCAGAAAGT	TGCCTTTACATCGTCTCCAA
Fabp4	CTTGTGGAAGTCACGCCTTT	AAGAGAAAACGAGATGGTGACAA
Ucp1	CACCTTCCCGCTGGACT	CCTGGCCTTACCTTGGAT
Pln1	AGCGTGGAGAGTAAGGATGTC	CTTCTGGAAGCACTCACAGG

DISCUSSION DU PAPIER

Durant ce travail, j'ai donc pu mettre en évidence l'effet inhibiteur de l'acide carnosique sur la formation de l'adipocyte thermogénique. La présence chronique du composé entraîne en effet une inhibition de l'expression des marqueurs clés du phénotype thermogénique comme UCP1 et CPT1M. Nous aurions pu supposer que la présence de l'acide carnosique freinait l'expression des marqueurs mais ses effets sont identiques sur un adipocyte thermogénique ayant déjà acquis le phénotype puisque lorsque les adipocytes hMADS beige sont traités entre les jours 18 et 22, on observe une inhibition des marqueurs, caractéristique du phénomène de blanchiment des adipocytes beiges. Cela signifie que l'acide carnosique exerce ses effets inhibiteurs sur un facteur essentiel pour l'acquisition du phénotype ainsi que son maintien. En outre, les niveaux de protéines d'UCP1 sont également diminués par le traitement par l'acide carnosique que ce soit durant la phase de brunissage (entre les jours 14 et 18) ou la période en aval, pendant laquelle il induit le blanchiment.

Un résultat étonnant était l'inhibition de l'expression de PPAR γ 2 lui-même. La littérature décrit l'acide carnosique comme un inhibiteur de C/EBP β qui est un facteur de transcription essentiel dans l'expression de PPAR γ au moment de l'induction de l'adipogenèse. Néanmoins, ce processus se déroule au début de la différenciation tandis que les effets que nous observons sur les cellules hMADS s'exercent lors d'une phase terminale de différenciation voire même après l'installation de phénotype puisque l'expression de PPAR γ 2 est également inhibée pendant la phase de blanchiment.

Ces observations nous ont amené à tester si une augmentation de la concentration de rosiglitazone pouvait surpasser les effets inhibiteurs de l'acide carnosique. Les résultats obtenus montrent que malgré de fortes concentrations de rosiglitazone, la présence d'acide carnosique freine considérablement l'expression du marqueur adipogénique FABP4 et thermogénique CPT1M et leur expression semble atteindre un plateau sans aboutir au niveau de la condition contrôle. Quant à l'expression d'UCP1, elle reste la plus touchée par l'inhibition de l'acide carnosique avec 70% d'inhibition.

Ces résultats suggèrent fortement que l'acide carnosique est en compétition avec la rosiglitazone et qu'il impacte directement l'activité de PPAR γ . Les expériences de transactivation ont confirmé cette hypothèse et ont mis en évidence que l'effet inhibiteur de l'acide carnosique est causé par une activité antagoniste de PPAR γ . D'après nos résultats, l'acide carnosique exerce son effet sur le domaine de liaison au ligand, le domaine AF-2 situé en C-terminal de la protéine. En effet, les expériences de transactivation utilisant une protéine chimérique constituée seulement du

domaine AF-2 de PPAR γ et du domaine de liaison à l'ADN de la Gal4, l'ajout d'acide carnosique inhibe de manière dose dépendante l'activation de la transcription par la rosiglitazone.

Les effets de l'acide carnosique sont en revanche réversibles, l'expression des marqueurs inhibés étant presque complètement restaurée en seulement 24h en absence du composé. Cela suggère que l'inhibition de PPAR γ par l'acide carnosique n'entraîne pas la perte du programme d'expression du phénotype thermogénique malgré qu'il ne soit pas encore installé. J'ai cependant eu l'occasion de tester les effets de l'acide carnosique sur des fenêtres de temps plus précoces, durant la période d'induction de l'adipogenèse et le traitement par l'acide carnosique des cellules hMADS pendant les 2 jours suivant la période d'induction inhibe la différenciation (**Figure 33**). Ces résultats confirment ce qui a été décrit dans la littérature sur les cellules 3T3-L1 dont l'adipogenèse est inhibée lorsque les cellules sont traitées de manière précoce par des extraits de romarin. Les mécanismes dans notre modèle adipocytaire humain sont donc similaires. Une exposition précoce empêche donc l'induction de la différenciation de manière irréversible. Nous pouvons également supposer que d'autres acteurs de la différenciation sont des cibles de l'acide carnosique puisque durant cette période, PPAR γ 2 n'est pas ou peu exprimé. La littérature décrit C/EBP β comme cible de l'inhibition par l'acide carnosique (Gaya et al., 2013) mais les études sur l'inhibition de C/EBP β (LIP dominant négatif ou absence de glucocorticoïdes dans le mélange d'induction) n'empêche pas l'adipogenèse en présence d'un ligand exogène des PPARs, notamment la troglitazone qui active PPAR γ 1 (Hamm et al., 2001). De manière surprenante, le traitement des adipocytes hMADS durant les 2 premiers jours d'induction semblent augmenter l'adipogenèse puisque les marqueurs PPAR γ 2, FABP4 et PLIN1 s'en trouvent augmentés à la fin de la différenciation (**Figure 38**).

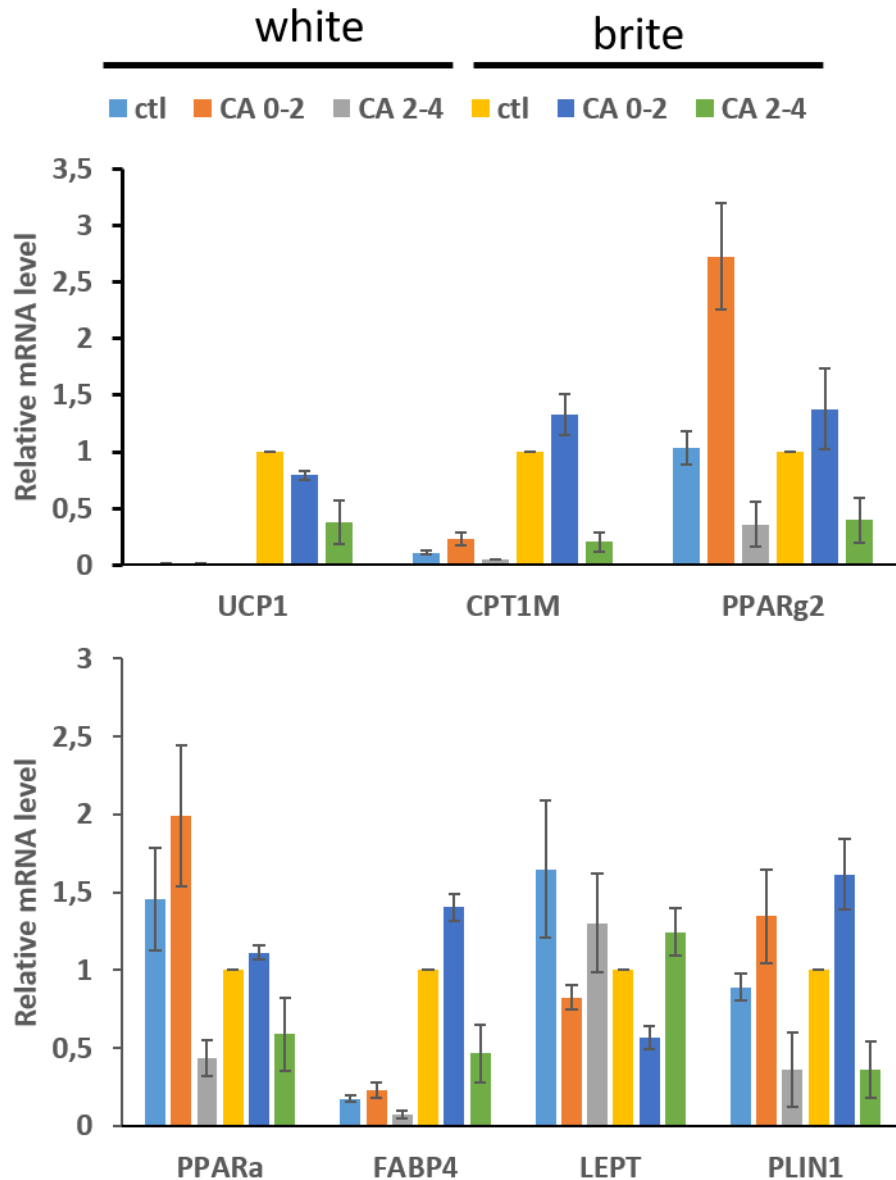


Figure 38: Les effets d'un traitement précoce par l'acide carnosique sur l'expression des marqueurs thermogéniques (panneau haut) et adipogéniques (panneau bas) des cellules hMADS. Les cellules hMADS ont été différenciées en adipocytes blancs (white) et beige pendant 18 jours après traitement à l'acide carnosique (CA) durant les 2 premiers jours d'induction (CA 0-2) ou les 2 suivants (CA 2-4) ou sans traitement (ctl). Les résultats d'expression d'ARNm sont normalisés à la condition beige ctl. Résultats préliminaires, n=2 expériences indépendantes.

Ensuite, l'acide carnosique semble inhiber la lipolyse basale mais pas la réponse β -adrénergique puisque la mesure indirecte de la lipolyse *via* la sécrétion de glycérol ramenée à son contrôle est identique en absence ou en présence d'acide carnosique. Il faudrait toutefois vérifier si l'expression d'UCP1 est augmentée lors d'une stimulation par un agoniste aux β -adrénergiques en présence d'acide carnosique. En effet, le promoteur d'UCP1 contient une séquence de réponse à CREB induit par l'augmentation d'AMPC caractéristique de la signalisation adrénérergique. J'ai réalisé une expérience préliminaire sur des adipocytes différenciés depuis la fraction stroma-

vasculaire de souris traités avec de l'acide carnosique puis stimulés avec de l'isoprotérenol, un agoniste des récepteurs β -adrénergiques. Les résultats préliminaires montrent que l'inhibition de l'expression d'UCP1 induite par l'acide carnosique est reversée après une stimulation à l'isoprotérenol (**Figure 39**). Néanmoins, l'inhibition des marqueurs par l'acide carnosique dans les adipocytes murins *in vitro* est déjà modérée comme nous le montrons dans la figure supplémentaire 2 de la publication. La présence de sérum pourrait expliquer l'effet modéré de l'inhibition et ainsi la réversion observée avec l'isoprotérenol.

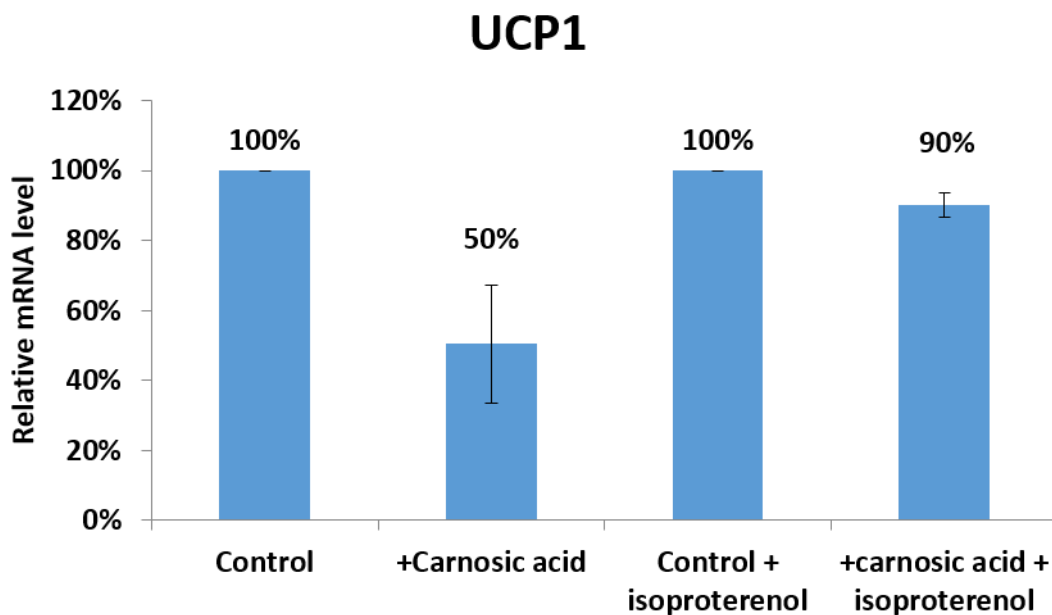


Figure 39: Les effets de la stimulation β -adrénergique par l'isoprotérenol sur des adipocytes beiges primaires de souris différenciés à partir de la fraction stroma-vasculaire de WAT sous cutané et traités à 10 μ M d'acide carnosique (carnosic acid) pendant les 3 derniers jours de différenciation. Les résultats sont exprimés en pourcentage (%) de leur condition contrôle respective (Control). Résultats préliminaires, n=1 et 3 réplicas par condition.

Enfin, l'acide carnosique a également été impliqué dans les réponses anti-oxydantes cellulaires ainsi que comme un inhibiteur potentiel de certaines enzymes, notamment les lipoxygénases. Malgré que les lipoxygénases ne semblent pas exprimées à des niveaux significatifs dans notre modèle en fin de différenciation, nous ne pouvons donc pas exclure que ses effets inhibiteurs soient liés à l'altération de la production de ligands endogènes des PPARs, PPAR γ bien sûr mais également PPAR α puisque d'après nos résultats, son activité est également diminuée en présence d'acide carnosique même en conditions basales sans l'agoniste GW7647. Néanmoins l'inhibition de l'activité de PPAR α reste modérée par rapport celle de PPAR γ .

Des effets de l'acide carnosique sur la production de ligands endogènes des PPARs peuvent également être suggérés par i) l'inhibition de certains marqueurs de l'adipogenèse dans les

adipocytes hMADS blancs au jour 18 (FABP4, CPT1M et PLIN1) et ii) l'irréversibilité de l'inhibition lors de traitements précoces durant l'induction.

En conclusion, ce travail a permis d'identifier un nouvel antagoniste de PPAR γ et de PPAR α dans une moindre mesure, et pour la première fois dans la formation de l'adipocyte thermogénique. Le rôle des PPARs a été énormément étudié dans le métabolisme et notamment le métabolisme adipocytaire et hépatique qui sont les voies les plus impliquées dans l'obésité et ses comorbidités. Il existe toutefois des conditions où l'activité thermogénique et le recrutement de nouveaux adipocytes thermogéniques sont exacerbés et peuvent mener au décès, comme chez les grands brûlés ou la cachexie entraînée par certains cancers. La consommation d'acide carnosique en supplément alimentaire est possible, et ne semble pas altérer ses propriétés anti-adipogéniques, comme le montrent certaines études d'enrichissement nutritionnel chez l'animal (Ibarra et al., 2011). L'acide carnosique peut ainsi représenter une stratégie intéressante pour lutter contre l'hyper-activation métabolique observée dans certaines pathologies, sous forme de complément alimentaire ou d'application topique par exemples.

DISCUSSION GENERALE ET PERSPECTIVES

L'épidémie d'obésité a pris de telles proportions ces dernières décennies qu'il devient urgent de développer des stratégies thérapeutiques. Il apparaît évident aujourd'hui que la lutte contre l'obésité doit se faire à plusieurs niveaux : le développement de stratégies pharmacologiques ne sera efficace qu'en association avec un mode de vie qui équilibre les apports et les dépenses énergétiques ; la modification du mode de vie ne pourra être réalisée sur le long terme qu'avec des méthodes de prévention adaptées et les méthodes de prévention ne seront efficaces qu'avec une compréhension des mécanismes de régulation du métabolisme énergétique.

Les études sur le développement et les fonctions du tissu adipeux sont abondantes et ont ouvert de nombreuses perspectives pharmacologiques et environnementales, sans effets positifs avérés jusqu'ici (Müller et al., 2018).

La mise en évidence de la capacité thermogénique du tissu adipeux brun chez l'adulte avait créé une nouvelle vague d'optimisme dans la lutte contre les pathologies métaboliques mais la complexité de sa régulation, les implications de la génétique et de l'épigénétique, du microbiote ou encore de l'alimentation, retarde la mise en place de thérapeutiques efficaces. Il serait pourtant intéressant de pouvoir utiliser ce tissu capable de participer à la dépense énergétique, notamment chez les obèses où l'exercice physique peut être compliqué à mettre en place et chez qui le tissu adipeux brun est peu voire pas actif.

L'alimentation est un des points centraux de la régulation du métabolisme puisqu'elle est à la fois un substrat énergétique et un facteur régulateur de l'homéostasie. Afin de permettre à l'organisme d'avoir accès en continu à cette énergie, elle est donc stockée, ce qui signifie que sa capacité d'intervenir dans l'homéostasie est également présente en continu. Ce stockage prend place dans le tissu adipeux et les études de ces dernières décennies se sont appliquées à comprendre le fonctionnement de ce tissu, ainsi que les mécanismes à l'origine de son développement dans les contextes physiologiques et pathologiques comme l'obésité (Cinti, 2018a).

Il est apparu clairement que le tissu adipeux participait activement à la régulation du métabolisme énergétique puisqu'en plus de gérer les ressources énergétiques, il produit et sécrète des hormones et des facteurs endocrines permettant un dialogue avec les autres acteurs du métabolisme comme le cerveau, le foie, le tube digestif ou encore les muscles. Une balance énergétique positive, où les apports énergétiques sont favorisés au détriment des dépenses, entraînera un stockage important des ressources excédentaires et conduira à des altérations du fonctionnement du tissu adipeux, d'où découlera l'obésité (Cinti, 2019).

Les études sur l'alimentation, en regard des pathologies métaboliques comme l'obésité et le diabète de type 2, ont pointé du doigt l'égale importance de la quantité et de la qualité des

nutriments ingérés, notamment les lipides et les acides gras (Simopoulos and DiNicolantonio, 2016). Les lipides sont des intervenants indispensables dans la physiologie puisqu'ils participent à l'organisation cellulaire et tissulaire en constituant les membranes, qu'au sein même de ces membranes ils sont essentiels aux plateformes enzymatiques, ils servent de précurseurs à la synthèse des hormones stéroïdes ou sont eux-mêmes des molécules de la signalisation cellulaire appelées lipokines (Blüher, 2014). En outre, la nature ou la qualité des nutriments nourrit la flore intestinale ou microbiote, qui produit des molécules impliquées dans la signalisation métabolique. Au niveau moléculaire, de plus en plus d'études montrent le rôle des nutriments dans la régulation de l'expression des gènes par des modifications épigénétiques (Ling and Rönn, 2019).

Parmi les lipides, les acides gras sont apparus comme primordiaux dans le métabolisme. Ce sont les travaux de Georges et Mildred Burr en 1930, qui ont mis en évidence le caractère essentiel de certains acides gras dans l'alimentation, notamment l'acide gras polyinsaturé (AGPI) $\omega 6$ linoléique (LA). Suite à leurs résultats, il a été observé une augmentation de la consommation de LA, particulièrement supplémenté dans les aliments préparés comme les laits infantiles et les nutriments médicaux parentéraux et une diminution de la quantité d'acides gras saturés, jugés défavorables pour la santé et notamment dans les cas d'obésité. Dans les années 1990, les études ont mené les chercheurs à mettre en évidence une augmentation de la prévalence globale de l'obésité et l'implication de l'utilisation presque unique du LA. Les études sur les régimes alimentaires de certaines populations présentant moins de cas d'obésité comme au Japon, en Europe du Nord ou encore dans le bassin méditerranéen ont permis de supposer qu'il fallait associer la consommation en LA avec des AGPI de la famille des $\omega 3$ comme l'acide α -linoléique (ALA). Aujourd'hui les organisations de santé publique comme l'OMS et l'ANSES préconisent de respecter un équilibre entre les quantités d'AGPI $\omega 6$ et $\omega 3$ (ratio $\omega 6/\omega 3$) de l'ordre de 4 (Kris-Etherton Penny M. et al., 2002). En effet, ces AGPI partagent des voies de synthèse et de métabolisation commune et l'équilibre du ratio $\omega 6/\omega 3$ permet des réponses physiologiques.

Les acides gras et plus particulièrement leurs dérivés ont été fortement étudiés dans la réponse inflammatoire, c'est-à-dire la défense de l'organisme contre les pathogènes, où ils apparaissent comme des facteurs majeurs. Les dérivés des acides gras que sont notamment les prostaglandines, les thromboxanes et les leucotriènes permettent au système immunitaire de s'organiser en augmentant ou en diminuant les flux sanguins, en régulant l'agrégation des plaquettes et en recrutant et activant les cellules immunitaires. D'autres dérivés d'acides gras qui sont les résolvines, les marésines et les protectines, permettent la résolution de cette inflammation lorsque le danger lié au pathogène n'est plus (Serhan et al., 2014).

Il se trouve que ces mêmes dérivés sont également impliqués dans le développement et le fonctionnement du tissu adipeux. Ils sont produits par les adipocytes eux-mêmes, ils possèdent

des récepteurs et activent une signalisation cellulaire dans les adipocytes. Les AGPI de la famille des $\omega 6$ et plus particulièrement l'acide arachidonique (ARA) et ses dérivés, ont été reliés à des mécanismes favorisant le stockage dans le tissu adipeux blanc et diminuant la formation et l'activité des adipocytes thermogéniques (Wada et al., 2007). Parmi eux, on trouve essentiellement l'ARA et la production de la prostaglandine $PGF2\alpha$ qui sont impliqués (Maehara et al., 2020). De la même manière, l'ARA permet la synthèse de la prostacycline 2, capable d'induire des voies cellulaires menant à l'activation du facteur majeur du développement de l'adipocyte ainsi que de la thermogénèse, $PPAR\gamma$ (Ghandour et al., 2016; Massiera et al., 2003).

Au cours de ma thèse, j'ai eu l'opportunité de travailler à la fois sur l'aspect régulateur des acides gras et de leurs dérivés sur la formation et l'activité thermogénique et également à un niveau plus moléculaire sur la régulation du facteur de transcription $PPAR\gamma$.

Dans un premier temps, j'ai pu constater l'importance du ratio $\omega 6/\omega 3$ dans la nutrition. *In vivo* sur un modèle murin et *in vitro* sur un modèle adipocytaire humain (hMADS), l'enrichissement en acides gras $\omega 6$ LA et ARA était défavorable au développement et à l'activité des adipocytes thermogéniques tandis que la supplémentation en acides gras $\omega 3$ LNA, EPA et DHA restaure une activité thermogénique (**Voir Annexe 1**). La simple supplémentation en $\omega 3$ n'a cependant jamais montré d'effets favorables, ce qui signifie que l'homéostasie n'est atteinte que dans le cadre d'un équilibre entre les deux familles d'AG. Cela s'explique notamment par le fait que les voies de synthèse de ces acides gras soient communes : une alimentation riche en acides gras $\omega 6$ seulement entraîne la production de métabolites à partir de l'ARA, qui sont pro-obésogéniques et anti-thermogéniques tandis que la présence d'acides gras $\omega 3$ diminue les métabolites des acides gras $\omega 6$ et augmente ceux des acides gras $\omega 3$.

Dans un deuxième temps, j'ai pu mettre en évidence le rôle inhibiteur de la thromboxane A2 (TxA2), un autre métabolite de l'ARA, sur la formation de l'adipocyte thermogénique. Le rôle de la TxA2 a longuement été étudié dans le cadre de la réponse inflammatoire où elle contrôle l'agrégation des plaquettes (la formation du thrombus). La TxA2 est sécrétée par les plaquettes et agit d'une manière paracrine pour augmenter l'agrégation des plaquettes et la constriction vasculaire. L'obésité est associée à une inflammation dite de bas grade dont les origines ne sont pas encore réellement claires même si les hypothèses semblent suggérer l'implication des métabolites d'acides gras ainsi qu'une situation de lipotoxicité causée par un excès de lipides et d'AG dans la circulation. Dans ce contexte de l'obésité, proche de l'inflammation dû à un pathogène, la production excessive de TxA2 entraînerait une inhibition de l'activité de l'adipocyte thermogénique et ainsi un frein à la dépense énergétique.

Parmi les métabolites de l'ARA, la PGI2 joue le rôle inverse de la TxA2 en entraînant la dilatation des vaisseaux sanguins et en inhibant la formation de thrombus. Elle a par ailleurs des effets favorables sur l'activité thermogénique et les études ont mis en évidence son action directe *via* son récepteur membranaire et *via* l'activation de PPAR γ sur l'expression de la protéine thermogénique par excellence qui est UCP1. Il est alors acceptable de supposer que la TxA2 emprunte les mêmes voies que la PGI2 pour exercer son action inhibitrice soit directement par son récepteur (TxAR2) qui est exprimé dans les adipocytes, soit indirectement en inhibant l'activation des facteurs permettant l'expression des marqueurs thermogéniques. On peut également envisager que la TxA2 entre en compétition avec la PGI2 au niveau de la signalisation cellulaire, comme par exemple interférer avec l'activation de PPAR γ . Toutefois, si la TxA2 est capable d'agir négativement sur les facteurs comme les PPARs, cela suggère qu'elle possède des propriétés anti-adipogéniques. Les mécanismes d'action de la TxA2 sur l'adipocyte et la thermogénèse ne sont toutefois pas décrits et restent à être identifiés.

Parmi les rôles décrits et supposés des acides gras et de leurs dérivés, il y a le rôle de ligands et activateurs des facteurs de transcription de la famille des PPARs.

La famille des facteurs de transcription PPARs a été également très largement étudiée dans le métabolisme, notamment pour leur implication dans la régulation énergétique, notamment PPAR γ qui est considéré comme le facteur essentiel de l'adipogénèse. Les ligands endogènes de PPAR γ ne sont pas précisément connus, seule leur appartenance à la famille des métabolites oxygénés des acides gras fait consensus et plusieurs études décrivent des activateurs potentiels au sein des métabolites des AGPI ω 6. On peut distinguer par exemple les 9- et 13-HODEs, produits de l'ALA par les LOX ou la 15d-PGJ2 produite de l'ARA par les COX. *In vitro* et dans des modèles de transactivation, ils sont capables d'interagir avec le domaine de liaison au ligand de PPAR γ et d'induire son activité transcriptionnelle mais leurs propriétés n'ont pas été démontrées *in vivo*. Ils n'ont également pas pu induire l'expression des marqueurs thermogéniques dans notre modèle cellulaire humain hMADS.

L'étude des mécanismes d'activation des PPARs est également très analysée et au cours de mon projet, j'ai pu identifier un antagoniste de PPAR γ et de PPAR α dans une moindre mesure. L'acide carnosique (AC) est un composé lipidique extrait du romarin et de la sauge, que la plante synthétise comme molécule anti-oxydante de défense. L'AC est utilisé pour ses propriétés anti-oxydantes dans les conservateurs alimentaires et comme antidiabétique sous forme d'infusions dans la médecine traditionnelle. *In vitro* et *in vivo*, il a été décrit comme un composé anti-adipogénique, empêchant le stockage des lipides dans un modèle cellulaire murin et diminuant la stéatose hépatique chez la souris. Les études montrent qu'il inhibe l'adipogénèse en inhibant indirectement l'expression de PPAR γ dans les adipocytes murins et j'ai pu montrer qu'il inhibait

également la formation de l'adipocyte thermogénique et ce, en exerçant une activité antagoniste sur la fixation de la rosiglitazone, un agoniste synthétique de PPAR γ .

Une caractéristique de l'obésité est la présence d'une inflammation de bas grade, impliquant le système immunitaire et favorisant notamment l'activation et l'infiltration de macrophages dans le tissu adipeux. En effet, les adipocytes sont capables de sécréter des cytokines inflammatoires qui entraînent la signalisation inflammatoire chez les macrophages résidents du tissu adipeux. Sous l'effets de cytokines pro-inflammatoires, les macrophages se polarisent vers un phénotype de type M1 inflammatoire et sécrètent à leur tour des cytokines inflammatoires (Cucak et al., 2014; Lumeng et al., 2007). Ces sécrétions induisent le recrutement et l'infiltration de macrophages neutres présents dans la circulation sanguine. Les cytokines pro-inflammatoires vont participer à la mise en place d'une résistance à l'insuline locale et ainsi à un dysfonctionnement des adipocytes. Par ailleurs, un environnement inflammatoire empêche l'activation de la thermogénèse (García-Alonso et al., 2016). Au cours d'une collaboration avec le Dr. Didier Pisani, j'ai pu mettre en évidence le rôle de deux autres composés, les 9- et 13-HODEs dans le maintien du phénotype anti-inflammatoire des macrophages, appelés de type M2 (**Voir Annexe 4**). Dans une lignée humaine de macrophages polarisés vers un type M1 ou M2, nous avons constaté que l'inhibition de PPAR γ par l'acide carnosique entraînait une modification du phénotype du macrophage, en potentialisant l'expression des marqueurs du type M1 ou en inhibant l'expression des marqueurs du type M2. La supplémentation en 9- et 13-HODE permettait de restaurer le phénotype du macrophage, en diminuant l'expression des marqueurs inflammatoires ou en augmentant ceux anti-inflammatoires. Les composés 9- et 13-HODEs ont été décrits comme des ligands de PPAR γ et capables de moduler l'expression des marqueurs des macrophages, notamment lorsqu'ils sont mis en contact avec des oxLDL (Lee et al., 2016; Nagy et al., 1998). Ces résultats suggèrent que les oxylipines et notamment les 9- et 13-HODEs contenues dans les vésicules lipidiques circulantes comme les oxLDL peuvent réguler la polarisation des macrophages résidents des tissus adipeux et favoriser la présence de macrophages anti-inflammatoires dans les tissus thermogéniques.

La famille des lipides est vaste et les possibilités d'y trouver des molécules régulatrices de l'adipogenèse et de la thermogénèse également. Au sein de la littérature il existe de nombreuses études mettant en avant des lipides et des acides gras qui vont améliorer l'homéostasie glucidique et favoriser la formation d'adipocytes thermogéniques comme par exemple la famille des acides gras hydroxylés sur un acide gras (FAHFAs) ou les métabolites décrits précédemment. L'utilisation de ces composés dans les études sur la régulation de l'adipogenèse blanche et brune reste cependant compliquée en raison de l'instabilité de ces molécules, de la diversité des modèles

dans lesquels elles ont été décrites et dans lesquels elles sont testées ou encore les différences avérées qui existent entre le modèle animal généralement utilisé qui est la souris, et la réalité chez l'Homme. En effet, la description et les études sur la thermogenèse chez l'animal font du BAT de souris un tissu aux propriétés vraiment intéressantes pour augmenter la dépense énergétique chez l'obèse mais les études chez l'Homme restent plus partagées (Nedergaard and Cannon, 2013). Il semble que le tissu thermogénique chez l'Homme est un tissu mixte, composé d'adipocytes thermogéniques et non thermogéniques (Cannon et al., 2020); que son pouvoir de thermogenèse est moins important, d'autant plus que chez l'Homme il faut prendre en compte les facteurs sociétaux et environnementaux comme la possibilité de chauffer son habitat et de porter des couches de vêtements protectrices. Il est alors difficile d'extrapoler les stratégies mises en place et efficaces chez la souris directement chez l'Homme. L'exposition au froid est compliquée à mettre en place et même si certaines méthodes de refroidissement sont à l'étude, comme les vestes réfrigérées, les différences interindividuelles doivent être prise en compte et nécessitent des protocoles personnalisés. Par ailleurs, la dépense énergétique réalisée par le BAT et par les adipocytes beiges est-elle suffisante pour entraîner des effets favorables sur la santé des personnes en situation d'obésité sévère ? Enfin, nous avons eu l'opportunité de comparer des profils d'oxylipines chez l'Homme et chez la souris dans les tissus adipeux blancs et bruns ainsi que dans les adipocytes thermogéniques se formant dans le WAT chez la souris (**Voir Annexes 2 et 3**). Et ces profils sont très différents, chez l'Homme ils ne permettent absolument pas de distinguer les tissus thermogéniques et non thermogéniques, peu de métabolites semblent donc caractéristiques et utilisables directement sous forme de compléments alimentaires par exemple.

CONCLUSION

En conclusion, mon travail de thèse a d'abord permis d'identifier des métabolites impliqués dans le développement et la fonction des adipocytes thermogéniques (**Figure 40**). Indirectement, les métabolites 9- et 13-HODEs participent à la fonction thermogénique car même s'ils n'induisent pas l'expression des marqueurs dans l'adipocyte, ils sont essentiels au fonctionnement des macrophages résidents du tissu adipeux et notamment dans le maintien de leur phénotype anti-inflammatoire. La production de TxA2 inhibe l'acquisition du phénotype thermogénique et, dans le contexte inflammatoire de l'obésité, ouvre de nouvelles possibilités thérapeutiques. En comprenant les mécanismes sous-jacents à la production de TxA2 dans l'obésité ainsi que ses mécanismes d'action sur l'adipocyte, il sera possible d'envisager des cibles pharmacologiques afin de réguler son action et de favoriser la dépense énergétique. Des expériences d'inhibition pharmacologique du récepteur et des voies de synthèse pourraient fournir des informations sur la signalisation de la TxA2 sur l'adipocyte. L'inhibition de PPAR γ à l'aide d'antagonistes ou de siRNA permettrait de répondre également en partie à cette question. L'utilisation de la transactivation permettrait de vérifier l'interaction potentielle entre la TxA2 ou PGI2 et PPAR γ . Mes résultats ont ensuite mis en évidence un nouvel antagoniste de PPAR γ , le principal acteur de l'acquisition du phénotype thermogénique par les adipocytes. Outre la mise au point d'un nouvel outil de travail *in vitro*, les effets inhibiteurs de l'acide carnosique sur l'adipocyte thermogénique permettent d'envisager des stratégies contre un autre versant des pathologies métaboliques qui est l'hyper activation du tissu adipeux thermogénique observé dans les formes cachexiques de certains cancers ainsi que chez les grands brûlés (Alipoor et al., 2020). Des études supplémentaires sont néanmoins nécessaires pour identifier plus précisément les mécanismes sous-jacents, notamment déterminer comment l'acide carnosique inhibe l'activité des PPARs, en se liant au site de liaison et en faisant compétition avec les ligands ? en modifiant la conformation du site de liaison ou en empêchant l'association aux cofacteurs des PPARs ?

Enfin, certains métabolites décrits pour jouer un rôle dans la thermogenèse n'ont pas montré d'effets sur l'expression des marqueurs principaux (**Figure 41**) mais d'autres marqueurs doivent être analysés comme ceux impliqués dans l'homéostasie glucidique ainsi que les fonctions des adipocytes, comme la respiration ou la lipolyse.

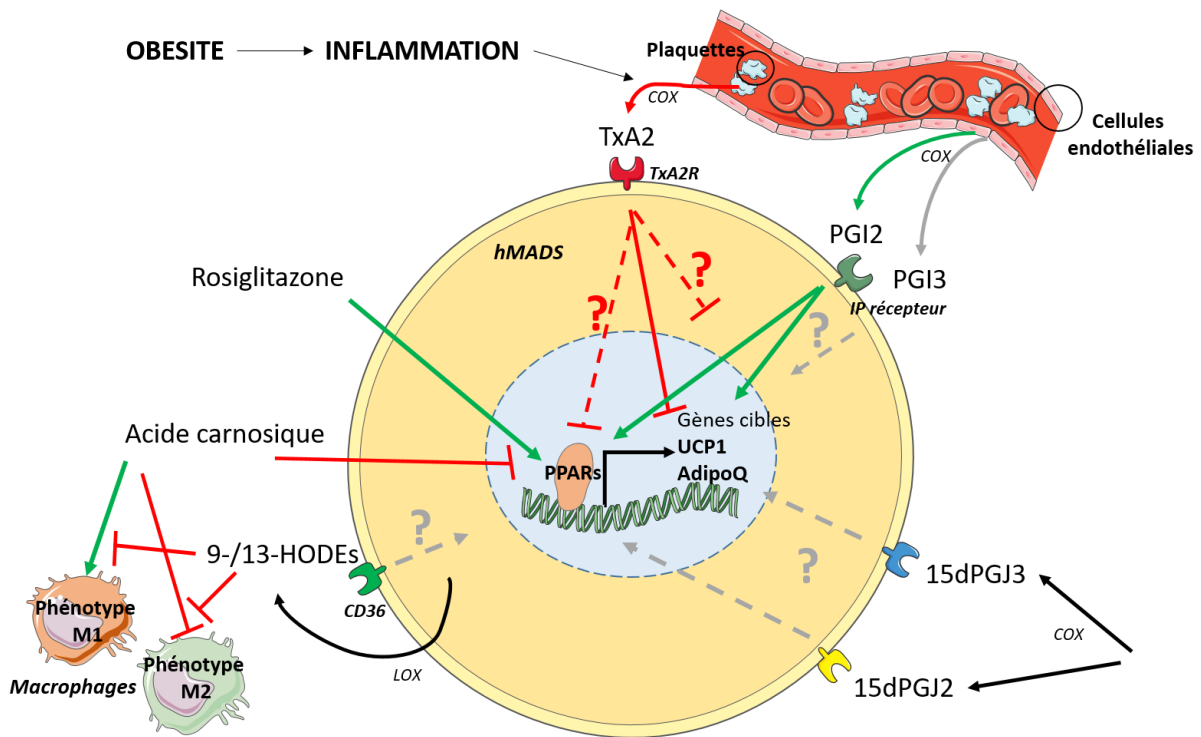


Figure 40 : Schéma récapitulatif. Les effets des métabolites d'acides gras et de l'acide carnosique sur la formation et l'activité de l'adipocyte thermogénique.

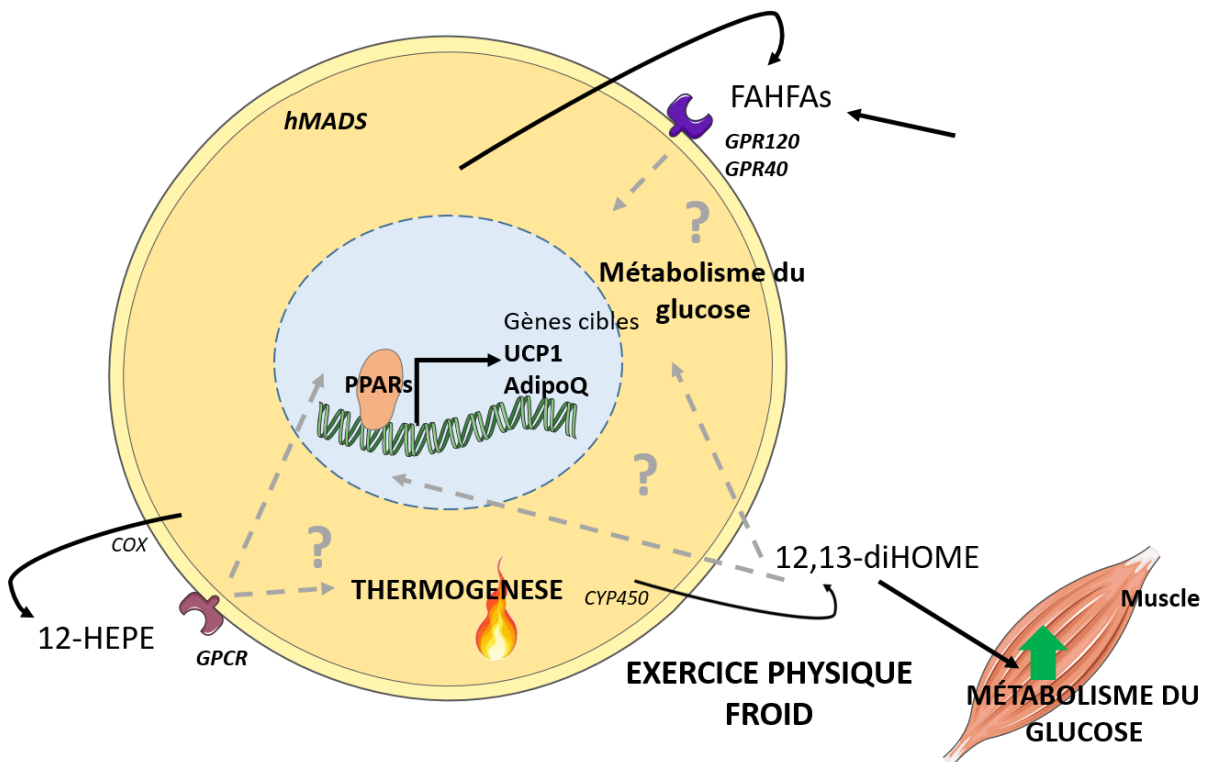


Figure 41 : Schéma récapitulatif. Les métabolites 12-HEPE, 12,13-diHOME ainsi que la nouvelle classe d'acides gras FAHFAs n'induisent pas le phénotype thermogénique dans les adipocytes hMADS.

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ANNEXE 1:

Impact of dietary ω 3 polyunsaturated fatty acid supplementation on brown and brite adipocyte function

Rayane A Ghandour, **Cécilia Colson**, Maude Giroud, Stefanie Maurer, Samah Rekima, Gérard Ailhaud, Martin Klingenspor, Ez-Zoubir Amri, Didier F Pisani

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Impact of dietary ω 3 polyunsaturated fatty acid supplementation on brown and brite adipocyte function^S

Rayane A. Ghandour,* Cecilia Colson,* Maude Giroud,[†] Stefanie Maurer,[§] Samah Rekima,* Gérard Ailhaud,* Martin Klingenspor,[§] Ez-Zoubir Amri,^{1,*} and Didier F. Pisani^{1,*}

Université Côte d'Azur,* CNRS, Inserm, iBV, Nice, France; Institute for Diabetes and Cancer (IDC),[†] Helmholtz Zentrum, München, Germany, Center for Nutritional Medicine,[§] Technical University Munich, Freising, Germany

ORCID IDs: 0000-0003-0596-2906 (G.A.); 0000-0002-4502-6664 (M.K.); 0000-0001-8426-5396 (E.A.); 0000-0001-5879-8527 (D.F.P)

Abstract The recent characterization of functional brown adipose tissue in adult humans has opened new perspectives for regulation of energy expenditure with respect to obesity and diabetes. Furthermore, dietary recommendations have taken into account the insufficient dietary intake of ω 3 PUFAs and the concomitant excessive intake of ω 6 PUFA associated with the occurrence of overweight/obesity. We aimed to study whether ω 3 PUFAs could play a role in the recruitment and function of energy-dissipating brown/brite adipocytes. We show that ω 3 PUFA supplementation has a beneficial effect on the thermogenic function of adipocytes. In vivo, a low dietary ω 6: ω 3 ratio improved the thermogenic response of brown and white adipose tissues to β 3-adrenergic stimulation. This effect was recapitulated in vitro by PUFA treatment of hMADS adipocytes. We pinpointed the ω 6-derived eicosanoid prostaglandin (PG)F 2α as the molecular origin because the effects were mimicked with a specific PGF 2α receptor agonist. PGF 2α level in hMADS adipocytes was reduced in response to ω 3 PUFA supplementation. The recruitment of thermogenic adipocytes is influenced by the local quantity of individual oxylipins, which is controlled by the ω 6: ω 3 ratio of available lipids. In human nutrition, energy homeostasis may thus benefit from the implementation of a more balanced dietary ω 6: ω 3 ratio.—Ghandour, R. A., C. Colson, M. Giroud, S. Maurer, S. Rekima, G. Ailhaud, M. Klingenspor, E-Z. Amri, and D. F. Pisani. **Impact of dietary ω 3 polyunsaturated fatty acid supplementation on brown and brite adipocyte function** *J. Lipid Res.* 2018. 59: 452–461.

Supplementary key words PUFA • oxylipins • prostaglandins • adipose tissue • UCP1

Dietary fats are the source of essential PUFAs; for example, ω 6 linoleic acid (LA), a precursor of ω 6 arachidonic

acid (ARA), and ω 3 α -linolenic acid, a precursor of ω 3 EPA and DHA. These long-chain PUFAs trigger a variety of biological responses, particularly in adipose tissue, and are required for healthy development (1, 2). New dietary recommendations take into account the insufficient intake of ω 3 PUFAs and the excess of ω 6 PUFAs, which correlate with overweight/obesity (3–5). Indeed, high ω 6: ω 3 ratios are positively associated with adiposity of infants at 6 months and 3 and 4 years of age (6–8), and ARA intake correlates positively with BMI and the associated metabolic syndrome (9–13). In fact, diets exhibiting a high ω 6: ω 3 ratio result in higher ARA bioavailability for the synthesis of ω 6 derived oxylipins due to an insufficient compensatory effect of EPA and DHA (14). These ω 6 oxygenated derivatives are known to favor inflammatory responses (15), promote energy storage (16), and inhibit energy expenditure (17). These effects are mainly triggered by oxylipins arising from the cyclooxygenase (COX) pathway.

In contrast to the white adipose tissue (WAT) involved in energy storage and release, brown adipose tissue (BAT) is endowed with thermogenic activity and regulates body temperature by dissipating energy through nonshivering thermogenesis (18). This mechanism is mediated by uncoupling protein 1 (UCP1), which uncouples mitochondrial oxygen consumption from energy production. Interestingly, a further population of UCP1-positive adipocytes is present in WAT and is termed brite for “brown in white” or beige adipocytes (19–21). In vivo, brite adipocytes stem from progenitors or emerge by direct conversion of mature white

Abbreviations: ARA, arachidonic acid; BAT, brown adipose tissue; COX, cyclooxygenase; eWAT, epididymal WAT; FP receptor, PGF 2α receptor; iBAT, interscapular BAT; LA, linoleic acid; LNA, alpha linolenic acid; OCR, oxygen consumption rate; PG, prostaglandin; PLN1, perilipin 1; qPCR, quantitative PCR; scWAT, subcutaneous WAT; UCP, uncoupling protein 1; WAT, white adipose tissue.

¹To whom correspondence should be addressed.

e-mail: pisani@unice.fr (D.F.P.); amri@unice.fr (E-Z.A.)

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adipocytes (22–24). Several studies have demonstrated the involvement of oxylipins in fat mass development through the modulation of white and brown/brite adipocyte differentiation and activity (25). Mice exposed to diets with high levels of ω 6 PUFA during the perinatal period display progressive accumulation of body fat across generations (26). This observation is in agreement with findings that overweight and obesity have increased and develop earlier in life within a given population consuming Westernized diets rich in ω 6 PUFAs during the last decades (26). Moreover, oxylipins derived from ω 6 PUFA inhibit brite and brown adipocyte activity both in vitro and in vivo (17). ARA lowers the expression and function of UCPI, thus affecting the dissipation of energy in these thermogenic adipocytes. These effects are mediated via COX activities that lead to increased synthesis and release of prostaglandin (PG)E2 and PGF2 α , which act as inhibitors of Ucp1-mediated thermogenesis via a calcium-dependent pathway.

It has been shown that healthy adult humans exhibit BAT in the cervical and thoracic part of the body, which consists of islets of energy-dissipating thermogenic adipocytes (27–31). These adipocytes display a gene expression signature comparable to either rodent brown or brite adipocytes depending on the localization and the depth of the analyzed tissue (21, 32–34). As brown and brite adipocytes represent important candidates for controlling body weight, investigations in humans of the regulation of brown/brite adipocyte recruitment and activation are in demand, particularly from a nutritional point of view, because quantitative and qualitative issues of dietary lipids are relevant to increased body weight (3). Because, in mammals, oxylipins govern a large part of the biology of adipose tissue, any dysregulation in their levels may disrupt tissue homeostasis. Thus, it is tempting to assume that prevention of excessive consumption of ω 6 fatty acids or reestablishment of a balanced ω 6: ω 3 PUFA ratio may contribute to reduce excessive adipose tissue development by controlling white adipocyte formation and enhancing brite adipocyte recruitment.

Herein, we aimed to study whether the inhibitory effect of ω 6-PUFA LA (via local action of ARA) on brite adipocyte recruitment could be reversed by supplementation with the ω 3-PUFA α -linolenic acid associated to its metabolites EPA and DHA. We show, in vivo in mice and in vitro in human cells, that adjustment of a ω 6: ω 3 PUFA ratio from 30 to 3.7 by supplementation of ω 6 PUFA-enriched diet with ω 3 PUFAs rescues the inhibitory effect of ω 6-derived oxylipins on the activity of brown and brite adipocytes. These effects are mediated through oxylipins via the decreased level of PGF2 α .

MATERIALS AND METHODS

Animals and diets

The experiments were conducted in accordance with the French and European regulations (directive 2010/63/EU) for the care and use of research animals and were approved by national

experimentation committees (MESR 01947.03). Ten-week-old C57BL/6J male mice from Janvier Laboratory (Le Genest Saint Isle, France) were maintained at thermoneutrality ($28 \pm 2^\circ\text{C}$) and 12:12 h light-dark cycles, with ad libitum access to food and water and euthanized between 10 and 11 AM. Mice were fed for 12 weeks with isocaloric ω 6- or ω 3-supplemented diets (12% energy content as lipids). These experimental diets were prepared from standard chow diets (ref. 2016, Harlan Laboratories, Madison WI). The ω 6-supplemented diet was enriched with 0.5% linoleate-ethyl-ester (LA) and 0.7% oleate-ethyl-ester (ratio ω 6: ω 3 = 30); the ω 3-supplemented diet comprised 0.5% LA, 0.54% α -linolenate-ethyl-ester (LNA), 0.08% eicosapentaenoate-ethyl-ester and 0.08% docosahexaenoate-ethyl-ester (ratio ω 6: ω 3 = 3.7) (see supplemental Table S1 for details) to follow human nutritional recommendations. Safflower oil (0.5%) was added to favor dispersion of ethyl esters in the diet. Fatty acid ethyl esters were from Nu-Chek-Prep (Waterville, MN) and diets were produced by Harlan. Chronic β ₃-adrenergic receptor stimulation was carried out during the last week of the diet treatment by daily intra-peritoneal injections of CL316,243 (1 mg/kg in saline solution). Control mice were injected with vehicle only. Blood, interscapular BAT (iBAT), epididymal WAT (eWAT), and inguinal subcutaneous WAT (scWAT) were sampled and used for different analyses.

hMADS cell culture

The establishment and characterization of hMADS cells has been described (35–37). In the experiments reported herein, hMADS-3 cells were used between passages 14 and 20. All experiments were performed at least three times using different cultures. Cells were cultured and differentiated as previously described (17, 35). Briefly, cells were induced to differentiate at day 2 postconfluence (designated as day 0) in DMEM/Ham's F12 media supplemented with 10 μ g/ml transferrin, 10 nM insulin, 0.2 nM triiodothyronine, 1 μ M dexamethasone, and 500 μ M isobutylmethylxanthine. Two days later, the medium was changed (dexamethasone and isobutylmethylxanthine omitted) and 100 nM rosiglitazone was added. At day 9, rosiglitazone was withdrawn to enable white adipocyte differentiation but was again included between days 14 and 17 to promote white-to-brite adipocyte conversion as previously described (17, 35). Fatty acids were bound to BSA (0.04% for 15 min at 37°C) prior to addition to culture media.

Oxylipin quantification

In vitro analysis of secreted PGF2 α was performed on differentiated cells at day 17 after incubation for 1 or 24 h in fresh culture media. PGF2 α was quantified by Elisa Immuno Assay following the manufacturer's instructions (Cayman, BertinPharma, Montigny le Bretonneux, France).

Quantification of oxylipins was performed at the METATOUL platform (MetaboHUB, INSERM UMR 1048, I2MC, Toulouse, France) by mass spectrometry analysis. All tissues were snap-frozen with liquid nitrogen immediately after collection and stored at -80°C until extraction. Extraction and analysis were performed as previously described (17, 38).

Measurement of oxygen consumption

Oxygen consumption rate (OCR) of hMADS adipocytes was determined at day 17 using an XF24 Extracellular Flux Analyzer (Seahorse Bioscience, Agilent). ATP synthase uncoupled OCR was determined by the addition of 1.2 μ M oligomycin A (ATP synthase inhibitor) and maximal OCR by addition of 1 μ M FCCP [Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone as a mitochondrial oxidative phosphorylation uncoupling agent]. Rotenone and Antimycin A (2 μ M each) were used to inhibit Complex I- and Complex III-dependent respiration, respectively. Parameters

were measured for each individual well using the OCR values as previously described (39).

Histology

Histological analysis was performed as previously described (17). Sections (4 μ m) were dewaxed and treated in boiling citrate buffer (10 mM, pH 6.0) for 6 min. Cooled sections were rinsed and then permeabilized in PBS 0.2% triton X-100 at room temperature for 20 min. Sections were saturated in the same buffer containing 3% BSA for 30 min, incubated with perilipin antibody (#RDI-PROGP29, Research Diagnostic Inc., Flanders, NJ) for 1 h, and TRITC-coupled anti-guinea pig antibody for 45 min. Nuclear staining was performed with DAPI.

UCP1 immunohistochemistry was performed following manufacturer's instructions (LSAB+ system-HRP, Dako, Les Ulis, France) and using goat anti-UCP1 (clone C-17, Santa Cruz, Teubio, Le Perray-en-Yvelines, France). Visualization was performed with an Axiovert microscope and pictures were captured with AxioVision software (Carl Zeiss, Jena, Germany). Lipid droplet diameters (visualized by perilipin staining) were measured using Fiji software (40). Displayed images are representative of the four mice per group analyzed.

Isolation and analysis of RNA

These procedures follow MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) recommendations (41). Total RNA was extracted using TRI-Reagent kit (Euromedex, Souffelweyersheim, France) according to the manufacturer's instructions. For RNA isolation from organs, tissues were homogenized in TRI-Reagent using a dispersing instrument (ULTRA TURRAX T25, Ika, Germany). RT-PCR was performed using M-MLV-RT (Promega). SYBR qPCR premix Ex TaqII from Takara (Ozyme, France) was used for quantitative PCR (qPCR), and assays were run on a StepOne Plus ABI real-time PCR machine (PerkinElmer Life and Analytical Sciences, Waltham, MA). The expression of selected genes was normalized to that of the TATA-box binding protein (TBP) and 36B4 housekeeping genes for human genes, and 36B4 and GAPDH for mouse genes, and then quantified using the comparative- Δ Ct method. Primer sequences are available upon request.

Statistical analysis

Data are expressed as mean values \pm SEM and were analyzed using InStat software (GraphPad Software). Data were analyzed by one-way ANOVA followed by a Student-Newman-Keuls posttest, or Student's *t*-test to assess statistical differences between experimental groups. Differences were considered statistically significant with $P < 0.01$.

RESULTS

ω 3 PUFA supplementation makes mice sensitive to β 3-adrenergic receptor agonist treatment

Ten-week-old mice were fed for 12 weeks with an isocaloric standard diet enriched in ω 6 PUFAs (ω 6 diet, ω 6: ω 3 = 30), or supplemented with ω 3 PUFAs (ω 3 diet, ω 6: ω 3 = 3.7) (supplemental Table S1). There was no difference in body weight (Fig. 1A) and food intake during 12 weeks (ω 6 diet, 4.49 g/day vs. ω 3 diet, 4.46 g/day per mouse). During the last week, mice received daily injections of the agonist CL316,243 (1 mg/kg) to activate the β 3-adrenergic receptor pathway. As expected, such treatment decreased body

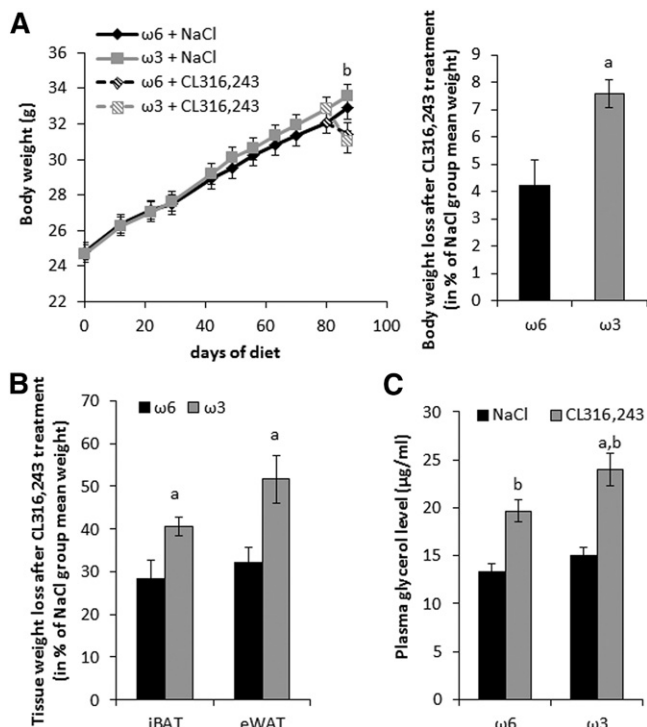


Fig. 1. A low dietary ω 6: ω 3 ratio enhances body weight loss in response to a β 3-adrenergic receptor agonist. Mice maintained at 28°C were fed with diets supplemented with ω 6 PUFAs or ω 3 PUFAs for 12 weeks. CL316,243 or vehicle (NaCl) treatment was performed daily during the last week of feeding. A: Body weight development of mice (left); body weight loss of mice after CL316,243 treatment relative to vehicle treatment (right). B: Interscapular brown adipose tissue (iBAT) and epididymal white adipose tissue (eWAT) weight loss after CL316,243 treatment. C: Plasma glycerol levels after NaCl or CL316,243 treatment. Data are mean \pm SEM, $n = 12$ mice/group. a, $P < 0.01$ ω 6 versus ω 3; b, $P < 0.01$ NaCl versus CL316,243.

weight (Fig. 1A) as well as iBAT and eWAT mass (Fig. 1B). Interestingly, ω 3 diet-fed mice showed a larger decrease in body mass and adipose tissue mass on CL316,243-treatment compared with ω 6 diet-fed mice (Fig. 1A,B). This observation is in line with higher plasma glycerol levels (Fig. 1C). Together, these results indicate a higher sensitivity of ω 3 diet-fed mice to CL316,243 treatment.

ω 3 diet-fed mice showed an improved response to thermogenic stimulation in BAT and scWAT

After CL316,243 treatment, mice fed the ω 3-diet displayed an increase in BAT mass loss and in plasma glycerol level, suggesting a higher lipolysis capacity. As expected, histological analysis of BAT showed clear activation of brown adipocytes after CL316,243 treatment as indicated by adipocyte morphological changes in the two groups of mice (Fig. 2A). This observation was confirmed by immunostaining for perilipin 1 (PLN1), a known lipid droplet surface protein, which allowed the measurement of droplet diameters. The data showed smaller lipid droplets in BAT of mice treated with CL316,243 (Fig. 2C). Interestingly, the droplet size was smaller in ω 3 compared with ω 6 diet-fed mice of both treatment groups (Fig. 2C). These morphological modifications were in line with elevated

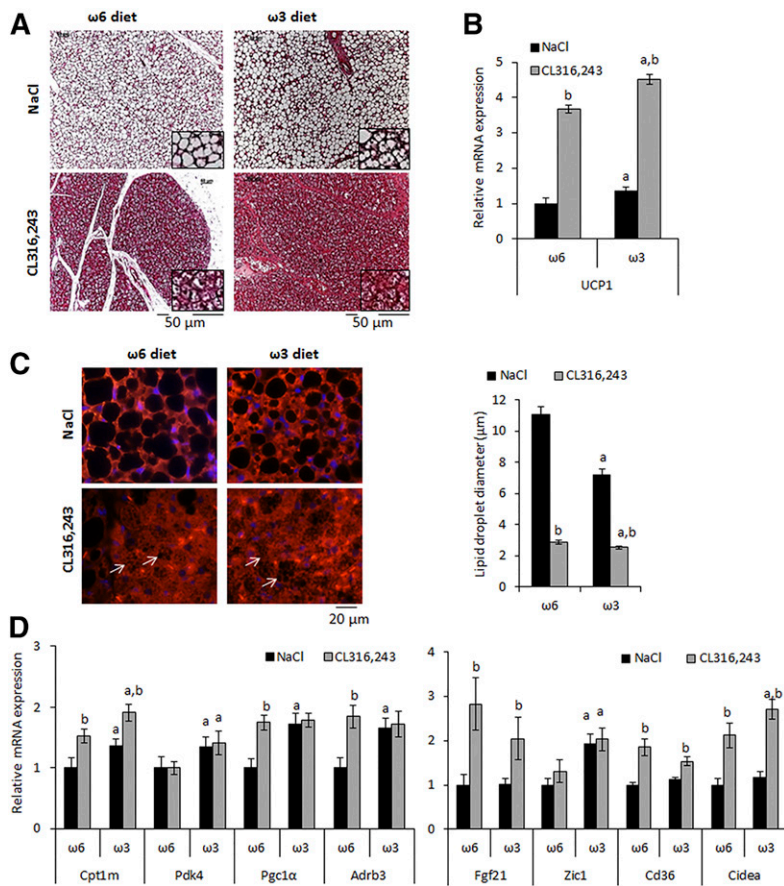


Fig. 2. Morphological and molecular analysis of brown adipose tissue. A: Hematoxylin and eosin staining of paraffin-embedded tissue sections and (B) expression of Ucp1 mRNA. C: PLN1 immunostaining of iBAT sections from mice fed ω6 or ω3-diet. Lipid droplet (white arrows) diameters were evaluated using PLN1 staining; data are mean ± SEM, n = 200 lipid droplets/mouse, 4 mice/group. D: Expression of brown adipocyte marker mRNAs was determined by RT-qPCR. mRNA expressions are shown as fold increase relative to “NaCl ω6” values. Data are mean ± SEM, n = 12 mice/group. a, $P < 0.01$ ω6 versus ω3; b, $P < 0.01$ NaCl versus CL316,243.

expression of brown adipocyte marker genes in ω3 versus ω6 diet-fed mice (Fig. 2B, D).

The β3-adrenergic agonist CL316,243 is a potent inducer of brown adipocytes and therefore we hypothesized a contribution of these thermogenic cells to CL316,243-induced plasma glycerol levels and body weight loss. No morphological or gene expression differences were found in scWAT between the two groups of mice in response to vehicle treatment (Fig. 3A, B). However, after CL316,243 treatment, visual examination of sections suggested increased Ucp1-positive adipocytes in scWAT of ω3 diet-fed mice compared with ω6 diet-fed mice (Fig. 3A). This observation was supported by the analysis of Ucp1 mRNA expression, which tended to be increased in the ω3 diet-fed group after CL316,243 treatment. Gene expression of other markers in response to CL316,243 was not affected by the diet (Fig. 3B). As shown in supplemental Fig. S1, eWAT did not display the multiloculated adipocytes that are characteristic of UCP1+ adipocytes. However, a decrease of adipocyte mean diameter after CL316,243 treatment was found in both groups, in agreement with eWAT weight decrease (Fig. 1B).

Together, these data indicate that the dietary ω6:ω3 ratio affects the induction/activation of thermogenic adipocytes in BAT and scWAT upon stimulation with a β-adrenergic agonist. As dietary fatty acid composition can considerably influence the quality and quantity of fatty acid metabolites (2, 5), this differential response to thermogenic

stimulation may be the consequence of modulated eicosanoid level in adipose tissues.

Dietary ω3 PUFA supplementation controls the level of ω6-derived oxylipins

To investigate diet-induced differences in the quantity of ω3- and ω6-derived oxylipins, we quantified the levels of more than 30 selected metabolites in scWAT and iBAT from ω3 and ω6 diet-fed mice. Depending on their origin, oxygenated metabolites derived from ω6 (ARA) or ω3 (EPA and/or DHA) PUFAs, due to COX, LOX, and CYP450 activities (supplemental Fig. S2A, B, left panels, oxylipins) or to COX activity only (right panels, eicosanoids: a group of oxylipins) were analyzed (analyzed oxylipins are detailed in supplemental Table S2).

As expected, ω3 PUFA supplementation allowed higher quantities of their metabolites in BAT and scWAT (supplemental Fig. S2A, B, black columns). In BAT, where the molecular effect of a ω3 diet was the most important, we did not find any significant impact of the diet on ω6-derived oxylipin levels (supplemental Fig. S2A, gray columns). In scWAT, similar data were obtained in untreated mice; however, when the mice were treated with the β3-adrenergic receptor agonist, the decrease in the levels of ω6-derived oxylipins was higher in ω3 diet-fed mice than in ω6-diet fed mice (supplemental Fig. S2).

Analysis of COX-derived eicosanoids showed an increase in EPA-derived eicosanoids in the ω3 diet-fed group and no

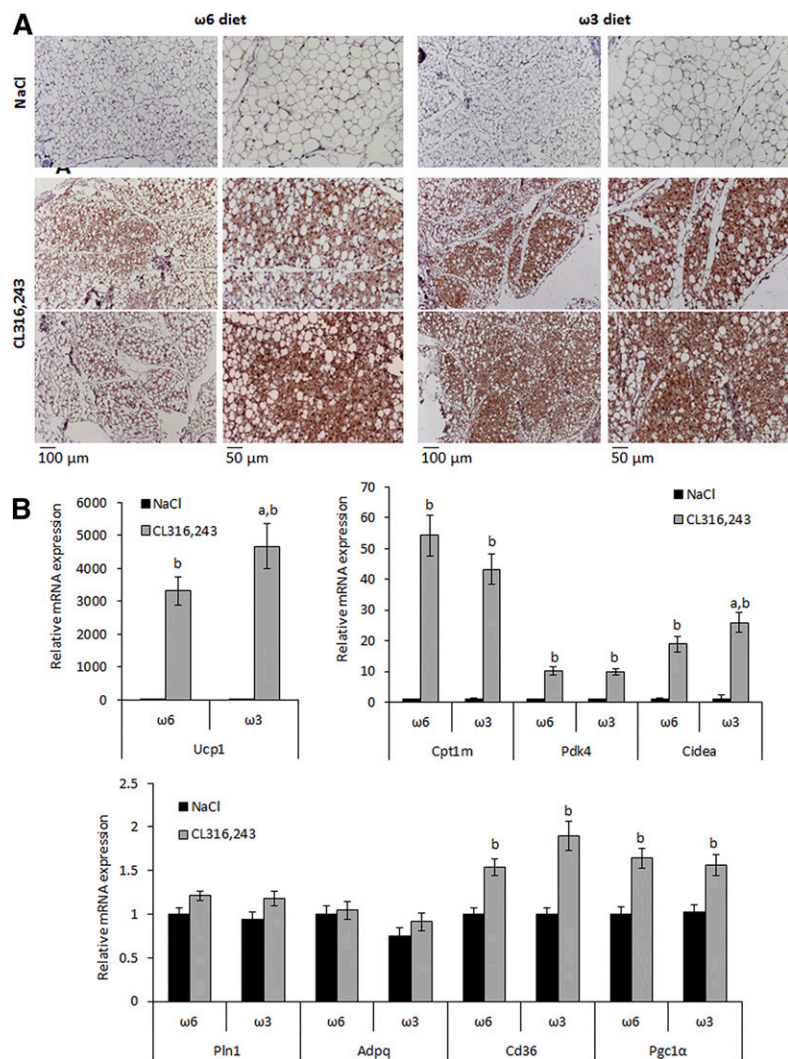


Fig. 3. Morphological and molecular analysis of subcutaneous white adipose tissue. A: UCP1 immunohistochemical analysis of subcutaneous WAT (scWAT) sections from mice fed an $\omega 6$ or $\omega 3$ diet. Slides were counterstained with hematoxylin and eosin. B: Expression of Ucp1 and brite/white adipocyte marker mRNAs was determined by RT-qPCR. mRNA expressions are shown as fold increase relative to “NaCl $\omega 6$ ” values. Data are mean \pm SEM, n = 12 mice/group. a, $P < 0.01$ $\omega 6$ versus $\omega 3$; b, $P < 0.01$ NaCl versus CL316,243.

significant decrease of $\omega 6$ -derived eicosanoids, except after CL316,243 treatment (supplemental Fig. S2A, B, right panels). Of note, the treatment with CL316,243, a situation where UCP1 activity is increased via the release of fatty acids, led to a decrease in the levels of the various oxygenated metabolites, possibly reflecting the preferential use of PUFAs as fuel for thermogenesis, thus limiting PUFA availability for oxylipin production (Fig. 4 and supplemental Fig. S2).

Previous work from our laboratory has demonstrated that COX-derived oxylipins are crucial for the formation and activation of thermogenic adipocytes (17, 42). Accordingly, further analyses focused on the levels of individual COX-derived oxylipins [i.e., 6kPGF1 α (representative of PGI $_2$), PGF2 α , PGE $_2$, TXB $_2$, PGD $_2$, and 15dPGJ $_2$] in BAT and scWAT. The levels of most individual metabolites were similar in iBAT and scWAT of vehicle-treated $\omega 6$ diet- and $\omega 3$ diet-fed mice, except for a decrease in TXB $_2$ levels in BAT and in PGE $_2$ levels in scWAT (Fig. 4A, B). Treatment with CL316,243 resulted in decreased quantity of these oxylipins in both diet groups in BAT and scWAT, although downregulation of TXB $_2$ and PGE $_2$ was more pronounced in scWAT than iBAT. Interestingly, PGF2 α was exempt from this mode of regulation: CL316,243-treatment resulted in a significant decrease in PGF2 α level in $\omega 3$ diet-fed

but not in $\omega 6$ diet-fed mice. Thus, the level of this eicosanoid in response to CL316,243-treatment is inversely correlated with Ucp1 expression in BAT and scWAT.

Our previous results demonstrated (17) that PGF2 α is a negative regulator of thermogenic adipocyte recruitment. Indeed, the $\omega 6$ -enriched diet PGF2 α level was not affected by CL316,243 treatment (Fig. 4A, B) and correlated with inhibition of brown and brite adipocyte recruitment and activation (Figs. 2, 3). In contrast, for an $\omega 3$ -enriched diet after CL316,243 treatment, no alteration in brown and brite adipocyte recruitment and activation was found, although a striking decrease in the PGF2 α level was observed (Fig. 4A, B).

Taken together, these results demonstrated that diet supplementation with $\omega 3$ PUFAs reversed the inhibitory effect of a $\omega 6$ -enriched diet. This effect could be due to competition between $\omega 6$ and $\omega 3$ PUFAs at the COX activity level leading in turn to a decrease in PGF2 α synthesis. To further investigate the effect of PUFAs and eicosanoids on adipocyte function, we used brite adipocytes derived from hMADS cells as a model system.

EPA reversed the ARA-inhibitory effect in vitro

hMADS cells are a human stem cell model that is able to differentiate into white adipocytes and to convert into

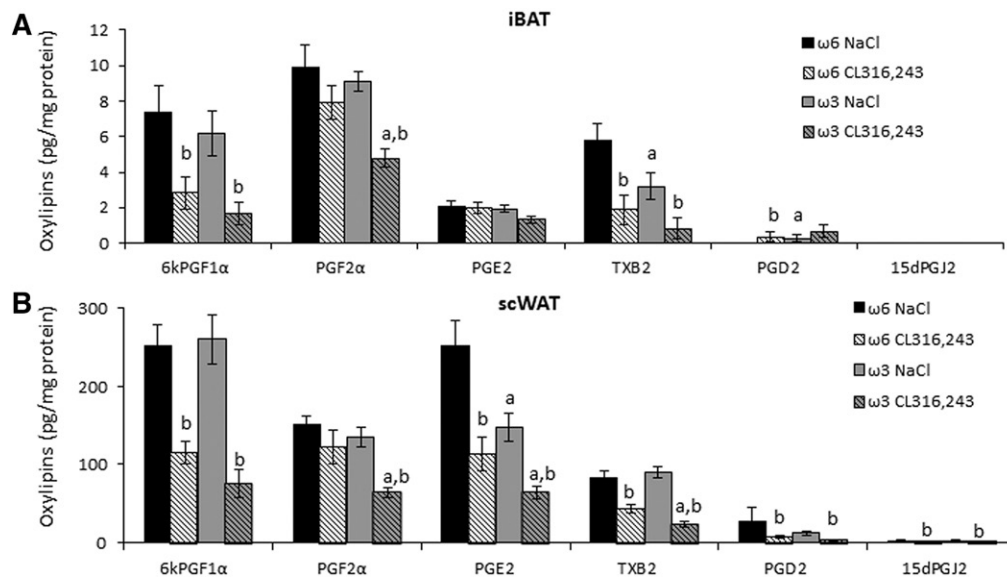


Fig. 4. Abundance of $\omega 6$ PUFA-derived eicosanoids in BAT and WAT. Eicosanoid levels were measured by LC-MS/MS in (A) iBAT and (B) scWAT of vehicle- and CL316,243-treated mice fed with an $\omega 6$ or $\omega 3$ diet. Data are mean \pm SEM, 8 mice/group. a, $P < 0.01$ $\omega 6$ versus $\omega 3$ and b, $P < 0.01$ NaCl versus CL316,243.

functional brite adipocytes upon rosiglitazone treatment [Fig. 5A (17, 35)]. As described previously (17), this process can be modified by $\omega 6$ -PUFA ARA; treatment of hMADS adipocytes during the conversion to brite adipocytes with ARA inhibited the expression of UCP1 and other brite adi-

pocyte markers (CIDEA, CPT1M, PLN5) (Fig. 5A). Such treatment did not affect adipogenesis per se, as the expression of PLN1 and ADIPQ was not affected (Fig. 5A). Treatment of hMADS adipocytes with $\omega 3$ -PUFA EPA (molar ARA:EPA ratio of 3) reversed the inhibitory effect of ARA

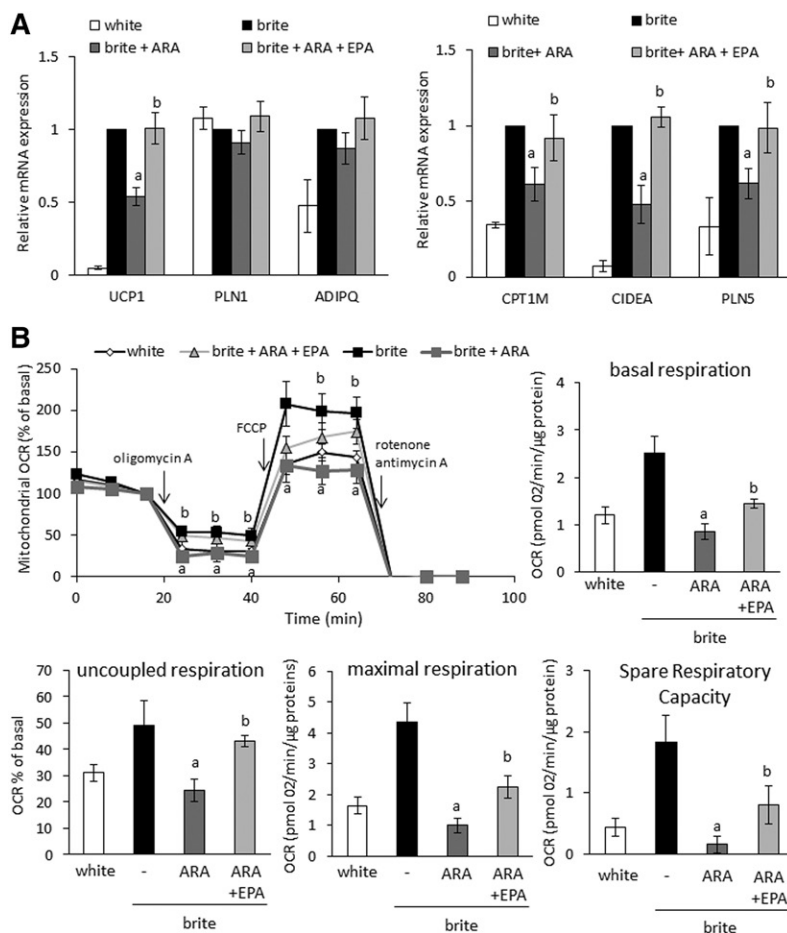


Fig. 5. EPA reversed the effect of ARA on adipocyte browning in vitro. hMADS cells were differentiated into white or brite adipocytes. Brite hMADS adipocytes were treated during the last 3 days of differentiation with 10 μ M ARA in the presence or absence of 3.3 μ M EPA. A: Expression of adipocyte markers was determined by RT-qPCR and is expressed as fold increase relative to “brite” group values. B: Basal, ATP synthase uncoupled respiration (% of residual respiration after addition of ATP synthase inhibitor oligomycin), and maximal respiration (obtained after addition of the chemical uncoupling agent FCCP) were assessed at the end of treatment to determine the oxygen consumption rate (OCR) of mitochondria. The spare respiratory capacity represents a measure for the full respiratory potential of mitochondria and is calculated as the difference between maximal and basal respiration. Data are mean \pm SEM of three (A) or six (B) independent experiments. a, $P < 0.01$ versus brite; b, $P < 0.01$ versus brite + ARA.

on brite adipocyte marker expression (Fig. 5A). This effect was further investigated at the functional level. Oxygen consumption analysis of hMADS brite adipocytes revealed that ARA inhibited all mitochondrial respiration parameters (Fig. 5B), (i.e., basal, uncoupled, and maximal respiration as well as spare respiratory capacity). EPA partially reversed this inhibitory effect of ARA on mitochondrial oxygen consumption, thus affecting the overall thermogenic capacity of these cells (Fig. 5B). No change in adipogenic marker expression was observed when DHA was added as an alternative ω 3 PUFA (supplemental Fig. S3) indicating that EPA specifically represents the effective compound within this class of molecules.

Taken together, these results indicate that the thermogenic function of adipocytes is positively and negatively affected by EPA (ω 3) and ARA (ω 6), respectively.

EPA reduced PGF2 α synthesis and secretion

To identify the pathway involved in the EPA effect, we used fluprostenol, an agonist of the FP receptor (PGF2 α receptor), instead of the precursor ARA during the conversion of white hMADS adipocytes into brite adipocytes. In agreement with our previous work (17), fluprostenol mimicked the effect of ARA by inhibiting the expression of UCP1 mRNA (Fig. 6A). Cotreatment with EPA did not reverse this effect (Fig. 6A), indicating that the UCP1 expression of brite hMADS adipocytes was directly affected by an interaction between the FP receptor and its ω 6-derived, endogenous ligand PGF2 α . Thus, we hypothesized that EPA reversed the ARA-induced effects in brite hMADS adipocytes by modulating the availability of PGF2 α as ligand for its receptor. Accordingly, we measured PGF2 α secretion after ARA treatment of brite hMADS adipocytes in the absence or presence of EPA. PGF2 α secretion was not altered by EPA during the first hour of treatment. However, a striking decrease in PGF2 α levels was observed after 24 h (Fig. 6B). This decrease in quantity was likely not due to a lower

expression of the major enzymes involved in PGF2 α synthesis. Indeed, the expression of COX-1, COX-2 (allowing the metabolism of ARA to PGG2), AKR1B1, and AKR1C3 (allowing the metabolism of PGG2 to PGF2 α) mRNAs was not altered by EPA treatment (Fig. 6C). As both ARA and EPA can be recognized and metabolized by COX-1 and COX-2, EPA most likely blocks PGF2 α synthesis by competing with ARA.

DISCUSSION

The obesogenic effect of ω 6 PUFAs, particularly ARA, is thought to originate from their metabolism to oxylipins, thus promoting fat storage and a reduction in energy expenditure (16, 17, 43, 44). However, this system and its regulation appear to underlie an unanticipated complexity. In fact, previous studies demonstrated the ω 6 eicosanoid prostacyclin (PGI2, which is derived from ARA via COX-dependent metabolism) as a positive modulator of UCP1 expression and brite adipogenesis in human and murine cellular model systems (42, 45–47). In line with these findings, transgenic mice with constitutive overexpression of COX-2 (the inducible isoform of COX) show enhanced browning of WAT and resistance to diet-induced obesity (46). The complexity of this system becomes apparent in IP receptor (PGI2 receptor) knockout mice, which are protected from ω 6 PUFA-induced body and fat mass gain (16). In a similar way, when mice were fed a high-fat diet, the inhibition of COX activities with indomethacin prevented body weight gain, due to decreased fat storage and enhanced recruitment of brite adipocytes in scWAT (15, 43). These examples emphasize the need to increase our current understanding of the pro- and anti-adipogenic properties of PUFAs on the level of oxylipin metabolism to modulate energy balance regulation.

In our previous work, we elucidated in more detail the relationship between ARA-derived eicosanoid level, browning

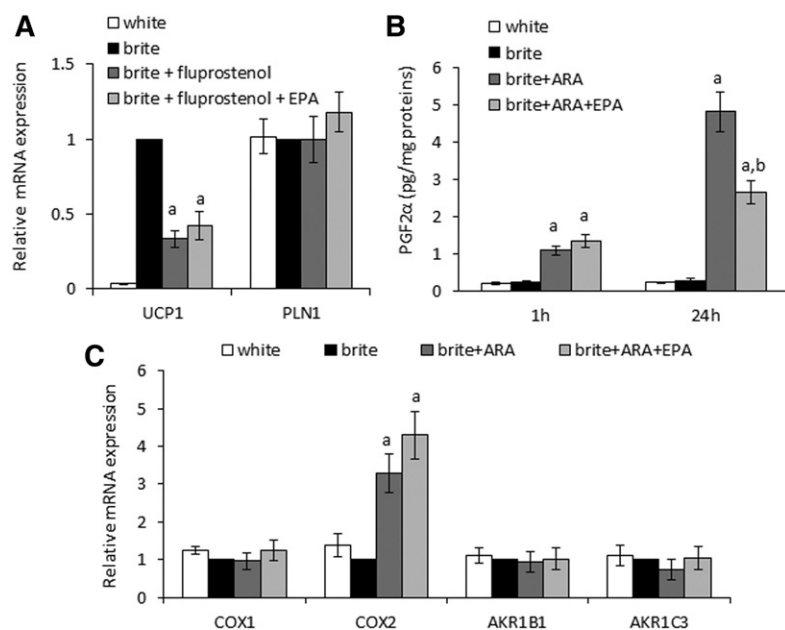


Fig. 6. EPA competes with ARA at the cyclooxygenase level. hMADS cells were differentiated into white or brite adipocytes. A: Brite hMADS adipocytes were treated with 10 nM fluprostenol (agonist of PGF2 α receptor) in the presence or absence of 3.3 μ M EPA during the last 3 days of adipogenic differentiation. UCP1 and PLN1 were evaluated by RT-qPCR. B: Brite hMADS were exposed to 10 μ M ARA in the presence or absence of 3.3 μ M EPA for 1 or 24 h. PGF2 α was quantified in culture media by Elisa Immuno Assay. C: Brite hMADS adipocytes were treated with 10 μ M ARA in the presence or absence of 3.3 μ M EPA during the last 3 days of differentiation. Expression of enzymes involved in the metabolism of ARA to PGF2 α was evaluated by RT-qPCR. mRNA expressions are expressed as fold increase relative to "brite" values. Data are mean \pm SEM of 3 independent experiments. a, $P < 0.01$ versus brite; b, $P < 0.01$ versus brite + ARA.

of WAT, and energy expenditure. Mice were fed an ARA-supplemented standard diet and showed impaired brown adipocyte activation and brite adipocyte recruitment in response to CL316,243 treatment, an effect that was attributed to the abundance of PGF2 α (17). Using hMADS adipocytes as an in vitro cell model, we demonstrated that this eicosanoid translates its inhibitory effect on the thermogenic capacity of adipocytes via an interaction with its natural cell surface receptor, the FP receptor, on its synthesis from ARA via COX activity (17). Herein we show that this ARA-dependent production of PGF2 α is attenuated in the presence of ω 3-PUFAs both in vitro in brite hMADS adipocytes and in vivo in thermogenically activated brown and brite adipocytes. We conclude that Ucp1 expression in these models is less affected by the elevated quantity of ω 3-derived metabolites but rather depends on the reduced level of individual ω 6-derived metabolites (such as PGF2 α) with EPA-treatment.

Both ω 6 and ω 3 PUFAs are transported in the bloodstream between tissues and are incorporated in plasma membrane under the form of phospholipids or as triglycerides within adipocytes (48–50). PUFAs are released into the cell by lipases and metabolized into oxylipins using similar pathways. ω 6 and ω 3 PUFAs are known to compete at different steps that modulate the availability of their respective metabolites. LA and LNA use the same Δ -desaturases and elongases to generate PUFA metabolites such as ARA, dihomo- γ -linolenic acid, EPA, and DHA (51). They can also be used as substrates via β -oxidation, ω 3 PUFAs being more rapidly oxidized compared with ω 6 PUFAs and mono-unsaturated fatty acids (52).

Although our data support a model in which dietary PUFAs act on the level of oxylipins, this mechanism is not exclusive in the context of thermogenic adipocyte recruitment, because ω 3-PUFAs per se are associated with browning properties (53, 54). For example, when mice or rats are fed an LA-enriched diet, the increase in fat mass can be prevented by LNA supplementation under isolipidic and isocaloric conditions (16, 55, 56). Using this strategy, we demonstrated herein that ω 3 PUFA diet supplementation ameliorates brown adipocyte function in response to β -adrenergic stimulation by promoting a more oxidative phenotype and, to a lesser extent, brite adipocyte recruitment. Analysis of PUFA metabolites associated with this improvement showed a decrease in n-2 series prostaglandins levels, especially PGF2 α . Altogether, in vivo and in vitro data show that the competition between ω 6 and ω 3 PUFAs takes place at the level of COX activities, favoring the production of EPA-derived metabolites instead of ARA-derived metabolites. DHA treatment was inefficient. The lack of effect could be due to the fact that DHA does not compete with ARA for metabolite production; however, DHA was described to inhibit activity and expression of COX-2 (57).

Interestingly, this competition between ω 6 and ω 3 PUFAs does not appear to contribute to the regulation of body weight when mice are fed under thermoneutral conditions but rapidly initiates the mobilization of fat as an energy substrate in response to thermogenic stimulation.

This observation may be of particular relevance to the treatment of overweight and obesity in human subjects as humans constantly live in a thermoneutral environment. Thus, adjusting the ω 6: ω 3-ratio of human nutrition according to dietary recommendations may benefit the recruitment of brown and brite adipocytes associated with other therapeutic strategies to promote a negative energy balance. Supporting the physiological relevance of ω 3 PUFA-induced effects, fish oil supplementation has been reported to induce UCP1 expression via the sympathetic nervous system, although this mechanism is restricted to BAT (58, 59).

In conclusion, herein we demonstrate that ω 3 PUFA supplementation compensates for the inhibitory effect of ω 6 PUFAs on the thermogenic function of adipocytes. We identified the eicosanoid PGF2 α as a causal effector, the level of which is influenced by the availability of ω 6 and ω 3 PUFAs competing at the level of their metabolization. Our data indicate that the dietary ω 6: ω 3 ratio is a major regulator of adaptive thermogenesis consequently affecting energy homeostasis. These findings are of particular importance in a human nutritional context as the dietary ω 6: ω 3 ratio is associated with the development of obesity and cardiovascular and inflammatory diseases. **FF**

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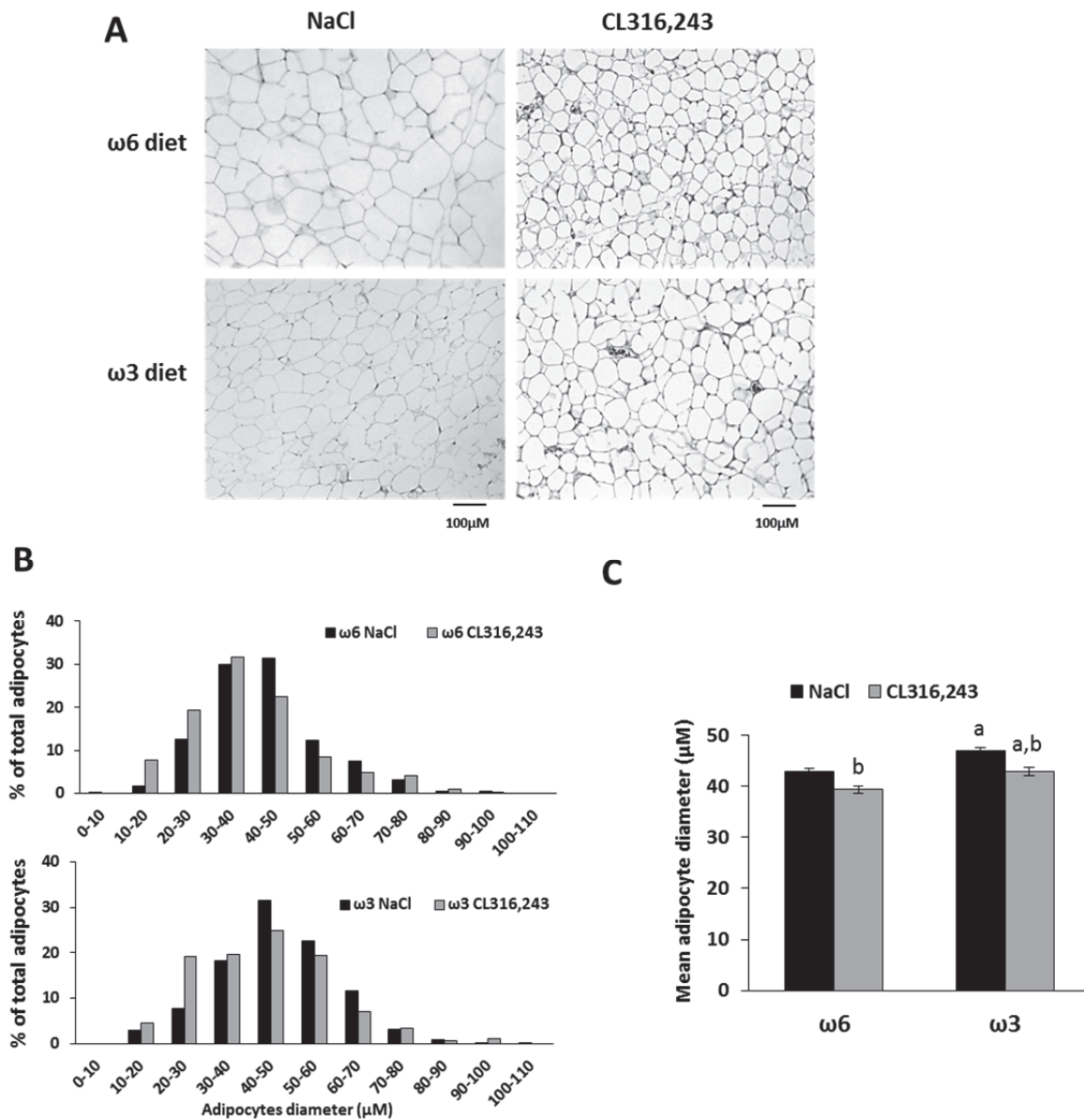
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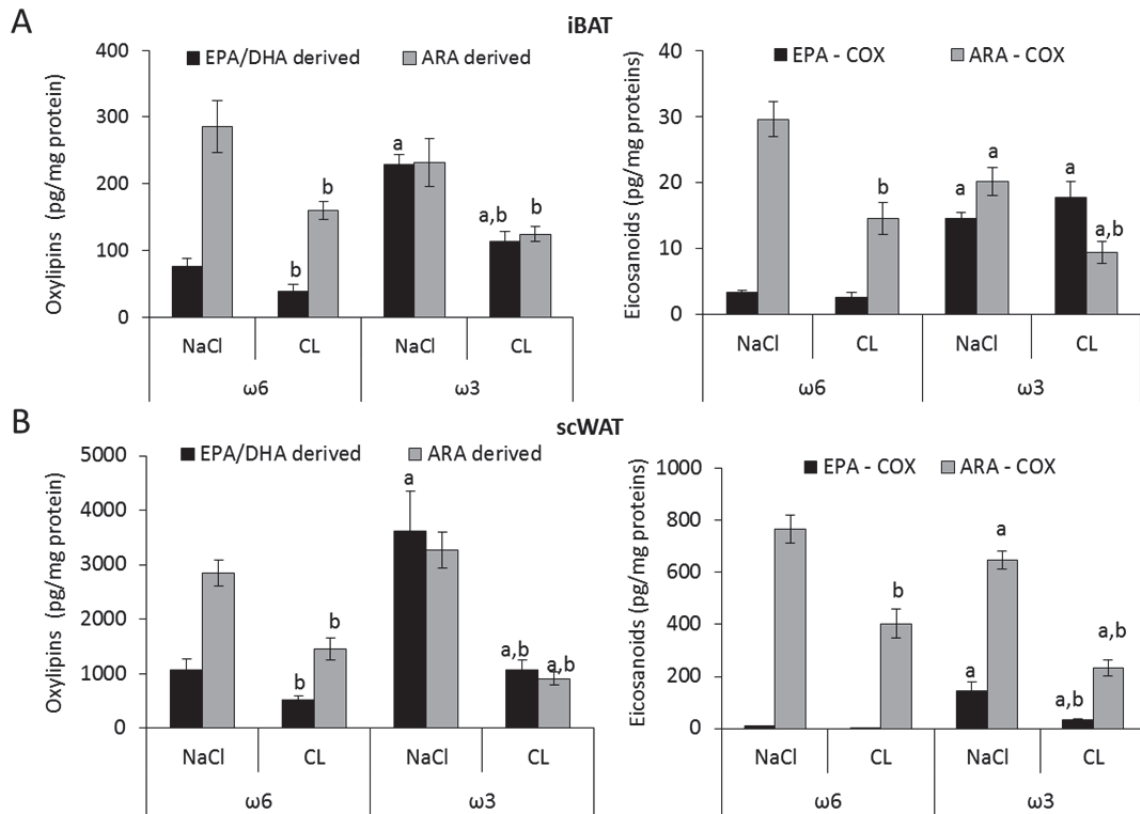
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Supplemental Figures and Tables



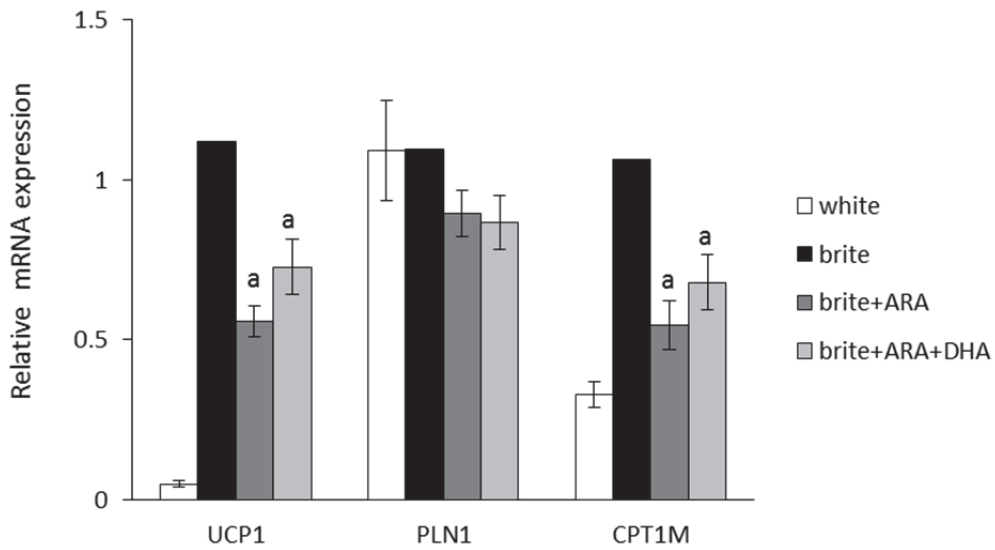
Supplemental Figure S1. Morphological analysis of epididymal white adipose tissue. A)

Histological analysis of epididymal WAT (eWAT) sections from mice fed $\omega 6$ or $\omega 3$ -diet stained with hematoxylin and eosin. B) Distribution and (C) mean diameter of adipocytes. Data are presented as % of total adipocytes or mean \pm SEM, n = 500 adipocytes from 4 mice per group. a = $p < 0.01$ $\omega 6$ vs. $\omega 3$ and b = $p < 0.01$ NaCl vs CL316,243.



Supplemental Figure S2. Abundance of ω6 and ω3 PUFA-derived oxylipins in BAT and WAT.

Oxylipin levels were measured by LC-MS/MS in iBAT and scWAT of vehicle and CL316,243-treated mice fed with ω6 and ω3 diet. Total abundance of oxylipins in (A) iBAT and (B) scWAT of ω6 diet and ω3 diet-fed mice in response to vehicle and CL316,243-treatment. Oxylipins were classified by their origin, *i.e.* derived from the metabolism of the ω3 PUFAs EPA and DHA, or from the ω6 PUFA ARA. Data are presented as mean ± SEM of 8 mice per group. a = p<0.01 ω6 vs ω3 and b = p<0.01 NaCl vs CL316,243.



Supplemental Figure S3. DHA did not compete with ARA. hMADS cells were differentiated into white or brite adipocytes. Brite hMADS adipocytes were treated with 10 μ M ARA in the presence or absence of 3.3 μ M DHA during the last 3 days of adipogenic differentiation. mRNA expressions were evaluated by RT-qPCR and expressed as fold relative to “brite” values. Data are presented as mean \pm SEM of 3 independent experiments. a = $p < 0.01$ vs brite and b = $p < 0.01$ vs brite + ARA.

Supplemental Table S1. Diet composition.

	ω6 diet	ω3 diet
Metabolizable energy [KCal/g]	3.2	
Crude protein [g/Kg]	159	
Digestible carbohydrate [g/Kg]	519	
Crude fat [g/Kg]	51	
Saturated FAs [% of total FAs]	12	
Monounsaturated FAs [% of total FAs]	26	14
Polyunsaturated FAs [% of total FAs]	62	74
Linoleic acid [g/Kg]	30	
Alpha-linolenic [g/Kg]	1	6.4
EPA [g/Kg]	0	0.8
DHA [g/Kg]	0	0.8
Σ ω6 [g/Kg]	30	
Σ ω3 [g/Kg]	1	8
ω6 / ω3 PUFA ratio	30	3.75

Supplemental Table S2. List of analyzed oxylipins

ARA-derived oxylipins			EPA/DHA-derived oxylipins		
ARA-COX	ARA-LOX	ARA-other	EPA-COX	EPA-LOX	DHA-LOX
11 β -PGF2 α	5-HETE	5,6-EET	18-HEPE	LTB5	14-HDoHE
15 Δ -PGJ2	8-HETE	8,9-EET	PGE3		17-HDoHE
6keto-PGF1 α	12-HETE	11,12-EET			Maresin 1
8iso-PGA2	15-HETE	14,15-EET			Protectin Dx
PGD2	5,6-DiHETE				Resolvin D1
PGE2	5oxoETE				Resolvin D2
PGF2 α	LTB4				
TxB2	LxA4				
	LxB4				

ANNEXE 2:

Fatty Acid Metabolite Profiling Reveals Oxylipins as Markers of Brown but Not Brite Adipose Tissue

Sebastian Dieckmann, Stefanie Maurer, Tobias Fromme, **Cécilia Colson**,
Kirsi A Virtanen, Ez-Zoubir Amri, Martin Klingenspor

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Fatty Acid Metabolite Profiling Reveals Oxylipins as Markers of Brown but Not Brite Adipose Tissue

Sebastian Dieckmann^{1,2,3}, Stefanie Maurer^{1,2,3}, Tobias Fromme^{1,2,3}, Cécilia Colson⁴, Kirsi A. Virtanen⁵, Ez-Zoubir Amri⁴ and Martin Klingenspor^{1,2,3*}

¹ Chair for Molecular Nutritional Medicine, TUM School of Life Sciences, Technical University of Munich, Freising, Germany, ² EKfZ - Else Kröner-Fresenius Center for Nutritional Medicine, Technical University of Munich, Freising, Germany, ³ ZIEL Institute for Food and Health, TUM School of Life Sciences, Technical University of Munich, Freising, Germany, ⁴ Université Côte d'Azur, CNRS, Inserm, IBV, Nice, France, ⁵ Turku PET Centre, Turku University Hospital, University of Turku, Turku, Finland

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*Correspondence:

Martin Klingenspor
mk@tum.de

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Metabolites of omega-6 and omega-3 polyunsaturated fatty acids are important signaling molecules implicated in the control of adipogenesis and energy balance regulation. Some of these metabolites belonging to the group of oxylipins have been associated with non-shivering thermogenesis in mice mediated by brown or brite adipose tissue. We aimed to identify novel molecules with thermogenic potential and to clarify the relevance of these findings in a translational context. Therefore, we characterized and compared the oxylipin profiles of murine and human adipose tissues with different abundance of brown or brite adipocytes. A broad panel of 36 fatty acid metabolites was quantified in brown and white adipose tissues of C57BL/6J mice acclimatized to different ambient temperatures and in biopsies of human supraclavicular brown and white adipose tissue. The oxylipin profile of murine brite adipose tissue was not distinguishable from white adipose tissue, suggesting that adipose tissue browning *in vivo* is not associated with major changes in the oxylipin metabolism. Human brown and white adipose tissue also exhibited similar metabolite profiles. This is in line with previous studies proposing human brown adipose tissue to resemble the nature of murine brite adipose tissue representing a heterogeneous mixture of brite and white adipocytes. Although the global oxylipin profile served as a marker for the abundance of thermogenic adipocytes in bona fide brown but not white adipose tissue, we identified 5-HETE and 5,6-EET as individual compounds consistently associated with the abundance of brown or brite adipocytes in human BAT and murine brite fat. Further studies need to establish whether these candidates are mere markers or functional effectors of thermogenic capacity.

Keywords: adipose tissue, browning, thermogenesis, PUFA (polyunsaturated fatty acid), n-6 fatty acid, n-3 fatty acid, oxylipin

INTRODUCTION

Obesity is one of today's major health burdens with a steadily increasing prevalence. It is characterized by excessive fat accumulation and unhealthy expansion of white adipose tissue (WAT) associated with severe comorbidities such as type 2 diabetes and cardiovascular diseases. Obesity is the consequence of a chronic positive energy balance, a state where energy intake

exceeds energy expenditure. A major obstacle of obesity management is the maintenance of a given body weight loss, since weight loss is accompanied by a notable and persistent decrease in energy expenditure (1, 2). This decrease in energy expenditure is hardly compensated by physical activity, the only available strategy to increase energy expenditure so far. Consequently, other means to increase energy expenditure are in demand. Thermogenic tissues such as brown (BAT) and brite adipose tissue are promising targets. Brite adipose tissue, in contrast to BAT, is an inducible type of fat originating from the recruitment of brown-like, so called brite (or beige) cells with thermogenic properties in WAT. Both tissues dissipate chemical energy from fatty acids and glucose to generate heat, thus increasing energy expenditure. This non-shivering thermogenesis is mediated by uncoupling protein 1 (UCP1). It is naturally activated upon cold exposure to defend body temperature and during eating to promote meal termination (3). Although the presence of functional BAT has been confirmed in adult humans (4–6), humans mostly live under thermoneutral conditions (7). Therefore, BAT activation in humans is mostly associated with food intake, whereas cold-induced activation is less prevalent. BAT volume and activity negatively correlate with BMI (8), suggesting a lower abundance of active BAT in overweight and obese compared to lean subjects. Consequently, the therapy of obesity by means of BAT and brite fat not only requires strategies to activate it but also to increase its abundance. Several natural compounds and drugs are associated with the activation and recruitment of BAT in mice and humans (9, 10). Among these are metabolites of omega-6 and omega-3 polyunsaturated fatty acids (PUFA). Oxygenated PUFA metabolites, belonging to the group of oxylipins, are important signaling molecules implicated in the control of adipogenesis and energy balance regulation (11). These potent and short-lived metabolites are generated by a series of enzymatic steps involving one of three enzyme classes—cyclooxygenase (COX), lipoxygenase (LOX) or cytochrome P450 (CYP) (12). Some oxylipins have been associated with the browning of adipose tissues. The COX derived ARA metabolites prostaglandin E2 (PGE2) and prostacyclin (PGI2) facilitate the formation of brite adipocytes *in vitro* (13–16). The oxylipin 12-hydroxyeicosapentaenoic acid (12-HEPE), identified in a PUFA metabolite screen in murine serum samples, facilitates glucose uptake into brown adipocytes (17). In a similar approach, increased levels of 12,13-dihydroxy-9Z-octadecenoic acid (12,13-diHOME) were identified in oxylipin profiles of human serum after cold acclimatization (18). This oxylipin increases fatty acid uptake into brown adipocytes and presumably UCP1 expression (18). Furthermore, a second class of PUFA derived metabolites, the endocannabinoids, are suggested to be involved in the negative regulation of BAT activity in mice (19). Thus, several lines of evidence suggest PUFA-derived metabolites to be involved in the recruitment and activity of thermogenic cells in mice and humans. The aim of the current study was to characterize the oxylipin profiles of murine and human adipose tissues with different abundance of brown and brite adipocytes to identify novel molecules with thermogenic potential in a translational context.

We quantified a panel of 36 fatty acid metabolites in brown and white adipose tissues of C57BL/6J mice acclimatized to different ambient temperatures and in biopsies of human supraclavicular brown and white adipose tissue. Our results reveal the global oxylipin profile of bona fide brown but not brite adipose tissue as a marker for the abundance of brown adipocytes. Moreover, we identified 5-HETE and 5,6-EET as individual compounds associated with the abundance of brown or brite adipocytes in both human BAT and murine brite fat.

MATERIALS AND METHODS

Animal Experiments

Eight-week-old male C57BL/6J mice were housed in climate cabinets (HPP750 life, Memmert) at 23°C and 55% humidity with a 12/12 h light/dark cycle. Mice were provided *ad libitum* access to water and a control diet (Sniff, Cat# S5745-E720). After an adaptation phase of 3 weeks, mice were assigned to one of two groups and transferred to preconditioned cabinets at 5 or 30°C. After 1 week, mice were killed by CO₂ exposure and tissues were immediately dissected, snap frozen in liquid nitrogen, and stored at –80°C until further processing. The experiment was performed according to the German animal welfare law with permission from the district government of Upper Bavaria (Regierung von Oberbayern, reference number ROB-55.2-2532.Vet_02-16-166).

Human Subjects

Paired biopsies of BAT and WAT were obtained from the supraclavicular region of 14 healthy male and female subjects. A detailed description of the biopsy procedure and of anthropometric characteristics of this study cohort has been published previously (20). Depending on the size of the specimens obtained, BAT and WAT were either entirely subjected to RNA isolation or grinded in liquid nitrogen to obtain aliquots used for both metabolite analysis and RNA isolation.

Oxylipin and Endocannabinoid Profiling

Murine interscapular BAT, inguinal WAT and human supraclavicular fat biopsies were grinded in liquid nitrogen. Aliquots of 23–140 mg were subjected to oxylipin and endocannabinoid analysis, which was conducted at the Metatoul lipidomic platform (INSERM UMR1048, Toulouse, France), certified to ISO 9001:2015 standards. Metabolite abundance was normalized to tissue mass.

RNA Isolation and Quantitative Real-Time PCR (qRT-PCR)

RNA isolation from murine inguinal WAT and supraclavicular BAT as well as human adipose tissues was performed with TRIsure™ (Bioline, Cat# BIO-38032) according to the manufacturer's instructions. Precipitated RNA was transferred to spin columns (SV Total RNA Isolation System, Promega, Cat# Z3105), centrifuged for 1 min with 12,000 × g and further processed according to the supplier's instructions. RNA concentration was determined spectrophotometrically (Infinite 200 PRO NanoQuant, Tecan). Generation of cDNA was performed with 1 µg RNA (SensiFAST™ cDNA Synthesis

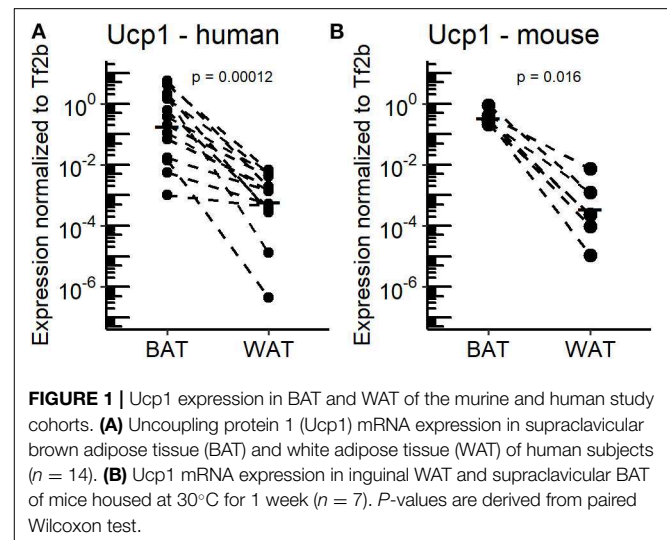
Kit, Bioline, Cat# BIO-65053). qRT-PCR was performed in a 384 well plate format with the LightCycler 480 system (Roche Diagnostics) in a total reaction volume of 12.5 μ l containing 6.25 μ l 2x SensiMix SYBR no-ROX (Bioline, Cat# QT650-05), 250 nM forward and reverse primers and 1 μ l template cDNA. Murine primers (Ucp1 5'-TCTCTGCCAGGACAGTACCC-3' and 5'-AGAAGCCAATGATGTTTCAG-3', Tf2b 5'-TGGAGATTGTGCCACCATGA-3' and 5'-GAATTGCCAAACTCATCAA-3') and human primers (UCP1 5'-GGAGGCCTTTGTGAAAAACA-3' and 5'-CTTGAAGAAAGCCGTTGGTC-3', TF2B 5'-GCTGTGGAAGTGGACTTGGT-3' and 5'-AGTTTGTCCACTGGGGTGTGC-3') were produced by Eurofins MWG Operon. Expression of Ucp1 was normalized to transcription factor 2b (Tf2b) expression.

Statistical Analysis

All statistical analyses were performed using R-Studio (version 1.2.5019) with R version 3.6.1. Principal component analysis was performed with the R packages factoextra (version 1.0.5) and FactoMineR (version 1.42). Other statistical tests were calculated with the R package ggpubr (version 0.2.3). Wilcoxon test was performed for all group comparisons, after checking the assumption of normal distribution with Shapiro–Wilk test. $P < 0.05$ were deemed statistically significant. The appropriate statistical test, paired, or unpaired is mentioned for each figure.

RESULTS

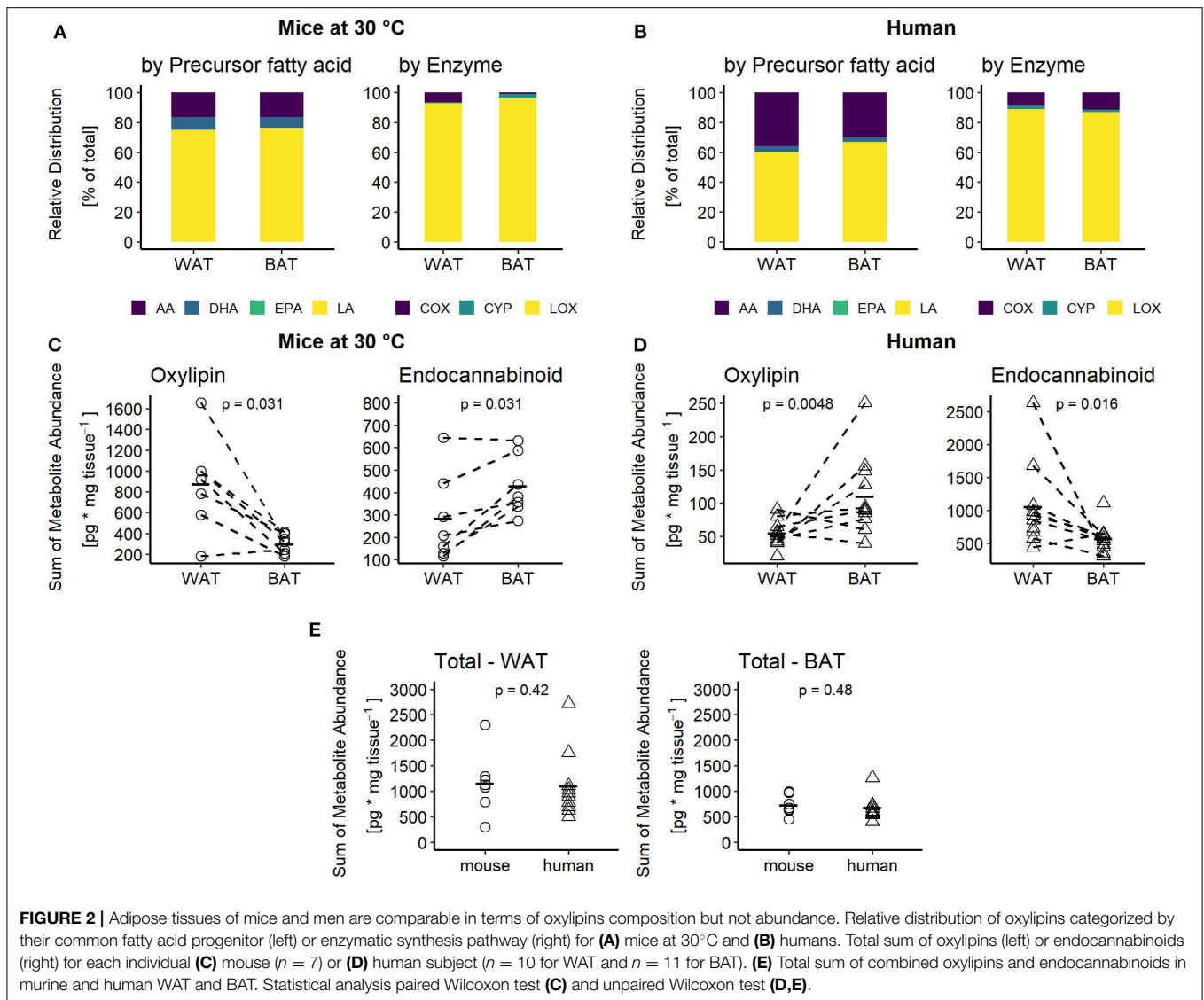
Adaptive, non-shivering thermogenesis is the key functional difference that discriminates mammalian BAT and WAT. Since almost a decade, the rediscovery of functional BAT in adult humans has intensified efforts to characterize the molecular properties of human adipose tissues and to identify novel thermogenic effectors intended for therapeutic use. Within this scope, oxylipins appear to be a promising class of endogenous compounds affecting the function and recruitment of thermogenic adipocytes in cultured cells of human and murine origin (11). In the course of this study, we further elucidated the association of these metabolites with the recruitment of thermogenic brown and white adipocytes in a translational context. To this end, we subjected BAT and WAT of murine and human origin to metabolite profiling and analyzed the data in consideration of the tissues' thermogenic properties. Human BAT and WAT biopsies were obtained from the supraclavicular region subsequent to PET imaging under cold-exposed conditions (20). Humans live within thermoneutral conditions most of their life (7). Thus, for a more appropriate comparison between mice and humans we acclimatized C57BL/6J mice to 30°C for 1 week to mimic the thermal environment of humans. In order to confirm the thermogenic potential of BAT vs. WAT in both humans and mice, Ucp1 mRNA expression was quantified as a surrogate marker for the abundance of thermogenic competent adipocytes. As expected, all BAT specimens were characterized by considerably higher Ucp1 mRNA levels compared to WAT with a wide range of inter-individual variation (Figures 1A,B). However, mean Ucp1 mRNA expression in human and murine BAT was 544- and 255-fold higher compared to WAT,



respectively. Consequently, human and murine BAT harbor more brown adipocytes than WAT.

The Abundance of Adipose Tissue Oxylipins Differs Between Mice and Humans

To elucidate the regulation of oxylipin production in BAT and WAT, we quantified a broad panel of 33 metabolites representing major oxylipin classes produced by mammalian tissues. Additionally, we quantified the levels of 3 AA-derived endocannabinoids. The oxylipin panel encompasses COX, LOX and CYP-derived metabolites generated by the conversion of arachidonic acid (AA), its n-6 precursor linoleic acid (LA), and the n-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Within this setting, LA-derived metabolites were most abundant, while n-3 derived metabolites had a relatively low abundance in inguinal WAT and interscapular BAT of mice (Figure 2A). This high abundance of LA-derived metabolites was reflected in a high percentage of LOX-derived metabolites (Figure 2A). In human adipose tissues, the relative abundance followed a slightly different pattern. In both human BAT and WAT, AA-derived metabolites produced via the COX-pathway accounted for a higher percentage of total oxylipin abundance compared to murine fat, which proportionally reduced the relative levels of LA and DHA-derived metabolites (Figures 2A,B). The contribution of EPA-derived oxylipins to the oxylipins pool was negligible in adipose tissues of both species. Of note, the contribution of CYP derived oxylipins in mice was higher in interscapular BAT compared to inguinal WAT while in humans no notable difference was observed. Despite this similar composition of the oxylipin pools in WAT and BAT, the total abundance of all oxylipins in BAT vs. WAT in mice was significantly lower while it was significantly higher in human BAT vs. WAT (Figures 2C,D). These differences are primarily attributed to changes in the abundance of the LA-derived oxylipins 9- and 13-HODE. These two oxylipins are the predominant species in WAT and BAT, accounting

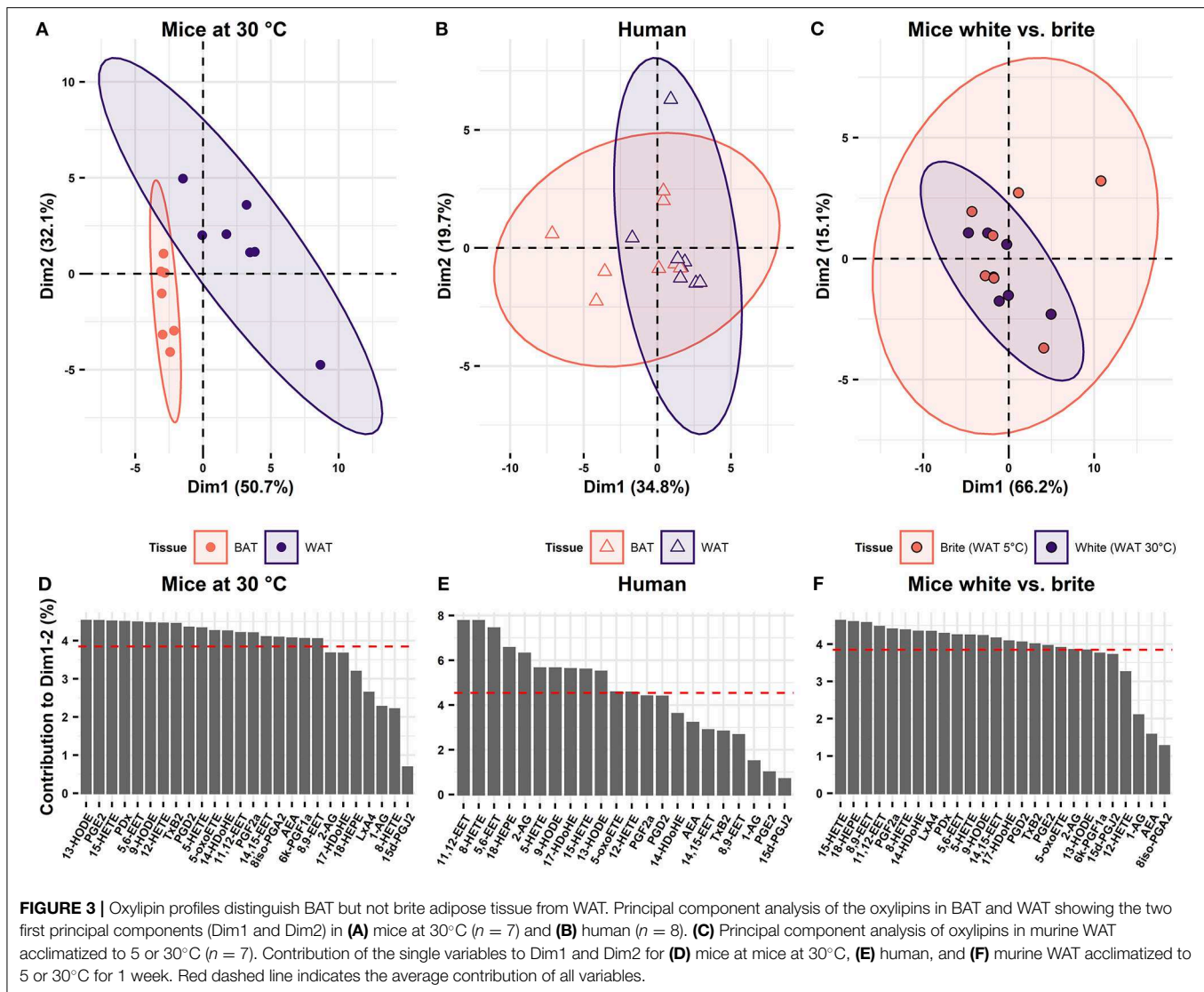


for at least 60% of the total oxylipin pool in adipose tissues of both species (**Figures 2A,B**). Endocannabinoids represent another class of fatty acid metabolites with potential effects on Ucp1 dependent thermogenesis (19). Interestingly, the total abundance of the three endocannabinoids was lower in murine WAT vs. BAT but higher in human WAT vs. BAT, while we observed the exact opposite for total oxylipin abundance (**Figures 2C,D**). Ultimately, considering the combined pool of oxylipins and endocannabinoids there was no difference in total metabolite abundance between murine and human WAT or BAT (**Figure 2E**). Conclusively, mice and humans are similar in terms of the total production of PUFA metabolites. However, these tissues seem to differ in the partitioning of PUFA metabolism.

The Global Oxylipin Profile Is a Surrogate Marker for the Abundance of Brown but Not Brite Adipocytes

As oxylipin abundance differs between WAT and BAT in both mice and humans, we investigated, whether oxylipins may serve

as discriminative markers for the two tissues. Therefore, we applied principal component analysis (PCA) on the metabolite data of BAT and WAT. In mice, principal components 1 and 2 together explained 82.8% of the variability between WAT and BAT. In a continuum of these principal components, murine WAT and BAT formed distinct and separate clusters (**Figure 3A**). Thus, murine BAT and WAT can be distinguished by their characteristic oxylipin patterns. In humans, principal components 1 and 2 explained considerably less of the variation between BAT and WAT (54.5%). A distinction of human BAT and WAT according to their specific oxylipin patterns was not possible (**Figure 3B**). This was reflected in the analysis of the combined human and murine data set. In this analysis the two species formed distinct clusters separating the murine tissues while human BAT and WAT could not be distinguished (**Supplementary Figure 1A**). In contrast to murine BAT, human BAT constitutes a complex, interwoven mixture of both brown and white adipocytes. Consequently, oxylipin patterns established from human tissues may not represent differences on the cellular level of individual brown



and white adipocytes and lack discriminative power (Figure 3B). To overcome this limitation, we transferred oxylipin patterns established from murine BAT and WAT and plotted human oxylipin levels according to these murine principal components. However, human BAT and WAT remained indistinguishable (Supplementary Figure 1B). Surprisingly, the reverse strategy, i.e., plotting murine oxylipin levels according to principal components of human oxylipin variation, murine BAT and WAT could be well-separated (Supplementary Figure 1C). This indicates that oxylipins in humans do not *per se* lack the variability observed in murine tissues but fail to sharply distribute into the categories BAT and WAT. The lack of discrimination of human supraclavicular BAT and WAT oxylipin patterns are in line with the observation that human supraclavicular BAT does not resemble the characteristics of classical BAT in conventional laboratory mice but rather displays a brite phenotype (7, 21). Indeed, brite adipose tissue obtained from mice housed at 5°C for 1 week and human supraclavicular BAT

were both characterized by increased UCP1 expression compared to WAT (Supplementary Figure 2 and Figure 1A). Murine brite adipose tissue contained a mixed population of unilocular white and multilocular brown/brite cells (Supplementary Figure 3), similarly to the phenotype reported from human supraclavicular BAT (7). We investigated this by comparing oxylipin profiles of inguinal WAT of mice acclimatized to either 30°C (white adipose tissue) or 5°C (brite adipose tissue). In this comparison, the principal components 1 and 2 explained a large proportion (81.3%) of the variation between brite and white adipose tissue (Figure 3C). However, murine brite and white adipose tissue could not be separated from one another by oxylipin patterns (Figure 3C), although both formed distinct populations separate from BAT (Supplementary Figure 1D). Interestingly, BAT of mice acclimatized to 5 or 30°C also formed distinguishable populations (Supplementary Figure 1D). This suggests the global BAT oxylipin profile as surrogate marker of the abundance of brown adipocytes, since BAT of 5°C acclimatized mice

contained more multilocular brown adipocytes than BAT of mice housed at 30°C. Conclusively, the oxylipin profiles of adipose tissues allow the discrimination of bona fide BAT and WAT composed of homogenous populations of brown and white adipocytes, respectively. However, it is either unsuitable to distinguish tissues harboring both types of cells or unable to distinguish brite from white adipocytes. Thus, the oxylipin profile can serve as a surrogate marker for brown adipocyte abundance in murine BAT but not murine brite fat or human BAT.

The Oxylipins 5-HETE and 5,6-EET Are Potential Markers of Brown Adipocyte Abundance in BAT in Mice and Humans

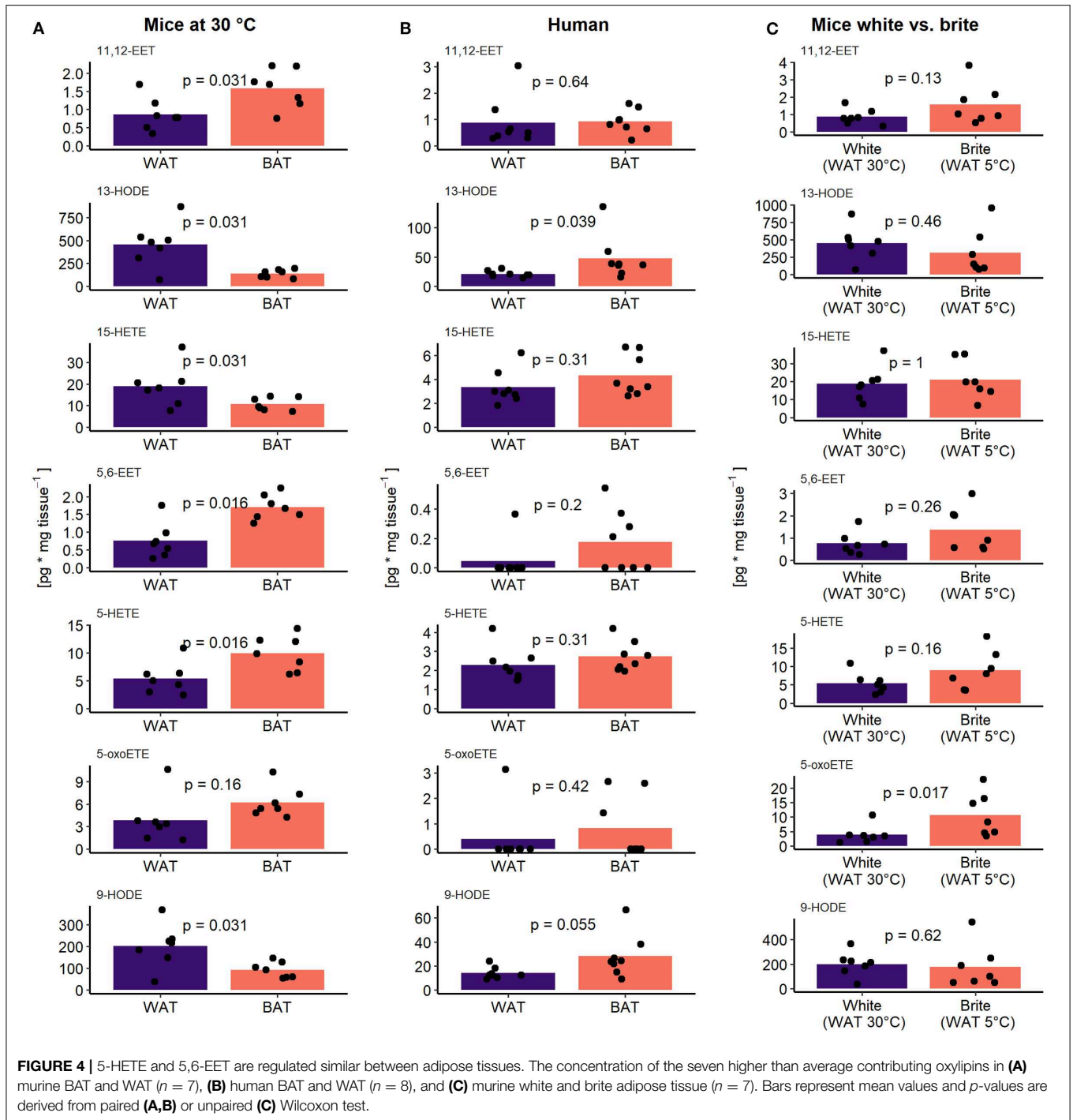
The oxylipin profile serves as a potential surrogate measure for the abundance of brown but not brite adipocytes. We asked which metabolites contributed the most to this phenomenon and whether we could identify novel oxylipins associated with the recruitment of brown and brite adipocytes. Therefore, we investigated the contribution of individual oxylipins to the principal components 1 and 2. In the murine adipose tissues, more than two-thirds of the measured fatty acid metabolites contribute higher-than-average to the first two principal components (**Figures 3D,F**). In contrast, less than half of the compounds did so in the human tissues (**Figure 3E**). Interestingly, several compounds previously associated with the recruitment of brown and brite adipocytes, namely 9- and 13-HODE (22), the PGI₂ degradation product 6k-PGF₁α (14, 15), 12-HETE (17) as well as PGE₂ (13, 14) contributed higher-than-average in the murine BAT/WAT comparison (**Figure 3D**). Among those, only 9- and 13-HODE consistently contributed to the discrimination of BAT and brite adipose tissue from WAT (**Figures 3D–F**), suggesting an association of individual metabolites with the abundance of thermogenic competent adipocytes in a translational context. In line with this notion, the AA-derivatives 11,12-EET and 5,6-EET generated by the CYP pathway, and the LOX pathway products 15-HETE, 5-HETE and its active form 5-oxoETE contributed above-average in all three conditions (**Figures 3D–F**). However, the abundance of most of these metabolites was exclusively different in murine BAT vs. WAT but not in the other comparisons (**Figures 4A–C**), confirming the limited discriminative potential of the oxylipin profile in these settings. Only the abundance of 13-HODE in humans and 5-oxoETE in the murine brite vs. white comparison were significantly different in brown and brite adipose tissue compared to WAT, respectively (**Figures 4B,C**). Interestingly, 11,12-EET, 5,6-EET, and 5-HETE were significantly higher in murine BAT than in WAT (**Figure 4A**), contradicting the overall trend toward higher total oxylipin abundance in WAT (**Figure 2C**). This suggests an involvement of these three metabolites in regulation of BAT function. We identified 5,6-EET and 5-HETE as the only two metabolites significantly more abundant in murine BAT compared to WAT that showed at least a similar trend toward a higher abundance in murine brite and human BAT vs. WAT (**Figures 4B,C**). In line with this regulation, 5-oxoETE, the oxidation product of 5-HETE, also

tended to be more abundant in these tissues. Consequently, 5-HETE and 5,6-EET constitute novel oxylipins associated with the abundance of brown and brite adipocytes in a translational context.

DISCUSSION

Activation of NST in BAT and brite adipose tissue increases energy expenditure and therefore is a potential therapeutic strategy to treat obesity. Within this scope, metabolites of PUFA (especially oxylipins) are discussed as potential effectors. Several studies have associated selected oxylipins with improved BAT functionality (17, 18) or the recruitment of brite adipocytes (13–15) in mice. However, evidence in the human context is scarce. Only the prostaglandin PGI₂ has been shown to increase UCP1 expression in cultured murine and human adipocytes (14, 16). Therefore, we screened the abundance of 36 omega-6 or omega-3 PUFA-derived metabolites in human and murine adipose tissues to identify novel compounds associated with abundance of brown and brite adipocytes in a translational context. Based on their respective PUFA metabolite pattern, murine but not human BAT could be distinguished from WAT. Indeed, it has been argued that human supraclavicular BAT resembles murine brite adipose tissue rather than murine BAT, thus comprising a mixture of white and brite adipocytes (7, 23). This is in line with our finding that in mice the oxylipin profiles of brite adipose tissue induced by cold exposure and WAT at thermoneutrality could also not be distinguished. We think of two possible explanations for this observation. First, the oxylipin profile could be a surrogate marker for the thermogenic activity of the respective adipose tissues, since only BAT but not WAT shows increased metabolic activity in cold acclimatized mice (24). However, we did not measure thermogenic activity in our study, and thus lack direct experimental evidence. Second, the oxylipin profile could be a surrogate marker for the abundance of brown or brite adipocytes that markedly increase in abundance upon cold exposure in both BAT and WAT of mice, respectively. However, we speculate that the relative abundance of interspersed brown or brite adipocytes in human BAT and murine brite adipose tissue, respectively, is not sufficient to notably alter the oxylipin metabolite profiles of the whole tissue.

We therefore checked individual oxylipins with discriminative potential between murine BAT and WAT. A set of seven oxylipins with high potential to explain variability between BAT or brite adipose tissue and WAT in mice and humans was identified. Within this set, we could confirm previously reported oxylipins associated with the recruitment of BAT and browning of WAT. As such, LA-derived 9- and 13- HODE were the most abundant oxylipins in both human and murine adipose tissues and had a high discriminative potential. When used at very high concentrations, both compounds sensitize murine white adipocyte progenitors to β₃-receptor agonist treatment, consequently increasing UCP1 expression in the presence of isoproterenol (22). In our study, the abundance of 9- and 13-HODE in murine adipose tissues gradually decreased with the



abundance of brown and brite adipocytes from white to brite to brown. This phenotype may indicate a coordinated regulation of 9- and 13-HODE production to contain thermogenic capacity on reasonable levels upon prolonged β -adrenergic stimulation (1 week at 5°C). However, the sensitizing effect of 9- and 13-HODE was achieved with supraphysiological concentrations of 68 μ M (22). Furthermore, the decreasing abundance of 9- and 13-HODE in increasingly thermogenic competent tissues might simply

reflect an increased consumption of precursor fatty acids caused by the higher lipolytic and oxidative activity. Consequently, the relevance of 9- and 13-HODE in a physiological context needs further experimental validation. Interestingly, 12-HETE and 14-HDoHE, two LOX products reported to be upregulated in murine interscapular BAT and inguinal WAT upon cold simulation (17), were also high contributors explaining variability between murine BAT and WAT in our study. In contrast to previously

published observations, 14-HDoHE was not only lower at 5°C compared to 30°C in BAT but also lacked regulation in WAT (**Supplementary Figure 4**). Additionally, 12-HETE concentrations were not different between 5 and 30°C in BAT or WAT (**Supplementary Figure 4**). We cannot exclude an effect of diets differing in the fatty acid composition altering the supply of oxylipin precursor fatty acids. However, we speculate that the lack of regulation of 12-HETE and 14-HDoHE especially in WAT upon cold stimulation indicates that both oxylipins are not implicated in the process of WAT browning *in vivo*.

In line with the scope of our study, we could identify two novel metabolites, which have not been associated with the recruitment of brown and brite adipocytes. Following a translational pattern, the oxylipins 5-HETE and 5,6-EET were more abundant in BAT and brite adipose tissue compared to WAT in mice and humans. 5,6-EET is directly synthesized from AA via the CYP pathway and can activate transient receptor potential vanilloid 4 (TRPV4) channels (25). In contrast, 5-HETE synthesis from AA is a multi-step process involving 5-LOX and glutathione peroxidases (26). Expression of CYP isoforms responsible for 5,6-EET production has been reported for murine adipocytes (27). Further 5-LOX is expressed in human and murine adipose tissue (28–30). Thus, it is possible that both 5-HETE and 5,6-EET are generated endogenously in adipose tissues, although their tissue-specific abundance may be influenced by plasma levels. Considering the different number of brown adipocytes and levels of Ucp1 expression in BAT and WAT, both compounds might be associated with thermogenic capacity. Indeed, both 5-HETE and 5,6-EET are linked to signaling pathways with the potential to regulate the recruitment of thermogenic capacity in adipose tissue. Although 5-HETE is a rather inactive metabolite that needs further conversion by 5-hydroxyeicosanoid dehydrogenase (5-HEDH) to the active metabolite 5-oxo-EETE, the latter one activates the OXE receptor and PPAR γ (31). Activation of PPAR γ is one of the strongest inducers of Ucp1 expression (32). Consequently, 5-HETE could by conversion to 5-oxo-EETE activate PPAR γ and regulate adipogenesis and Ucp1 expression. Nevertheless, not all PPAR γ agonists are able to increase Ucp1 expression in brown or white adipose tissue. As such, the oxylipin and PPAR γ agonist 15d-PGJ2 has no effect on the recruitment of Ucp1 in human adipocytes (33). This is in line with our finding that 15d-PGJ2 was virtually undetectable in BAT as well as WAT and did not contribute to the variation in human adipose tissue samples. In contrast to 5-HETE, 5,6-EET could have a negative regulatory effect on the browning of adipose tissues by binding to the TRPV4 channel. This receptor constitutes a negative regulator of thermogenic capacity as mice with a knockout of TRPV4 show increased expression of Ucp1 in WAT in addition to increased total energy expenditure (34). Although 5-HETE and 5,6-EET have the potential to affect browning of adipose tissues, these effects remain to be demonstrated in *in vitro* and *in vivo* studies aiming to clarify the role both oxylipins for the recruitment and function of brown and brite adipocytes.

In summary, we show that bona fide BAT vs. WAT are distinguishable by their global oxylipin profile. Furthermore, we identify 5-HETE and 5,6-EET as novel compounds associated with the recruitment of brown and brite adipocytes in mice and humans. Further studies need to establish whether these oxylipins are mere markers or functional effectors of thermogenic capacity.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the ethical review board of the Hospital District of Southwest Finland. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by the district government of Upper Bavaria (Regierung von Oberbayern) Reference number ROB-55.2-2532.Vet_02-16-166.

AUTHOR CONTRIBUTIONS

SD performed the molecular analysis, data analysis, interpreted the results, and drafted the manuscript. SM conceived and designed the study, performed the mouse experiment, contributed to the molecular analysis and data interpretation, and revised the manuscript. MK conceived and designed the study, contributed to data interpretation, and revised the manuscript. TF contributed to data analysis and interpretation and revised the manuscript. E-ZA conceived and designed the study, contributed to data interpretation, and revised the manuscript. CC contributed to data interpretation and revised the manuscript. KV provided human tissue samples and revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fendo.2020.00073/full#supplementary-material>

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling Editor declared a past co-authorship with the E-ZA.

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ANNEXE 3:

Fatty Acid Metabolites as Novel Regulators of Non-shivering Thermogenesis

Stefanie F Maurer, Sebastian Dieckmann, Karin Kleigrewé, **Cécilia Colson**,
Ez-Zoubir Amri, Martin Klingenspor

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Fatty Acid Metabolites as Novel Regulators of Non-shivering Thermogenesis

Stefanie F. Maurer, Sebastian Dieckmann, Karin Kleigrew, Cécilia Colson, Ez-Zoubir Amri, and Martin Klingenspor

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S. F. Maurer (✉) · S. Dieckmann · M. Klingenspor

Molecular Nutritional Medicine, Else Kröner-Fresenius Center for Nutritional Medicine, Technical University of Munich, Freising, Germany

ZIEL Institute for Food and Health, TUM School of Life Sciences, Technical University of Munich, Freising, Germany

e-mail: stefanie.maurer@tum.de

K. Kleigrew

Bavarian Center for Biomolecular Mass Spectrometry (BayBioMS), Technical University of Munich, Freising, Germany

C. Colson · E.-Z. Amri

Université Côte d'Azur, CNRS, Inserm, iBV, Nice, France

Abstract

Fatty acids are essential contributors to adipocyte-based non-shivering thermogenesis by acting as activators of uncoupling protein 1 and serving as fuel for mitochondrial heat production. Novel evidence suggests a contribution to this thermogenic mechanism by their conversion to bioactive compounds. Mammalian cells produce a plethora of oxylipins and endocannabinoids, some of which have been identified to affect the abundance or thermogenic activity of brown and brite adipocytes. These effectors are produced locally or at distant sites and signal toward thermogenic adipocytes via a direct interaction with these cells or indirectly via secondary mechanisms. These interactions are evoked by the activation of receptor-mediated pathways. The endogenous production of these compounds is prone to modulation by the dietary intake of the respective precursor fatty acids. The effect of nutritional interventions on uncoupling protein 1-derived thermogenesis may thus at least in part be conferred by the production of a supportive oxylipin and endocannabinoid profile. The manipulation of this system in future studies will help to elucidate the physiological potential of these compounds as novel, endogenous regulators of non-shivering thermogenesis.

Keywords

Brite adipocytes · Brown adipocytes · Endocannabinoids · Oxylipins · PUFAs · Thermogenesis · Ucp1 · ω -3 · ω -6

1 Introduction

The storage of dietary lipids is a hallmark common to brown (BAT) and white adipose tissue (WAT). Based on the primary functions of these tissues, deposited lipids are predominantly released as fatty acids from triglycerides via lipolytic pathways to fuel energy-consuming processes in place or throughout the body. Besides their function as potent energy source, fatty acids are stored as structural components within biological membranes and act as metabolic regulators. The latter quality is significantly influenced by their conversion into bioactive metabolites within manifold cell types and tissues. Oxylipins and endocannabinoids represent two major metabolite classes. Both classes encompass compounds derived from polyunsaturated fatty acids (PUFAs) via designated pathways following their release from membrane phospholipids. Altogether, these metabolites are well-known for a long time to affect countless physiological processes including various adipocyte-related functions. Evidence pointing toward a novel role of these metabolites in mammalian brown/brite adipogenesis and thermogenic adipocyte function has only recently accumulated. Facing the global burden of obesity, an increase in the abundance or activity of thermogenic cells is considered a possibility to increase energy expenditure, thus reducing fat deposition and related metabolic impairment [for a recent review, see Betz and Enerback (2018)]. The potential of novel effectors and pathways targeting brown or brite adipocyte-based thermogenesis therefore

seems highly valuable to explore and is currently one focus of research related to energy balance regulation (Giordano et al. 2016; Vargas-Castillo et al. 2017; Pradhan et al. 2017).

We here provide a concise summary of the potential of individual fatty acid-derived candidate compounds to affect the abundance or activity of murine and human thermogenic adipocytes, give an overview of their biosynthesis pathways and mechanisms of action, describe state-of-the-art methods for their extraction from adipose tissues and subsequent quantification, and discuss the potency of nutritional intervention studies to modulate their abundance and consequently the capacity for non-shivering thermogenesis.

2 Origin and Formation of Oxylipins and Cannabinoids

2.1 Oxylipins

Oxylipins are oxygenated metabolites primarily derived from the enzymatic oxidation of ω -3 and ω -6 PUFAs, but they can also be generated through nonenzymatic pathways such as autoxidation. Fatty acids serving as oxylipin progenitors include the ω -3 PUFAs eicosapentaenoic acid (C20:5, EPA), docosahexaenoic acid (C22:6, DHA), and α -linolenic acid (C18:3, ALA) as well as the ω -6 PUFAs arachidonic acid (C20:4, ARA) and linoleic acid (18:2, LA) (Fig. 1). The 18-carbon molecules LA and ALA are essential fatty acids, as mammals in contrast to plants lack the enzyme ω -3-desaturase and are therefore unable to endogenously produce these PUFAs. Consequently, plant oils constitute rich sources for LA and ALA. Long-chained PUFAs such as ARA, EPA, and DHA can be produced by mammalian cells through elongation and desaturation of either LA or ALA, respectively. However, as both LA and ALA compete for the same desaturase enzymes, endogenous production of DHA, EPA, and ARA is limited (Simopoulos 2016). Consequently, their individual production may be insufficient to cover needs, which makes their nutritional uptake essential (Plourde and Cunnane 2007). The composition of PUFAs in tissues and the production of their bioactive oxylipin-derivatives can be altered by dietary lipid composition and targeted supplementation of certain lipids such as ω -3 PUFAs (Balvers et al. 2012; Ostermann and Schebb 2017; Schebb et al. 2014). Accordingly, diet is a major factor influencing oxylipin levels in the body. This is important as dietary patterns have changed toward an increased uptake of ω -6 PUFAs at the expense of ω -3 PUFAs, resulting in a dietary ω -6/ ω -3 ratio of 20:1 in modern days compared to a ratio of 1:1 during the Paleolithic period (Simopoulos 2001). Furthermore, both a high ω -6 PUFA intake and a high ω -6/ ω -3 ratio are associated with obesity and inflammation [reviewed by (Simopoulos 2016)]. After being resorbed from the diet, PUFAs are either esterified and stored as triglycerides or incorporated into biological membranes as phospholipids. Prior to the generation of oxylipins, PUFAs must be released from these compartments upon cell activation. The cytosolic phospholipase A₂ (cPLA₂) enzyme is involved in the release of PUFAs from membrane phospholipids, which is traditionally perceived as the

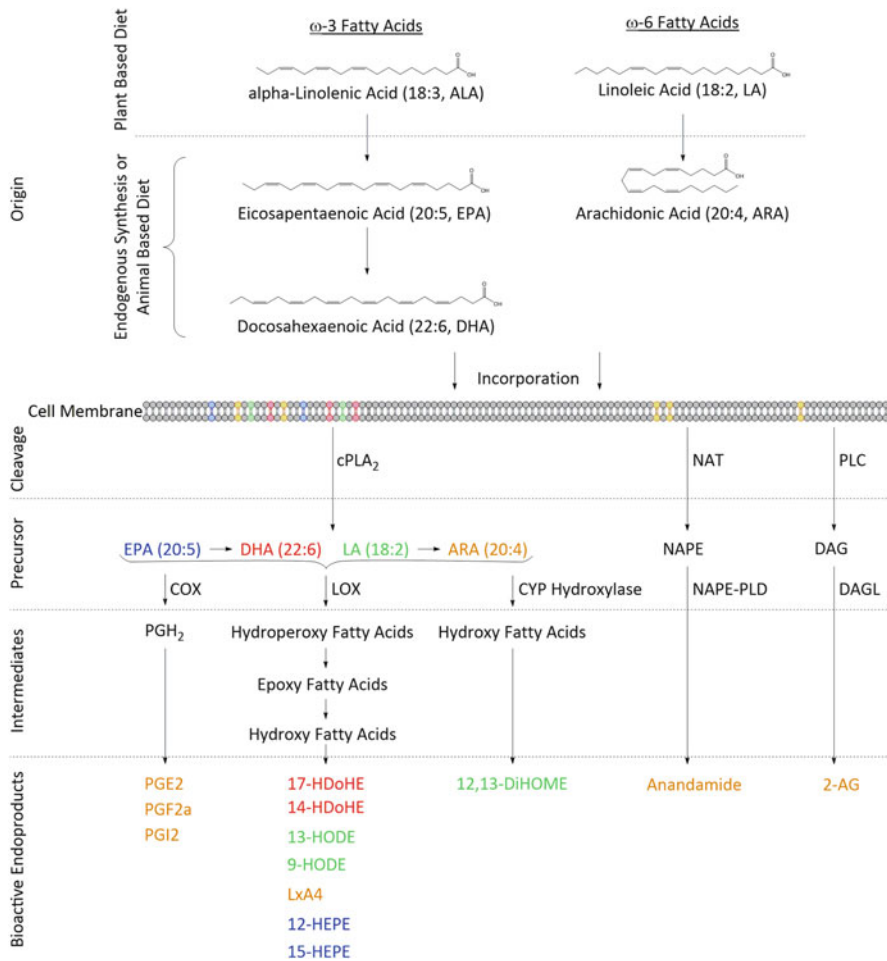


Fig. 1 Synthesis of oxylipins and endocannabinoids from membrane phospholipids. Diet-derived ω -3 and ω -6 fatty acids are incorporated into biological membranes. Through the action of various enzymes, membrane phospholipids are cleaved to release free fatty acids, *N*-arachidonoyl-phosphatidylethanolamine (NAPE) or diacylglycerols (DAG), into the cytosol. These precursors serve as substrates for different enzymatic pathways involved in the synthesis of oxylipins or endocannabinoids. Individual compounds derived from EPA, DHA, LA, and ARA are highlighted in blue, red, green, and orange, respectively. *cPLA₂* cytosolic phospholipase A2, *NAT* *N*-acyltransferase, *PLC* phospholipase C, *COX* cyclooxygenase, *LOX* lipoxygenase, *CYP* cytochrome P450, *NAPE-PLD* NAPE-phospholipase D, *DAG* diacylglycerol, *DAGL* DAG lipase

major origin of oxylipin precursor PUFAs. Deficiency of *cPLA₂* is, however, not sufficient to entirely attenuate oxylipin production in mice and humans, suggesting the existence of alternative origins (Uozumi and Shimizu 2002; Adler et al. 2008). In fact, the adipose triglyceride lipase-mediated release of PUFAs from triglyceride-rich lipid droplets appears to constitute a novel route for the release of oxylipin

precursor fatty acids (Dichlberger et al. 2014; Schlager et al. 2015; Riederer et al. 2017). Subsequently, a vast diversity of oxylipins can be generated depending on the progenitor fatty acid released and on the metabolizing enzymes present in the cells. Based on the precursor PUFA, there are three classes of oxylipins to be distinguished: C18-derived octadecanoids, eicosanoids derived from C20 PUFAs, and C22-derived docosanoids. Fatty acids are converted to oxylipins by three types of enzymes, thus generating distinct oxylipin subclasses (Fig. 1).

The cyclooxygenase (COX) enzyme is responsible for the production of prostanoids and therefore an important mediator of inflammatory processes. Up to date, two COX isoforms have been identified. Although it is still a matter of debate, COX-1 is generally believed to be constitutively expressed in mammalian tissues, while COX-2 is regarded the inducible isoform being upregulated during states of inflammation. The COX-enzyme mainly facilitates the cyclization of its C20-PUFA substrate between C8 and C12, leading to the generation of the intermediary product prostaglandin H (PGH). Therefore, COX-1 and COX-2 are also known as prostaglandin H synthases (PGHS) 1 and 2, respectively. Depending on the substrate involved, PGH₁, PGH₂, or PGH₃ are generated from the C20 PUFAs dihomo- γ -linolenic acid (an intermediate in the synthesis of ARA from LA), ARA, or EPA, respectively. These intermediates serve as precursors for the formation of individual prostanoids of the 1-, 2-, and 3-series upon action of subsequent enzymes such as prostaglandin synthases, for instance. Among the different COX-substrates, ARA serves as the main substrate and precursor of 2-series prostanoids comprising various prostaglandins and thromboxanes [see Gabbs et al. (2015) for a comprehensive review about the generation of COX-derived oxylipins].

The lipoxygenase (LOX) isozymes represent a second oxylipin-producing enzyme class. There are six different human LOX genes (ALOX15, ALOX15B, ALOX12, ALOX12B, ALOXE3, and ALOX5), encoding for six functional isoforms. In contrast, ALOX15B is not found within the murine genome, which comprises the genes ALOX8 and ALOX12E instead. The common property of these enzymes is the introduction of dioxygen into PUFAs, thereby generating hydroperoxy, epoxy, and hydroxy fatty acids (Fig. 1). This includes the formation of hydroxyoctadecadienoic acids (HODEs), hydroxyeicosatetraenoic acids (HETEs), hydroxyeicosapentaenoic acids (HEPEs), and hydroxydocosahexaenoic acids (HDoHEs) from LA, ARA, EPA, and DHA, respectively. Additional products of the LOX pathway are leukotrienes, resolvins, and lipoxins (Kuhn et al. 2015; Gabbs et al. 2015).

Members of the cytochrome P450 (CYP450) system constitute the third class of enzymes able to generate oxylipins. In humans, there are 57 functional genes encoding for enzymes with hydroxylase or epoxygenase activity, while 102 functional genes have been identified in mice (Nelson et al. 2004). The hydroxylase activity of the CYP450 enzymes produces similar metabolite classes as LOX such as HODEs and HETEs, while epoxy fatty acids like ARA-derived epoxyeicosatetraenoic acids (EETs) are generated via epoxygenase activity (Konkel and Schunck 2011).

2.2 Endocannabinoids and Endocannabinoid-Like Compounds

Like oxylipins, endocannabinoids and endocannabinoid-like compounds are derivatives of PUFAs. The endocannabinoids *N*-arachidonoyl-ethanolamide (anandamide, AEA) and 2-arachidonoylglycerol (2-AG) are derived from ARA via two distinct enzymatic pathways (Fig. 1). The synthesis of 2-AG is initiated via the transformation of the membrane phospholipid 2-arachidonoyl-phosphatidylinositol into diacylglycerol by phospholipase C. Subsequently, diacylglycerol is converted by diacylglycerol lipase into 2-AG, which is a short-lived molecule that is either isomerized to 1-arachidonoylglycerol or degraded to ARA by monoacylglycerol lipase. Anandamide is generated by the transfer of ARA from 1-arachidonoyl-phosphatidylcholine to phosphatidylethanolamine, a process catalyzed by the enzyme *N*-acyltransferase. *N*-arachidonoyl-phosphatidylethanolamine (NAPE) is the product of this reaction, which is further converted to anandamide via NAPE-phospholipase D (NAPE-PLD). The degradation of AEA into ARA is subsequently mediated by the action of the fatty acid amide hydrolase. Both AEA and 2-AG are ligands of the cannabinoid receptors 1 and 2 (CB1 and CB2) and therefore considered endocannabinoids.

Endocannabinoid-like compounds represent *N*-acylated-ethanolamides (NAEs) that are generated by the same enzymatic pathways as endocannabinoids. They are, however, not derived from ARA but from other PUFAs or saturated fatty acids. As such, docosahexaenylethanolamide, eicosapentaenylethanolamide, or oleoyl-ethanolamide (OEA) are derived from DHA, EPA, or oleic acid, respectively. Their structural similarities with the endocannabinoid AEA constitute their denomination as endocannabinoid-like compounds (although these metabolites barely bind to CB receptors).

3 Extraction and Analysis of Oxylipins and Endocannabinoids in Adipose Tissues

Over the years, several methods were developed for the quantitation of oxylipins and endocannabinoids in biological samples. The first analytical methods were based on gas chromatography coupled to mass spectrometry. Due to laborious sample preparation and the alteration of the lipid pattern based on degradation of specific compounds, these methods were mostly replaced by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Advancements of analytical instruments allow the detection of hundreds of oxylipins and endocannabinoids simultaneously from the low nanomolar to the high millimolar range by mass spectrometry-based methods nowadays. Nevertheless, analyzing these metabolite classes, sample preparation, chromatographic separation, and detection of the desired analytes needs to be validated and optimized. For example, the oxylipin pattern can be altered through autoxidation mechanisms during sample storage. Therefore, antioxidants like butylated hydroxytoluene or ethylenediaminetetraacetic acid are added to the biological samples to minimize the change of the oxylipin pattern. The stable isotope dilution

assay is the method of choice for mass spectrometric methods; thus deuterated or ^{13}C -labeled oxylipins or endocannabinoids are added to the samples as internal standards to correct for losses during sample preparation and matrix effects during measurements. One bottleneck is the limited availability of these standards and their high price. Therefore, a compromise often is to use one isotopically labeled standard per lipid class. The lipids are extracted by liquid-liquid extraction, solid-phase extraction, or a combination of both. For example, frozen adipose tissues can be crushed in balanced salt solution, extracted with cold methanol, and subjected to polymeric solid-phase extraction to quantify arachidonic acid metabolites in adipose tissue with LC-MS/MS (Pisani et al. 2014; Le Faouder et al. 2013). Most methods nowadays, where around 100 oxylipins and endocannabinoids can be quantified simultaneously, use LC-MS/MS with negative electro-spray ionization or atmospheric pressure chemical ionization (Strassburg et al. 2012; Dumlao et al. 2011; Krott et al. 2016). Usually, quantitative mass spectrometry is performed on a triple quadrupole mass spectrometer, which is operated in the multiple reaction monitoring mode (MRM) (Schuchardt et al. 2013). Here, the parent ion, often $[\text{M}-\text{H}]^-$, is fragmented and a specific fragment is detected. The biggest challenge of oxylipins and endocannabinoids analysis is the differentiation of stereoisomers. Chiral chromatography methods have been developed to differentiate, for example, between (R)- and (S)-isomers (Mesaros and Blair 2012). Another example, where good chromatographic separation is needed, is the quantitation of the prostaglandins PGE_2 and PGD_2 . These prostaglandins have the same chemical formula ($\text{C}_{20}\text{H}_{32}\text{O}_5$) and therefore the same mass-to-charge ratio and also the same MS/MS fragmentation pattern. In some cases, stereoisomers can be differentiated by a clever choice of mass transitions. For example, product ions of HETEs are formed through α -cleavage adjacent to a double bond, and therefore the individual oxylipins 5-HETE and 8-HETE can be distinguished by their fragment ions (Murphy et al. 2005). In general, MRM methods are sensitive, selective and the method of choice for quantitation of oxylipins and endocannabinoids in adipose tissues (Willenberg et al. 2015). One restriction of these LC-MRM-MS methods is that only compounds can be detected where reference material is available. To identify new lipids and to resolve additional structural information, liquid chromatography coupled to high-resolution mass spectrometry (LC-HRMS/MS) with data-dependent fragmentation is used (Lynes et al. 2017). These methods are less sensitive than the MRM methods but bear the advantage to obtain more structural information due to the recording of complete MS/MS spectra. MS/MS spectra are compared to lipid databases, and the lipid classes are tentatively annotated (Fahy et al. 2007). To verify the correct annotation and to quantify the candidates, reference standards are necessary (Masoodi et al. 2010). To use benefits of the targeted (LC-MRM-MS) and the untargeted (LC-HRMS/MS) approaches, the SWATH (sequential window acquisition of all theoretical mass windows) technology was recently applied to lipidomics and will in the future generate deeper insight into the complex regulation of lipids (Prasain et al. 2015).

Independent of the analysis technique, the interpretation of quantitative metabolite data requires their normalization to a reference quantity. A normalization to the applied mass is easily applicable when tissues are used as starting material but presumes the determination of the dissected tissue weight prior to the analysis. Metabolites expressed per milligram tissue can be extrapolated to depot size, thus providing information on the contribution of a tissue to the physiological metabolite production (“per animal”) under the prerequisite that entire depots were dissected and weighed. Similarly, cell number may serve as reference quantity when cultured cells are subject to analysis. Additionally, the protein or lipid content of the material may be quantified to serve as alternative reference. However, the percentage of proteins and lipids may not only vary between different types of tissues but may also be influenced by treatment, which poses limitations to the interpretation of the results. This becomes particularly obvious in an exemplary, comparative analysis of oxylipins between WAT and BAT depots, which are well-known to differ in tissue mass (e.g., 250 mg vs. 65 mg depot mass for the inguinal WAT and interscapular BAT, respectively) and protein content (e.g., 2.5% vs. 7.25% of the respective, dissected tissue masses). Assuming that an oxylipin of interest is quantified with around 100 pg/mg protein in both tissues, a similar production rate would be a straightforward interpretation. However, when this production rate is extrapolated to the indicated tissue masses, the WAT depot is interpreted as a stronger contributor to the systemic synthesis of this oxylipin. In contrast, when the production rate is expressed per milligram tissue, BAT has an approx. threefold higher potency to produce this oxylipin. All normalization options provide valuable information but may result in over- or underestimation of metabolite production, which is particularly crucial when the analysis is applied to tissues of unknown mass or an undefined number of cells. Thus, oxylipin and endocannabinoid data should be analyzed in the light of all available normalization options to select the most reasonable option with regard to the scientific question.

4 Mechanisms of Action

Oxylipins and endocannabinoids are thought to act locally in autocrine or paracrine manner, which is based on their short half-life resulting from rapid degradation of the free, active compounds. However, their occurrence within the blood circulation suggests a possible, endocrine function. Blood oxylipins predominantly occur as esterified or albumin-bound forms (Raz 1972a, b; Maclouf et al. 1980; Shearer and Newman 2008; Shearer et al. 2018). Lipoprotein particles comprise a large quantity of esterified oxylipins, which can be released by lipoprotein lipase (Shearer and Newman 2008; Schebb et al. 2014). Thus, blood lipoproteins may represent a storage compartment for oxylipins to facilitate their delivery and uptake by distant tissues. In contrast, the majority of blood endocannabinoids and endocannabinoid-like compounds does not seem to require lipoproteins as transport vehicle but may bind to serum albumin (Bilgin et al. 2015; Bojesen and Hansen 2003).

The transcellular exchange of oxylipins is likely mediated by plasma membrane transporters rather than diffusion. Several transporters have been identified, which mediate the exchange of prostaglandins and leukotrienes. These proteins regulate the secretion of these compounds into the extracellular space as well as their uptake, which serves the activation of intracellular signaling pathways or their degradation (Schuster et al. 2015; Zhou et al. 2008). The influx and efflux of endocannabinoids is discussed to involve different mechanisms such as specialized transporters, passive diffusion, or endocytosis (Fowler 2013).

Effects of oxylipins and endocannabinoids within the target cell are evoked by activation of receptor-mediated pathways. These compounds interact with different types of receptors located intracellularly or at the cell surface. The latter class includes several members of the transient receptor potential (TRP) ion channel family [for a comprehensive summary, see Kaneko and Szallasi (2014)]. Moreover, many oxylipin receptors as well as the endocannabinoid receptors CB1 and CB2 represent G-protein-coupled receptors of the plasma membrane that signal via intracellular calcium (G_q -protein) or cyclic adenosine monophosphate (cAMP). The cAMP response is triggered by inhibition (G_i -protein) or activation (G_s -protein) of the adenylyl cyclase. Elevation of cAMP levels activates protein kinase A, which subsequently triggers the p38 mitogen-activated protein kinases pathway to stimulate the expression of selected genes. Signaling via this pathway stimulates expression of the uncoupling protein 1 (Ucp1) gene, which classically occurs upon activation of β -adrenoreceptors under conditions of non-shivering thermogenesis (Cao et al. 2001). The same pathway is repressed upon inhibition of the adenylyl cyclase, e.g., upon activation of the CB1 and CB2 receptors (Howlett et al. 2002).

The occurrence of cannabinoid receptors is not restricted to the plasma membrane. Following their synthesis, cannabinoid receptors can be incorporated into endolysosomes of human and rodent cells. These receptors are functional, couple to G_i and/or G_q , and elicit calcium release from intracellular stores (Brailoiu et al. 2011, 2014; Rozenfeld and Devi 2008; Boon et al. 2014). The significance of intracellular vs. plasma membrane cannabinoid receptors for the mediation of physiological effects has not yet been elucidated.

Peroxisome proliferator-activated receptors (PPARs) are a family of ligand-activated nuclear receptors occurring as subtypes α , β/δ , and γ . Oxylipins and endocannabinoids serve as endogenous ligands for the γ -subtype (Bouaboula et al. 2005; Barquissau et al. 2017), which is the central regulator of adipose tissue development, thus regulating white and brown adipogenesis and browning of WAT [reviewed by Harms and Seale (2013) and Seale et al. (2009)]. After its heterodimerization with the retinoic x receptor, PPAR γ is able to bind to the PPAR response element representing a DNA-binding domain. Such a PPAR response element is located in the promoter region of the Ucp1 gene, whose product essentially enables non-shivering thermogenesis in brown and brite adipocytes (Enerback et al. 1997; Li et al. 2014). Consequently, treatment of mice or cultured pre-adipocytes with the PPAR γ agonist rosiglitazone induces the development of brown and brite adipocytes with marked Ucp1 expression (Carmona et al. 2007; Petrovic et al. 2010).

5 Effects of Individual Compounds on Brown/Brite Adipocytes

Brown and brite adipocytes are functionally thermogenic and provide Ucp1-dependent non-shivering thermogenesis within BAT and WAT (Li et al. 2014). The role of fatty acids for adipocyte-derived thermogenesis is not restricted to their quality as fuel for heat production, but comprises their function as important signaling molecules. Thermogenic signaling cascades may be initiated by fatty acids acting as primary effectors (Quesada-Lopez et al. 2016; Hu et al. 2016; Kim et al. 2016) or by their property to function as precursors for the formation of secondary thermogenic metabolites. The latter is contributed by their conversion into oxylipins and endocannabinoids representing a novel function of these fatty acid derivatives. Here we review the candidate compounds reported to influence the capacity for Ucp1-dependent non-shivering thermogenesis in BAT and WAT.

5.1 Prostacyclin

The two COX enzymes are an important hub of oxylipin synthesis in numerous cell types and tissues. Their abundance in murine WAT is under adrenergic control, rendering these enzymes crucial regulators of brite adipocyte formation (Madsen et al. 2010; Vegiopoulos et al. 2010). This function has been ascribed to the production of prostacyclin (PGI₂), which is a derivative of ARA obtained by the specific action of prostacyclin synthase on COX-derived PGH₂. As a ligand for its G_s-protein-coupled cell surface receptor (the IP receptor) expressed by numerous tissues and cell types, this prostaglandin is well-known to potently act on vasodilation and platelet aggregation. Browning of WAT constitutes a novel function affected by this oxylipin.

The production of PGI₂ in WAT under conditions of non-shivering thermogenesis is likely mediated by mature adipocytes as these cells show increased expression of COX-2 upon adrenergic stimulation (Vegiopoulos et al. 2010). As part of a straightforward signal transduction, the released PGI₂ likely acts in paracrine manner to stimulate Ucp1 expression and the sensitivity toward noradrenergic stimulation during the differentiation of adipocyte precursors (Vegiopoulos et al. 2010). This brite adipogenesis seems to specifically originate from the action of PGI₂ on committed precursor cells of the stromal vascular fraction as the effect is *in vitro* most efficiently inducible by treatment of purified Lin⁻Sca1⁺ primary cultures of the posterior subcutaneous fat depot with carbaprostacyclin (cPGI₂) (Babaei et al. 2017). The latter represents a chemically stable PGI₂-analogue as PGI₂ itself is quickly converted into the inactive metabolite 6-keto-PGF_{1α}.

The cPGI₂-induced conversion of committed precursors into brite adipocytes *in vitro* is most efficient when the compound is added to the adipogenic culture medium during eight consecutive days (Babaei et al. 2017; Bayindir et al. 2015). Thermogenic maturation of these cells in response to cPGI₂ treatment is initiated

during an advanced stage of differentiation (Bayindir et al. 2015). This effect likewise seems to originate from an interaction of $cPGI_2$ with the cell surface IP receptor as well as with the nuclear receptor $PPAR\gamma$ since $cPGI_2$ -induced *Ucp1* expression is blunted during the differentiation of primary progenitors obtained from WAT of mice with heterozygous $PPAR\gamma$ ablation or in the presence of an IP receptor antagonist (Vegiopoulos et al. 2010). In fact, expression of the IP receptor is highest in pre-adipocytes and follows a reduction during the adipogenic differentiation of murine and human cells (Vassaux et al. 1992; Borglum et al. 1999). Thus, $cPGI_2$ may orchestrate brite adipogenesis via an initial activation of the IP receptor of committed pre-adipocytes to promote their thermogenic maturation in the further progress of differentiation via a predominant interaction with $PPAR\gamma$.

Intriguingly, $cPGI_2$ -induced brite adipogenesis is not restricted to murine adipocytes but reproducible in human mesenchymal stem cells involving similar signal transduction, either during a continuous treatment or upon 3 days of exposure in the final stage of the adipogenic differentiation (Vegiopoulos et al. 2010; Ghandour et al. 2016). The $cPGI_2$ -induced expression of *Ucp1* in human and murine cells is rather moderate compared to the potent effect of the $PPAR\gamma$ agonist rosiglitazone (Ghandour et al. 2016; Babaei et al. 2017). This relative difference in potency may be counterbalanced under physiological conditions since the naturally occurring PGI_2 is considered ten times more potent than its synthetic analogue (Aiken and Shebuski 1980; Whittle et al. 1980). However, the physiologic contribution of this system to the recruitment of brite adipocytes *in vivo* is to date virtually unexplored. The production of PGI_2 in WAT of COX-2 overexpressing mice correlates well with their increased abundance of brite adipocytes. This phenotype is associated with thermogenesis and resistance to diet-induced obesity (Vegiopoulos et al. 2010). Moreover, adrenergically induced browning of WAT is impaired in IP receptor knockout mice and in mice with heterozygous ablation of $PPAR\gamma$, indicating PGI_2 signaling to be a modulator of systemic energy expenditure (Vegiopoulos et al. 2010).

The effect of PGI_2 on browning of murine adipocytes remains to be corroborated by more studies. The author's laboratories were not able to fully reproduce these findings using our own proven protocols. Prostacyclin-mediated browning of murine cells appears to require very precise culture conditions and may thus not constitute a common, robust effect. Moreover, IP receptor knockout mice appear to be protected from the adipogenic effect of a diet rich in ARA precursors, indicating PGI_2 signaling to promote energy storage and fat mass expansion (Massiera et al. 2003). This observation is well in line with enhanced adipogenic differentiation of cultured cells in the presence of PGI_2 (Negrel et al. 1989; Catalioto et al. 1991). Altogether, these findings indicate a bifunctional role of PGI_2 for adipocyte identity with an effect on both brite and white adipogenesis. The underlying reasons remain to be clarified but may involve, for instance, the PGI_2 target cell itself, the abundance of committed precursors within a tissue or a culture, or the presence of other adipogenic factors in blood or cell culture sera, respectively. Further studies are needed to corroborate the browning potential of PGI_2 and to further explore its specific effect on energy balance regulation under *in vivo* conditions.

5.2 Prostaglandin E₂

As member of the various prostaglandins produced via the COX pathway, prostaglandin E₂ (PGE₂) specifically arises from the action of the PGE synthase isozymes on ARA-derived PGH₂. As for other prostaglandins, the physiological effects of PGE₂ originate from its interaction with at least four cognate cell surface PGE receptors (EP1-EP4). The individual physiological responses evoked by PGE₂ are not only the result of cell type-dependent variations in receptor expression but also influenced by the specific signaling cascade of the receptor subtypes. While the EP1 subtype is G_q-coupled, the EP2 and EP4 subtypes couple to G_s, whereas the EP3 subtype couples to G_i. The latter is involved in mammalian febrile response (Ushikubi et al. 1998; Lazarus et al. 2007), which is an indirect consequence of central PGE₂ signaling on BAT function. In a healthy organism, the hypothalamic preoptic area (POA) translates the environmental temperature sensed by cutaneous thermoreceptors into an appropriate physiological response to maintain normothermia. In a cold environment, this involves an inhibition of efferent POA neurons culminating in the excitation of sympathetic nerves to activate BAT thermogenesis. The activity of these efferent POA neurons is likely influenced by the EP3 receptor [for a comprehensive review, see Morrison and Madden (2014)]. During febrile response (e.g., to infection), elevated levels of central PGE₂ act on the same neuronal pathways mediating an increase in body temperature and energy expenditure via the stimulation of BAT activity (Morrison 2016; Nakamura et al. 2002; Scammell et al. 1996).

Reduced systemic levels of PGE₂ have been hypothesized to participate in the afebrile regulation of body temperature (Foster et al. 2015). Besides centrally mediated mechanisms, there is evidence for a direct interaction of PGE₂ with adipocytes affecting thermogenesis. In fact, exogenous PGE₂ acutely increases the oxygen consumption of brown adipocytes (Nagai et al. 1996). The administration of 16,16-dimethyl-PGE₂ (a stable analogue of PGE₂) to C57BL/6J mice enhances the expression of Ucp1 mRNA in WAT suggesting an effect on brite adipocyte recruitment (Madsen et al. 2010; Xue et al. 2016). This PGE₂-mediated browning appears to involve a coordinated interaction of distinct molecular effectors (Garcia-Alonso and Claria 2014; Garcia-Alonso et al. 2013). Mice with an adipocyte-specific knockout of PPAR γ have elevated adipose tissue levels of PGE₂ besides an increased expression of its synthesizing enzymes COX and microsomal PGE synthase 1 (mPGES1). This indicates a repressive effect of PPAR γ on the PGE₂-synthesis pathway, confining the physiological origin of adipose tissue PGE₂ production to a cell type with low or absent PPAR γ expression. In line with this, cells of the stromal vascular fraction from murine subcutaneous adipose tissue show higher PGE₂ secretion compared to mature adipocytes (Ying et al. 2017). Exogenous PGE₂ is able to suppress PPAR γ expression in murine cells and to stimulate COX-2 and mPGES1 expression of WAT explants, indicating the existence of an autocrine positive feedback loop within the secreting cell to further elevate its own PGE₂ production (Garcia-Alonso et al. 2013). Whereas the presence of PGE₂ during the adipogenic differentiation of primary epididymal pre-adipocytes enhances Ucp1

expression over that of vehicle-treated wild-type cells, Ucp1 expression is unaltered in PPAR γ -ablated cells (Garcia-Alonso et al. 2013). Thus, the browning effect of PGE₂ appears to require the presence PPAR γ within the target cell, although the underlying mechanism is not yet conclusive in the light of its suppressive effect on PPAR γ expression. Moreover, the specific cell type responding to PGE₂ has not yet been determined and may differ between species. Adipocyte progenitors seem to be a plausible target for the murine tissue as an acute treatment with PGE₂ is capable of increasing Ucp1 mRNA levels in cells of the stromal vascular fraction (Vegiopoulos et al. 2010). Conversely, primary mature adipocytes derived from human omental fat respond to acute PGE₂ treatment with an increase in Ucp1 mRNA expression, suggesting PGE₂-mediated browning to arise from trans-differentiation of white adipocytes (Garcia-Alonso et al. 2016). Such hypothesis is, however, not supported by experiments with human multipotent adipose-derived stem (hMADS) cells that serve as an established model system for human brite adipogenesis in culture (Elabd et al. 2009; Pisani et al. 2011). The white-to-brite adipocyte conversion of these cells is dose-dependently inhibited when 16,16-dimethyl-PGE₂ is present in the culture medium during an advanced stage of the adipogenic differentiation (Pisani et al. 2014).

The physiological effects of PGE₂ are mediated via its interaction with the four EP receptors, all of which are expressed in murine adipose tissue. The EP4 receptor has been suggested to be responsible for the browning effect (Madsen et al. 2010), which was further explored in EP4 receptor knockout mice recently (Ying et al. 2017). In fact, these mice show a more pronounced delipidation of their subcutaneous WAT depot than wild-type littermates after 10 days of repeated β_3 -adrenergic stimulation. Interestingly, this phenotype is not related to the abundance of Ucp1 but rather originates from mitochondrial biogenesis and a disequilibrium between lipid droplet breakdown and synthesis. Thus, ablation of the EP4 receptor enhances murine WAT remodeling, suggesting this receptor as a negative regulator of adrenergically stimulated WAT browning. In contrast, the inhibitory effect of 16,16-dimethyl-PGE₂ on brite adipogenesis of hMADS cells is even more pronounced in the presence of an EP4 receptor inhibitor, indicating a supportive effect on the trans-differentiation of human adipocytes (Pisani et al. 2014).

A question of profound interest is whether PGE₂ is of any physiological relevance for the recruitment of brite adipocytes *in vivo*. Pertaining to mice, reported effect sizes on Ucp1 mRNA expression are rather small, and none of the aforementioned studies provides information on Ucp1 protein expression, PGE₂-induced oxygen consumption of brite cultures or tissues, or physiological estimates of thermogenic capacity depending on the size of the affected depot. Studies in COX-2 transgenic mice or in mice with genetically or pharmacologically induced deficiency of COX-2 only provide limited information as physiological effects associated with brown/brite adipocyte-derived thermogenesis (e.g., body temperature, whole-body energy expenditure, body mass development) may be influenced by altered production of various COX-metabolites (Vegiopoulos et al. 2010; Madsen et al. 2010). Altogether, the observations currently available suggest PGE₂ to constitute a candidate compound that may mediate the recruitment of non-shivering thermogenesis in WAT, whose

potential requires comprehensive characterization *in vitro* and *in vivo* with particular focus on species-specific effects.

5.3 Prostaglandin F_{2α}

Together with PGI₂ and PGE₂, prostaglandin F_{2α} (PGF_{2α}) represents another COX-derived prostaglandin produced by adipose tissues. The synthesis originates from the metabolization of ARA into PGH₂, which serves as a direct or indirect (via the intermediate production of PGE₂ and PGD₂) substrate for the downstream production of PGF_{2α} by members of the aldo-keto reductase enzyme family. Its cognate receptor (the FP receptor) is abundant in adipose tissues (Volat et al. 2012), located at the cell surface, and couples to G_q, thus mediating the physiological effects of PGF_{2α} via an increase in intracellular calcium. This signaling pathway attenuates Ucp1 expression during brite adipogenesis, thus rendering this compound a negative regulator of thermogenic adipocyte function (Pisani et al. 2014). Intriguingly, the effect of PGF_{2α} on adipocyte browning has not yet been investigated in murine cells but is evident in hMADS cells. These cells can be differentiated into white or brite adipocytes under serum-free conditions depending on the presence (brite) or absence (white) of the PPARγ-activator rosiglitazone during the last days of the differentiation phase. Co-administration of the stable PGF_{2α}-analogue fluprostenol during that phase dose-dependently blunts the levels of Ucp1 mRNA and protein in brite hMADS adipocytes (Pisani et al. 2014). The effect is mediated via activation of the FP receptor resulting in intracellular calcium oscillations with subsequent phosphorylation of extracellular signal-regulated kinases (ERK). In line with this, differentiation of brite hMADS in the presence of an ERK inhibitor or a calcium-chelator restores fluprostenol-induced effects on Ucp1 expression. As PGF_{2α} signaling in adipocytes inhibits the activity of PPARγ (Reginato et al. 1998), the repressive effect of this compound on Ucp1 expression may be explained by an antagonization of rosiglitazone during the conversion of white into brite hMADS adipocytes.

Physiologic effects of PGF_{2α} in adipose tissue may comprise both mature and pre-adipocytes as responsive cell types. In fact, PGF_{2α} signaling in cultured 3T3-L1 pre-adipocytes results in an inhibition of adipogenesis (Annamalai and Clipstone 2014; Liu and Clipstone 2007; Ueno and Fujimori 2011), which could be hypothesized to limit the *de novo* maturation of thermogenic cells *in vivo* in the long term. Genetically induced reduction of PGF_{2α} production in mice, however, results in the excessive accumulation of adipose tissue and adipocyte hypertrophy (Volat et al. 2012) and thus appears to primarily promote general adipogenesis. As brite adipogenesis in hMADS cells can be interpreted as a model system for trans-differentiation, the thermogenic function of adipocytes may rather be affected by reduced conversion of mature white into brite adipocytes in the presence of PGF_{2α} (Pisani et al. 2014). The increased production of this compound by adipose tissues may antagonize the browning effect of other oxylipins and therefore limit adaptive heat production under conditions of non-shivering thermogenesis.

5.4 Lipoxin A₄

The ARA-derivative lipoxin A₄ (LXA₄) is an eicosanoid belonging to the subclass of lipoxins, whose biosynthesis is mediated via alternative routes within the LOX pathway. The formation of LXA₄ via the LOX-15 isozyme involves the intermediate formation of 15-hydroperoxyeicosatetraenoic acid, whereas the metabolization of ARA via LOX-5 results in the intermediate synthesis of leukotriene A₄ serving as precursor for the subsequent formation of both leukotriene B₄ and LXA₄. The activity of LOX-5 is tightly controlled by interaction with its activating protein ALOX5AP (Dixon et al. 1990; Miller et al. 1990). Overexpression of this protein in adipose tissue of mice increases the levels of LXA₄ but not leukotriene B₄ in BAT, subcutaneous WAT, and within the circulation (Elias et al. 2016). The subcutaneous WAT of these mice is characterized by an increased abundance of multilocular cells and elevated expression of Ucp1 protein under obesogenic and non-obesogenic conditions. Although elevations in WAT Ucp1 expression are reproducible on mRNA level during a 2-day injection of LXA₄ in wild-type mice, it remains to be determined whether adipose tissue browning in ALOX5AP transgenic mice is entirely related to elevated LXA₄ levels. In fact, LOX-5 is involved in the synthesis of a number of oxylipins, some of which may complement the effect of LXA₄ via a direct or indirect interaction with cells of white adipose tissue.

A possible, direct action of LXA₄ on cultured adipocytes has not been investigated, but this oxylipin appears to influence the number and activity of Ucp1-positive cells in ALOX5AP transgenic mice via a secondary mechanism. Cultivation of the liver cell line HepG2 in the presence of LXA₄ results in higher mRNA levels of bile acid synthesizing enzymes. In line with this, ALOX5AP transgenic mice have higher levels of circulating bile acids as well as a higher fecal bile acid excretion (Elias et al. 2016). Intriguingly, systemic bile acid levels have been associated with elevated energy expenditure and reduced propensity to diet-induced obesity (Watanabe et al. 2006, 2011, 2012; Teodoro et al. 2014; da-Silva et al. 2011; Zietak and Kozak 2016). These effects have been hypothesized to originate from Ucp1-dependent thermogenesis based on the ability of bile acids to activate brown adipocytes and to increase the abundance of brite adipocytes (Velazquez-Villegas et al. 2018; Zietak and Kozak 2016; Teodoro et al. 2014; Watanabe et al. 2006). In line with these studies, ALOX5AP transgenic mice have a higher BAT expression of the G-protein-coupled bile acid receptor 1 and display higher energy expenditure that translates into attenuated body mass accumulation upon high-fat diet feeding (Elias et al. 2016). However, the physiological relevance of Ucp1-dependent thermogenesis, activated or recruited via such LXA₄-bile acid-mediated mechanism, requires careful elucidation in further studies. Bile acid-mediated effects on energy expenditure seem to require the presence of Ucp1 (Zietak and Kozak 2016), while such relationship was not reproduced in our own laboratory (manuscript submitted). Thus, the currently available data do not support a direct thermogenic function of LXA₄ for brown and brite adipocytes, while its possible secondary effect on Ucp1-dependent energy expenditure may be of minor physiological relevance.

5.5 12,13-Dihydroxy-9Z-Octadecenoic Acid

Besides the eicosanoids described above, there are several compounds belonging to the class of octadecanoids recently associated with an impact on thermogenic adipocyte function. Among them, 12,13-dihydroxy-9Z-octadecenoic acid (12,13-DiHOME) represents a potent modulator of non-shivering thermogenesis as recently demonstrated by Lynes and coworkers (Lynes et al. 2017). This compound occupies a unique role among the oxylipins described herein as it exclusively influences the thermogenic function of brown but not brite adipocytes. The metabolite is generated in a two-step reaction from LA firstly forming its epoxide-precursor 12,13-epoxy-9Z-octadecenoic acid via the CYP450 pathway, which is subsequently converted into the diol 12,13-DiHOME via four epoxide hydrolase isozymes (Ephx1–4). Transcript levels of the Ephx1 and 2 genes are upregulated in murine BAT in a cold (4°C) environment, rendering BAT an important source of circulating 12,13-DiHOME levels under conditions of non-shivering thermogenesis. In vivo, the cold-induced release of 12,13-DiHOME appears to initiate an autocrine/paracrine feedback reaction to enhance the thermogenic function of BAT. The causative signal transducer has not yet been addressed but may involve PPAR γ since such an interaction has been described for its isomer 9,10-DiHOME (Lecka-Czernik et al. 2002). Interestingly, the thermogenic effect of 12,13-DiHOME does not result from a direct effect on Ucp1 expression but rather originates from an immediately augmented membrane translocation of adipocyte fatty acid transporters facilitating the lipid absorption of mature brown adipocytes. Consequently, repeated administration of 12,13-DiHOME to diet-induced obese mice results in enhanced BAT-specific uptake of triglycerides and free fatty acids, thus affecting oral lipid tolerance and serum triglyceride levels. This facilitated lipid uptake appears to improve adaptive heat production as 12,13-DiHOME-treated cold-exposed mice show an attenuated reduction of body temperature, an increased oxygen consumption, and a reduction of the respiratory exchange ratio indicative of lipid oxidation. Thus, 12,13-DiHOME supports non-shivering thermogenesis by increasing the supply of BAT with circulating lipids. The molecular fate of internalized lipids, however, remains subject to further experiments since fatty acids do not exclusively serve as fuel for thermogenesis but also as direct activators of Ucp1. Moreover, further studies are required not only to clarify whether this thermogenic mechanism is dependent on the presence of Ucp1 but also to determine the most effective therapeutic dosage of the oxylipin. In fact, supraphysiological concentrations of LA-diols interfere with the mitochondrial function of mammalian cells (Moran et al. 1997; Sisemore et al. 2001). Although the chronic treatment of diet-induced obese mice with 12,13-DiHOME does not affect body mass development (Lynes et al. 2017), this compound may have therapeutic relevance in the context of dyslipidemia. Intriguingly, circulating 12,13-DiHOME levels in humans correlate with BAT activity in response to acute cold exposure (14°C), emphasizing the translational significance of this novel effector (Lynes et al. 2017).

5.6 Hydroxyoctadecadienoic Acids

Recruitment of brite adipocytes in response to cold exposure involves interleukin-4 (IL-4) dependent, adipose-directed migration of alternatively activated M2 macrophages. This brite adipogenic mechanism has been ascribed to the local production of macrophage-derived catecholamines (Nguyen et al. 2011; Qiu et al. 2014), but this was questioned lately (Fischer et al. 2017). A recent publication suggests the octadecanoids 9-hydroxyoctadecadienoic acid (9-HODE) and 13-HODE as alternative mediators of macrophage-dependent adipose tissue browning (Lee et al. 2016). In their study, Lee and coworkers observed a transiently increased number of lipid-laden, cluster of differentiation 44 positive (CD44⁺) F4/80 macrophages in the stromal vascular fraction of murine gonadal WAT upon 3 days of β_3 -adrenergic treatment. This particular macrophage fraction is characterized by an increased production of 9-HODE and 13-HODE. In vivo, this macrophage-derived secretion of 9-HODE and 13-HODE could be supported by adipocyte-derived fatty acids as CD44⁺ macrophages accumulate their triglycerides during the clearance of dying adipocytes. This hypothesis is supported by the observation that β_3 -adrenergic treatment causes F4/80 macrophages to form crown-like structures in adipose tissue with elevated expression of the genes cluster of differentiation 36 (Cd36) and arachidonate 15-lipoxygenase (Alox15) in the CD44⁺ fraction. Whereas the first encodes a fatty acid transporter that may catalyze the uptake of adipocyte-derived fatty acids, the second encodes a LOX-enzyme involved in the biosynthesis of HODE from LA. The expression of macrophage Alox15 protein increases during co-culture with dying adipocytes along with the accumulation of markers typical for alternative macrophage activation. As these events occur in the presence but not absence of IL-4, the production of 9-HODE and 13-HODE likely represents a downstream event mediated by CD44⁺ macrophages following their targeted M2 polarization.

Alternatively activated M2 macrophages not only form crown-like structures to clear the tissue from dying adipocytes but also recruit adipocyte progenitors expressing the platelet-derived growth factor receptor A (Lee et al. 2013, 2014). This particular fraction is capable of undergoing brite adipogenesis (Lee et al. 2012). Indeed, adipogenic differentiation of these precursor cells in the presence of 9-HODE, 13-HODE, or rosiglitazone for 3 days results in an increased sensitivity toward β -adrenergic stimulation characterized by elevated Ucp1 mRNA levels, lipolysis, and Ucp1-dependent oxygen consumption following isoproterenol treatment (Lee et al. 2016). It remains to be determined whether these effects originate from an interaction with either PPAR γ , the cation channel TRP vanilloid 1 (TRPV1), or the G-protein-coupled receptor 132, all of which serving as receptors for these oxylipins (Nagy et al. 1998; Obinata et al. 2005; Patwardhan et al. 2009; Huang et al. 1999).

Thus, 9-HODE and 13-HODE represent candidate compounds for the browning of WAT likely acting in a paracrine manner following their orchestrated production from immune cells. The latter fraction complements mature and pre-adipocytes as source of adipose-derived oxylipins with brite adipogenic potential.

5.7 10-Oxo-12(Z)-Octadecenoic Acid

The intestinal microbiome has been repeatedly hypothesized to be a significant contributor to systemic energy balance regulation [see Moran-Ramos et al. (2017) and Zhang et al. (2017) for recent reviews]. This relationship appears to be contributed by the bacterial turnover of dietary LA affecting energy expenditure. Lactic acid bacteria are capable of producing the octadecanoid 10-oxo-12(Z)-octadecenoic acid (KetoA) and further structurally related LA metabolites (Kishino et al. 2013). Considerably higher levels in colon, small intestine, and plasma of conventional vs. germ-free mice render the intestinal microbiome a significant source of these compounds (Kishino et al. 2013). In their recent study, Kim and coworkers show that KetoA represents an activator of TRPV1 (Kim et al. 2017). Physiological effects of oral KetoA administration investigated in the same study rely on the TRPV1-dependent initiation of an afferent signaling cascade within the gastrointestinal tract as demonstrated by the use of TRPV1 knockout mice and mice subjected to gastric vagotomy. Afferent signaling of KetoA via the nervus vagus results in the excitation of efferent sympathetic pathways. KetoA-induced, sympathetic signaling may selectively target BAT and WAT but not the cardiovascular system as the turnover of norepinephrine is elevated locally in these tissues but not in the heart. Moreover, this sympathetic norepinephrine release translates acutely and chronically into elevated Ucp1 expression in BAT and WAT under obesogenic and non-obesogenic conditions. In line with an elevated capacity for non-shivering thermogenesis, KetoA-supplemented mice of the C57BL/6 and KK-Ay strains but not TRPV1 knockout mice show reduced propensity to diet-induced obesity, elevated rectal body temperature and energy expenditure.

The TRPV1 receptor is expressed by adipose tissue, and treatment of cultured cells with the TRPV1 agonist capsaicin affects brown and white adipogenesis (Kida et al. 2016; Baboota et al. 2014). KetoA may thus be able to complement Ucp1-dependent non-shivering thermogenesis via a direct interaction with TRPV1 of adipocytes upon spillover into the bloodstream. Such direct effect may be supported by its property to interact with PPAR γ (Goto et al. 2015), although the knockout of TRPV1 is sufficient to explain the majority of physiologic effects induced by oral KetoA (Kim et al. 2017). In fact, Kim and coworkers report KetoA to be undetectable in plasma of KetoA-supplemented mice (Kim et al. 2017), indicating the physiologic effects induced by this compound to entirely rely on secondary signaling upon activation of TRPV1 in the intestine. The effect of KetoA, however, has not been investigated upon administration via other peripheral routes (e.g., intraperitoneal or subcutaneous) or with cultured adipocytes.

Although the actual relevance of Ucp1-dependent thermogenesis remains to be assessed, the oxylipin KetoA appears to be a potent modulator of systemic energy expenditure of microbial origin. The physiological relevance of this microbiome-host-interaction should be further addressed in studies using KetoA-supplemented conventional and germ-free mice under conditions (e.g., cold exposure) that promote Ucp1-dependent non-shivering thermogenesis.

5.8 Endocannabinoids

The endocannabinoid system is well-known for its implication in energy homeostasis [reviewed by Matias and Di Marzo (2007), Silvestri and Di Marzo (2013), Richard et al. (2009)]. The best studied entity in this regard is the CB1 receptor expressed by various brain areas associated with the regulation of food intake and energy turnover. Its activation increases body mass by elevated food intake as well as reduced energy expenditure and thus centrally signals a positive energy balance (Kirkham et al. 2002). In line with this, treatment of mice with the synthetic CB1 antagonist rimonabant decreases food intake and body mass (Jbilo et al. 2005) and increases glucose and lipoprotein clearance by BAT (Bajzer et al. 2011; Boon et al. 2014).

While the importance of the endocannabinoid system for the regulation of energy homeostasis is strongly supported by the aforementioned studies, it is still a matter of debate (1) whether effects on energy expenditure are exclusively or only partially mediated by central cannabinoid receptors and (2) whether effects on energy expenditure originate from brown and brite adipocyte-derived thermogenesis. On the one hand, exclusive stimulation of peripheral CB1 in diet-induced obese mice is sufficient to reduce their body mass and to increase their energy expenditure, suggesting the occurrence of cannabinoid-dependent thermogenesis in the absence of central signaling (Boon et al. 2014). On the other hand, a genetically induced decrease of 2-AG levels (an endogenous CB1 agonist) in the murine forebrain results in elevated mitochondrial abundance and Ucp1 protein expression in BAT, increased body temperature upon adrenergic stimulation, and reduced body mass accumulation under obesogenic and non-obesogenic conditions (Jung et al. 2012), thus arguing for a central regulation of peripheral energy expenditure by the cannabinoid system. This is further supported by studies in mice with forebrain-specific CB1 deletion, which are resistant to diet-induced obesity and show higher body temperature under cold stimulation (Quarta et al. 2010). Elevated Ucp1 mRNA expression and glucose uptake in BAT of these mice indicates the recruitment of BAT-derived thermogenesis via central cannabinoid signaling (Quarta et al. 2010). Moreover, treatment of cultured murine white and brown adipocytes with a synthetic CB1 agonist decreases Ucp1 protein levels, suggesting a direct regulation of adipocyte-derived thermogenesis by the peripheral endocannabinoid system (Perwitz et al. 2006; Boon et al. 2014). In line with this, the adipose-specific knockout of the CB1 receptor is sufficient to induce a lower body mass in mice and to reduce the propensity to diet-induced obesity (Ruiz de Azua et al. 2017). Rimonabant-treated mice show increased Ucp1 mRNA in BAT independent of sympathetic innervation (Bajzer et al. 2011), providing additional evidence for the importance of peripherally expressed CB1. Hypothesizing the adipocyte CB1 receptor as direct repressor of Ucp1-dependent thermogenesis, ablation of its ligands should be expected to mimic the effect of the CB1 knockout. Such relationship was further investigated in mice with an adipocyte-specific knockout of NAPE-PLD, representing the enzyme that synthesizes the endocannabinoid AEA and endocannabinoid-like compounds (Geurts et al. 2015). As expected, the knockout translates into reduced levels of

the respective metabolites. Surprisingly, these mice have reduced Ucp1 mRNA expression and elevated body mass under obesogenic and non-obesogenic conditions, suggesting the endocannabinoid system as a positive regulator of adipocyte-derived thermogenesis. Thus, the significance of the endocannabinoid system as a direct or indirect regulator of Ucp1-dependent thermogenesis requires further deciphering as it appears to involve a complex regulation of the implicated effectors.

The potential of individual endocannabinoids or endocannabinoid-like compounds to activate or recruit Ucp1-dependent non-shivering thermogenesis is largely unexplored. Stimulation of mice by cold exposure or with a β_3 -adrenergic agonist increases the levels of endocannabinoids and NAEs like OEA in BAT and especially in WAT (Krott et al. 2016). Krott and coworkers hypothesize this regulation of the endocannabinoid system to mediate an autocrine negative feedback mechanism limiting the capacity for Ucp1-dependent thermogenesis during the recruitment of brown and brite adipocytes. However, mice supplemented with OEA exhibit increased thermogenic capacity in BAT, energy expenditure, and BAT temperature after β_3 -adrenergic treatment, suggesting this compound to potentiate the recruitment of Ucp1-dependent heat production (Suarez et al. 2014). As OEA is unable to bind cannabinoid receptors (Fonseca et al. 2013), this effect may be mediated by an interaction with PPAR α of brown and brite adipocytes (Fu et al. 2003). Further studies are needed to manifest the positive regulatory role of OEA on Ucp1-dependent non-shivering thermogenesis and to elucidate the potential of other endocannabinoids and endocannabinoid-like compounds.

6 Nutritional Interventions to Modulate Oxylipin Formation and the Thermogenic Capacity of Adipose Tissues

Dietary PUFAs constitute an important part of the human nutrition. The dietary ω -6/ ω -3 ratio has shifted from approx. 1:1 during the Paleolithic period to 15:1 and higher in modern societies, which is associated with the occurrence of obesity related to adipose tissue dysfunction (Simopoulos 2016). Concomitantly, the activity of BAT among adult humans is highest in lean and lowest in obese subjects (Saito et al. 2009; Cypess et al. 2009; van Marken Lichtenbelt et al. 2009; Vijgen et al. 2011). Treatment of hMADS cells with ARA blunts Ucp1 expression during brite adipogenesis, which is prevented upon co-treatment with EPA (Ghandour et al. 2018; Pisani et al. 2014). Thus, a causal relationship between dietary PUFA-uptake, the abundance and activity of thermogenic adipocytes, and energy expenditure may be hypothesized.

Evidence supporting a role of fatty acids and their metabolites in the activation of BAT and the recruitment of brite adipocytes has accumulated during recent years (Okla et al. 2017). Several studies have particularly investigated the effects of ω -3 fatty acids and show that supplementation of mice with fish oil comprising diets rich in EPA and DHA results in an elevated expression of Ucp1 in BAT and/or WAT (Kim et al. 2015, 2016; Bargut et al. 2016a, b). These in vivo findings are further

supported by *in vitro* experiments. Treatment of murine primary white and brown pre-adipocytes with EPA increases thermogenic gene expression and concomitantly enhances markers of mitochondrial abundance and function (Kim et al. 2016; Zhao and Chen 2014). Similarly, EPA elevates Ucp1 expression of human primary adipose tissue-derived stem cells, but the same effect is not observed in the presence of DHA (Fleckenstein-Elsen et al. 2016). Thus, ω -3 fatty acids and particularly EPA appear to beneficially affect the recruitment of Ucp1-positive cells *in vivo* and *in vitro*. Mechanistically, such effect may be mediated by a direct interaction of EPA with free fatty acid receptors (Quesada-Lopez et al. 2016; Kim et al. 2016), for instance, or via their conversion to bioactive metabolites. In fact, the fatty acid composition of membranes and thus the endogenous oxylipin profile changes upon nutritional interventions. Accordingly, the systemic production of ω -3-derived metabolites is enhanced upon dietary supplementation of ω -3 PUFAs [for a review, see Ostermann et al. (2017)].

The potential of nutritional interventions to modulate the abundance or activity of Ucp1-positive adipocytes as a direct consequence of altered oxylipin production is virtually unexplored. Recent studies support the hypothesized role of the dietary ω -6/ ω -3 ratio in this context (Pisani et al. 2014; Ghandour et al. 2018). Mice were subjected to chow diets supplemented with ω -6 or ω -3 PUFAs to achieve a dietary ω -6/ ω -3 ratio of 30 or 3.7, respectively. After 11 weeks of feeding, mice were treated with a β_3 -adrenergic agonist for another week to stimulate the recruitment of non-shivering thermogenesis in BAT and WAT. The expression of Ucp1 is more pronounced in BAT and WAT of ω -3-supplemented mice. This effect is hypothesized to originate from $\text{PGF}_{2\alpha}$, whose production is reduced in both tissues of ω -3- vs. ω -6-supplemented mice upon β_3 -stimulation. This reduced production is reproducible in ARA-treated brite hMADS cells upon co-incubation with EPA accompanied by a restoration of Ucp1 expression. As described above (Sect. 5.3), $\text{PGF}_{2\alpha}$ is a negative regulator of Ucp1-dependent thermogenesis in hMADS cells. In line with this, the capacity for non-shivering thermogenesis is impaired under conditions that favor the production of this compound. Mice on a chow diet supplemented with ARA have a higher WAT-production of $\text{PGF}_{2\alpha}$ compared to mice receiving oleic acid supplementation. This production rate of $\text{PGF}_{2\alpha}$ remains elevated following β_3 -adrenergic treatment. This regulation is well in line with the impaired recruitment of Ucp1 protein and the reduced abundance of multilocular adipocytes in WAT of ARA-supplemented vs. oleic acid-supplemented mice. Thus, these studies support the hypothesis that non-shivering thermogenesis is modifiable by the oxylipin pattern produced in response to a dietary intervention. These studies suggest this effect to primarily result from an antagonistic mechanism that reduces the production of individual ω -6-derived compounds rather than originating from an elevated abundance of ω -3-derived compounds acting as thermogenic effectors. This regulation may constitute one possible mechanism to explain the beneficial effect of dietary ω -3 supplementation on energy expenditure and cold tolerance (Kim et al. 2015, 2016). A possible relationship between dietary PUFA supplementation, changes in the endocannabinoid profile of adipose tissues, and the induction or activation of thermogenic adipocytes remains to be assessed.

7 Conclusion

Oxylipins and endocannabinoids represent two metabolite classes originating from the metabolic conversion of ω -3 and ω -6 PUFAs. These bioactive compounds affect a plethora of physiological processes constituting their role as essential regulators of metabolic control. Since only a few years, there is accumulating evidence for a novel role for these effectors as regulators of Ucp1-dependent non-shivering thermogenesis. Several individual compounds with the potential to affect brown and brite adipocytes have been identified (Fig. 2). These compounds appear to modulate thermogenic adipocytes on multiple levels by acting as (1) upstream effectors influencing non-shivering thermogenesis via a secondary mechanism, (2) direct effectors of adipocyte identity and function, or (3) sensitizers to enhance Ucp1-derived heat production in response to a thermogenic stimulus. Thus, they can act directly in adipose tissues or indirectly via central or peripheral circuitries. Accordingly, the production of thermogenic effector compounds is not restricted to local entities such as mature adipocytes, pre-adipocytes, or immune cells, but involves distant sites such as the central nervous system or the intestinal microbiota, which may in turn facilitate the delivery of effectors via the blood circulation. Their production and their biological effect are regulated in a complex manner affected

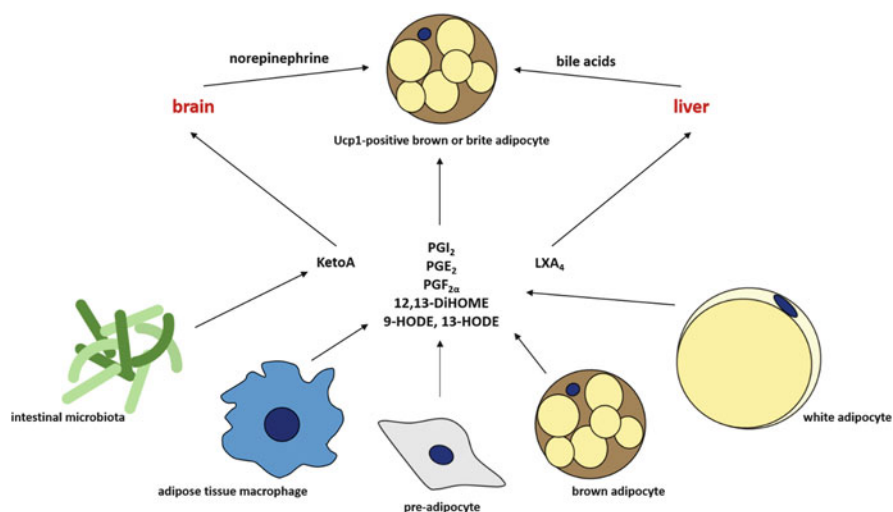


Fig. 2 Schematic summary of oxylipins affecting the recruitment or function of Ucp1-positive brown and brite adipocytes. The compounds PGI₂, PGE₂, PGF_{2α}, 12,13-DiHOME, 9-HODE, and 13-HODE likely act in autocrine or paracrine manner upon their local production in adipose tissues by mature adipocytes, pre-adipocytes, or immune cells. The compound LXA₄ produced in BAT and WAT is thought to affect hepatic bile acid production, which is in turn hypothesized to signal Ucp1-dependent thermogenesis. The microbial-derived compound KetoA stimulates gastrointestinal afferences to signal thermogenesis via sympathetic pathways. Endocannabinoids and endocannabinoid-like compounds (not depicted) may complement oxylipin-mediated effects on brown and brite adipocytes via direct or centrally mediated pathways.

by multiple variables such as the abundance of the precursor fatty acid, the enzymatic pathway involved, the responding cell type, and the implicated receptor and signaling pathway. Future studies may not only focus on the physiological potential of such compounds to affect parameters associated with Ucp1-dependent energy expenditure (such as adrenergically induced heat production, cold tolerance, or the regulation of body mass) but also on the manipulation of their production and signaling pathways. Nutritional interventions seem to represent a useful tool, although their effect is not restricted to the modulation of a single compound but may trigger the systemic generation of a profile that favors the abundance or activity of thermogenic adipocytes. Sophisticated analysis techniques with a high sensitivity will not only help to decipher effects of oxylipins and endocannabinoids on brown and brite adipocyte function in the future but also facilitate the identification of further compounds with thermogenic potential.

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ANNEXE 4:



Diet Supplementation in ω 3 Polyunsaturated Fatty Acid Favors an Anti-Inflammatory Basal Environment in Mouse Adipose Tissue

Cécilia Colson, Rayane A Ghandour, Océane Dufies, Samah Rekima, Agnès Loubat, Patrick Munro, Laurent Boyer, Didier F Pisani

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Article

Diet Supplementation in ω 3 Polyunsaturated Fatty Acid Favors an Anti-Inflammatory Basal Environment in Mouse Adipose Tissue

Cecilia Colson ¹, Rayane A. Ghandour ¹, Océane Dufies ², Samah Rekima ¹, Agnès Loubat ¹, Patrick Munro ² , Laurent Boyer ² and Didier F. Pisani ^{1,3,*} 

¹ Université Côte d'Azur, CNRS, Inserm, iBV, 06107 Nice, France; Cecilia.Colson@unice.fr (C.C.); ghandourrayane@hotmail.com (R.A.G.); Samah.Rekima@unice.fr (S.R.); Agnes.Loubat@unice.fr (A.L.)

² Université Côte d'Azur, Inserm, C3M, 06107 Nice, France; oceane.dufies@unice.fr (O.D.); Patrick.Munro@unice.fr (P.M.); Laurent.Boyer@unice.fr (L.B.)

³ Didier Pisani, Laboratoire de Physiomédecine Moléculaire—LP2M, Univ. Nice Sophia Antipolis, 28 Avenue de Valombrose, 06107 Nice CEDEX 2, France

* Correspondence: pisani@unice.fr; Tel.: +33-0493377037

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Abstract: Oxylipins are metabolized from dietary ω 3 and ω 6 polyunsaturated fatty acids and are involved in an inflammatory response. Adipose tissue inflammatory background is a key factor of metabolic disorders and it is accepted that dietary fatty acids, in terms of quality and quantity, modulate oxylipin synthesis in this tissue. Moreover, it has been reported that diet supplementation in ω 3 polyunsaturated fatty acids resolves some inflammatory situations. Thus, it is crucial to assess the influence of dietary polyunsaturated fatty acids on oxylipin synthesis and their impact on adipose tissue inflammation. To this end, mice fed an ω 6- or ω 3-enriched standard diet (ω 6/ ω 3 ratio of 30 and 3.75, respectively) were analyzed for inflammatory phenotype and adipose tissue oxylipin content. Diet enrichment with an ω 3 polyunsaturated fatty acid induced an increase in the oxylipins derived from ω 6 linoleic acid, ω 3 eicosapentaenoic, and ω 3 docosahexaenoic acids in brown and white adipose tissues. Among these, the level of pro-resolving mediator intermediates, as well as anti-inflammatory metabolites, were augmented. Concomitantly, expressions of M2 macrophage markers were increased without affecting inflammatory cytokine contents. In vitro, these metabolites did not activate macrophages but participated in macrophage polarization by inflammatory stimuli. In conclusion, we demonstrated that an ω 3-enriched diet, in non-obesogenic non-inflammatory conditions, induced synthesis of oxylipins which were involved in an anti-inflammatory response as well as enhancement of the M2 macrophage molecular signature, without affecting inflammatory cytokine secretion.

Keywords: oxylipins; brown adipose tissue; white adipose tissue; macrophages; inflammation

1. Introduction

ω 6 linoleic acid (LA), a precursor of dihomo- γ -linolenic acid (DGLA) and arachidonic acid (ARA), and ω 3 α -linolenic acid, a precursor of eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids are essential polyunsaturated fatty acids (PUFAs) only supplied by food. These PUFAs are required for healthy development from embryonic steps to adult life and are involved in a variety of biological processes, especially, in adipose tissue [1,2]. It is now well accepted that insufficient intakes of ω 3 PUFAs, as well as an excess of ω 6 PUFAs, correlate with various diseases; especially, metabolic diseases [3–5]. For example, ARA intake correlates positively with being overweight/obese, inflammatory diseases, and associated metabolic syndrome [6–10]. Indeed, ω 6 oxylipins (oxygenated

derivatives of PUFAs) are known to favor inflammatory responses [11], as well as to promote energy storage [12] and to inhibit energy expenditure [13,14]. The dietary $\omega 6/\omega 3$ PUFAs ratio is more important than the total amount of PUFA intake as it determines the level of synthesized $\omega 6$ -derived oxylipins. Indeed, $\omega 3$ PUFAs modulate $\omega 6$ -derived oxylipins synthesis [15]. Mechanistically this is characterized by (i) the capacity of $\omega 6$ and $\omega 3$ PUFAs to compete at the level of lipoxygenase (LOX) and cyclooxygenase (COX), their two major metabolization pathways and ii) the capacity of various $\omega 3$ PUFAs to inhibit these pathways.

The increase in the number of overweight or obese people has reached an epidemic stage in the 21st century. More than 2 billion adults are overweight (body mass index (BMI) > 25 kg/m²) and at least 600 million are clinically obese (BMI > 30 kg/m²). Obesity and being overweight are the consequences of a positive energy balance that leads to an increase in the mass of subcutaneous and visceral white adipose tissue. White adipocytes are storing energy under the form of triglycerides whereas brown adipocytes dissipate energy from triglycerides by producing heat (=thermogenesis). In addition, white and brown adipocytes are able to secrete molecules acting on their environment, and especially, on immune cells [16]. For example, white adipocytes secrete adipokines (e.g., adiponectin) and pro-inflammatory factors (e.g., PAI-1, MCP-1, or IL-6) which are able to recruit and activate macrophages [17]. Furthermore, it has also been shown that the white adipose tissue of obese subjects is characterized by low-grade inflammation that can lead to metabolic disorders such as insulin resistance [18]. This inflammation, characterized by an increase in inflammatory markers such as TNF α , PAI-1, or interleukins 1 and 6 (IL-1, IL-6), promotes the macrophage infiltration of adipose tissue and the polarization of macrophages of the alternative M2 type in classic pro-inflammatory M1 type [19].

The macrophages respond to environmental cues by acquiring specific functional phenotypes. Pro-inflammatory M1 macrophages are involved in the fight against many infections. They are activated by Toll-like receptor (TLR) ligands such as lipopolysaccharide and saturated fatty acids, but also by IFN γ and TNF α . They participate in the inflammatory environment by secreting many cytokines such as IL-1, IL-6, IL-12, IL-23, and TNF α , and by participating in the chemo-attraction of other immune cells [20]. M2 macrophages are more heterogeneous at functional and secretory levels. Considered as anti-inflammatory or inactive, they normally reside in tissues and are involved in tissue homeostasis by participating in the remodeling, repair, and activation of certain metabolic functions. They can be activated by cytokines such as IL-4, IL-10, and IL-13, but also by more specific signals from the tissue environment [21].

The accumulation of immune cells, especially that of macrophages, as well as their inflammatory phenotype, affect adipose tissue homeostasis and, more specifically, the recruitment and function of adipocytes in white and brown adipose tissues [16]. It has been shown that TNF α secreted by M1 macrophages inhibited adipocyte differentiation [22] and that IL-1 β blocked insulin signaling [23], thus favoring insulin-resistance. Recently, it has also been shown that IL-1 β and TNF α can affect the thermogenic function of brown adipocyte [24–26]. These inflammatory cytokines thus participate in the deregulation of tissue homeostasis by limiting its ability to dissipate an excessive supply of substrate in the form of heat. On the contrary, it was shown that M2 macrophages, via the secretion of factors such as IL-4 or IL-13 favored the formation of brown adipocytes and their activation [27,28]. In addition, immune cells can modulate insulin sensitivity and local secretion of catecholamines [29]. This secretion, that represents the preferential inducer of lipolysis and thermogenesis through the activation of the β -adrenergic pathway, appears to be crucial during prolonged exposure to cold or aging [28,30].

Similarly to adipokines, the oxygenated derivatives of $\omega 6$ PUFAs such as the $n-2$ series prostaglandins or the $n-4$ series leukotrienes, which are synthesized and secreted by adipocytes, participate in the inflammatory state of the tissue [31,32]. Furthermore, adipocytes are able to metabolize $\omega 3$ PUFAs, in the same way as $\omega 6$, to produce oxygenated anti-inflammatory derivatives such as $n-3$ series prostaglandins (PG), $n-5$ series leukotrienes (LT), as well as resolvins (Rv) and

protectins (PD) [32]. For example, the administration of ω 3 PUFAs to obese mice as well as resolvin D1 (RvD1), an oxygenated derivative of DHA, limits macrophage infiltration, favors their polarization toward the M2 phenotype, and rescues adipocyte metabolic dysfunction [33,34]. Thus, ω 6- and ω 3-derived oxylipins are able to modulate the inflammatory phenotype of immune cells, especially macrophages [11,35]. As dietary ω 6 and ω 3 PUFAs directly affect the quality and the quantity of oxylipins synthesized and secreted by the adipocytes, it is of high interest to characterize the impact of ω 3 PUFA diet supplementation on the inflammatory state of adipose tissue.

2. Materials and Methods

2.1. Reagents

Culture media and buffer solutions were purchased from Lonza (Ozyme, St-Quentin en Yvelines, France), fetal bovine serum (FBS) from Eurobio (Courtaboeuf, France), insulin and trypsin from InVitrogen (Cergy Pontoise, France). Oxylipins and inhibitors were purchased from Cayman (BertinPharma, Montigny le Bretonneux, France). Other culture reagents were from Sigma-Aldrich Chimie (Saint-Quentin Fallavier, France).

2.2. Animals and Diets

The experiments were conducted in accordance with the French and European regulations (Directive 2010/63/EU) for the care and use of research animals and were approved by national experimentation committees (MESR 01947.03). Ten-week-old C57BL/6J male mice from Janvier Laboratory (France) were maintained at thermoneutrality (28 ± 2 °C) and 12:12-h light-dark cycles, with ad libitum access to food and water to not hide any behavioral modification. Mice were fed for 12 weeks with isocaloric isoenergetic (3.2 kCal/g–13.5 kJ/g) ω 6- or ω 3-enriched diets (12% energy content as lipids). The diets were prepared by Harlan (WI, USA) from standard chow diets (reference number 2016) by the addition of specific fatty acid ethyl-esters from NuChekPrep (Elysian, MIN, USA). Detailed compositions are displayed in Table 1. Blood, interscapular brown adipose tissue (iBAT), epididymal (eWAT), and inguinal subcutaneous (scWAT) white adipose tissues were sampled and used for different analyses.

Table 1. Diet compositions.

	ω 6-Enriched Diet	ω 3-Enriched Diet
Protein [% by weight]		16
Carbohydrate [% by weight]		52
Fat [% by weight]		5
Saturated fatty acids (FAs) [% of total FAs]		12
Monounsaturated FAs [% of total FAs]	26	14
Polyunsaturated FAs [% of total FAs]	62	74
Linoleic acid [% by weight]		3
α -linolenic acid [% by weight]	0.1	0.64
EPA [% by weight]	-	0.08
DHA [% by weight]	-	0.08
ω 6/ ω 3 PUFA ratio	30	3.75

2.3. Cell Culture

THP-1, a human pro-monocytic cell line, was cultured in RPMI GlutaMax medium, supplemented with 10% FBS and 10 mM sodium pyruvate, at 37 °C and 5% CO₂. Differentiation in macrophages-like cells was induced by treatment with 20 nmol/L phorbol 12-myristate 13-acetate (PMA) for 72 h. Then, media were replaced and polarization was induced for 48 h either with lipopolysaccharides (LPS, 100 ng/mL) for M1 like-phenotype or with IL-4/IL-10 (10 ng/mL each) for M2 like-phenotype acquisition. Treatments with a LOX inhibitor (=carnosic acid (CA), 10 μ M), and/or with 9-HODE and 13-HODE (50 nmol/L + 50 nmol/L), were performed during the 48 h polarization step.

2.4. Oxylipin Quantification

For quantification of unesterified oxylipins, tissues were snap-frozen with liquid nitrogen immediately after retrieval and stored at -80°C . Extraction and analysis by mass spectrometry were performed at METATOUL platform (MetaboHUB, INSERM UMR 1048, I2MC, Toulouse, France) as previously described [13,36].

2.5. Cytokine Quantification

For blood analysis, plasmas were diluted twice and analysis following manufacturer's instructions using the mouse V-PLEX Proinflammatory Panel 1 Kit (Meso Scale Discovery, # K15048D) on a QuickPlex SQ 120 apparatus (Meso Scale Discovery).

For tissue analysis, proteins were extracted from frozen organs using an ULTRA TURRAX T25 (Ika, Germany) and lysis buffer (25 mM Tris-Cl (pH 7.4), 100 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% Nonidet P40 and protease inhibitors (Roche Diagnostics, Meylan, France)). Protein concentration was evaluated by BCA assay (Sigma-Aldrich Chimie, Saint-Quentin Fallavier, France). 10 μg proteins were used to evaluate cytokine concentration using the same kit and apparatus as those used for blood cytokine analysis.

2.6. Histology

Freshly sampled tissues were fixed in 4% paraformaldehyde overnight at RT and then paraffin-embedded. Embedded tissues were cut into 5- μm sections and dried overnight at 37°C . For immunohistochemistry, sections were then deparaffinized in xylene, rehydrated using alcohol, and washed in phosphate-buffered saline (PBS).

For histology analysis, sections were stained with hematoxylin-eosin and mounted in Mowiol.

For immunohistochemistry analysis, antigen unmasking was performed in boiling citrate buffer (10 mM, pH 6.0) for 6 minutes. Sections were then permeabilized in PBS with 0.2% Triton X-100 at room temperature for 20 minutes and blocked in the same buffer containing 3% BSA for 30 min. Sections were co-incubated with rat anti-F4/80 antibody (Biorad, clone Cl:A3-1, dilution 1:100) and rabbit anti-Arginase-1 (ThermoFisher Scientific, #PA5-29645, dilution 1:100) overnight at 4°C .

Following a 30-min incubation with biotinylated anti-rat and TRITC-coupled anti-rabbit secondary antibodies, the sections were incubated for another 30 min at room temperature with avidin-biotin complex (Vector Lab, VECTASTAIN ABC Kit, PK-4000), and were then labeled with 3,3'-diaminobenzidine solution (Vector Lab, DAB, SK-4100). Nuclear staining was performed with DAPI and sections were mounted in Mowiol.

Visualization was performed with an Axiovert microscope. Pictures were captured using AxioVision software (Carl Zeiss, Jena, Germany).

2.7. Isolation and Analysis of RNA

Procedures follow MIQE recommendations [37]. Total RNA was extracted using a TRI-Reagent kit (Euromedex, Souffelweyersheim, France) according to the manufacturer's instructions. For RNA isolation from organs, tissues were homogenized in TRI-Reagent using a dispersing instrument (ULTRA TURRAX T25). A reverse transcription-polymerase chain reaction (RT-PCR) was performed using M-MLV-RT (Promega). SYBR qPCR premix Ex TaqII from Takara (Ozyme, France) was used for quantitative PCR (qPCR), and assays were run on a StepOne Plus ABI real-time PCR machine (PerkinElmer Life and Analytical Sciences, Boston). The expression of selected genes was normalized to that of the TATA-box binding protein (TBP) and 36B4 housekeeping genes and then quantified using the comparative- ΔCt method. Primer sequences are available upon request.

2.8. Statistical Analysis

Data were expressed as mean values \pm standard error of the mean (SEM). Data were analyzed using InStat software (GraphPad Software) by one-way ANOVA followed by a Mann-Whitney (for in vivo experiments) or a Student-Newman-Keuls (for in vitro experiments) post-test to assess statistical differences between experimental groups. Differences were considered statistically significant with $p < 0.01$.

3. Results

3.1. Impact of $\omega 3$ PUFA Supplementation on General Parameters of Mice

3.1.1. General Metabolic Parameters

Ten-week-old male mice were fed for 12 weeks with an isocaloric isoenergetic standard diet enriched in $\omega 6$ PUFAs ($\omega 6$ -enriched diet, $\omega 6/\omega 3 = 30$), or supplemented with $\omega 3$ PUFAs ($\omega 3$ -enriched diet, $\omega 6/\omega 3 = 3.7$), see Table 1. Mice were housed at 28 °C, near thermoneutrality, in order to limit energy expenditure due to thermogenic metabolism and to avoid any effect of this activity on inflammatory response, as demonstrated previously [38].

Mice body weight, see Figure 1a, as well as food intake ($\omega 6$ -enriched diet, 4.49 g/day; $\omega 3$ -enriched diet, 4.46 g/day per mouse) were similar between the two groups. Epididymal white adipose tissue mean weight, see Figure 1b, and fed glycaemia, see Figure 1c, were not different after 12 weeks of the diets. Altogether, these results indicated that the $\omega 6/\omega 3$ ratio of a standard diet, equilibrated in carbohydrate, protein, and fat quantities (respectively, 20.1%, 65.4%, and 14.5% of energy supply), did not modify general metabolic parameters of mice.

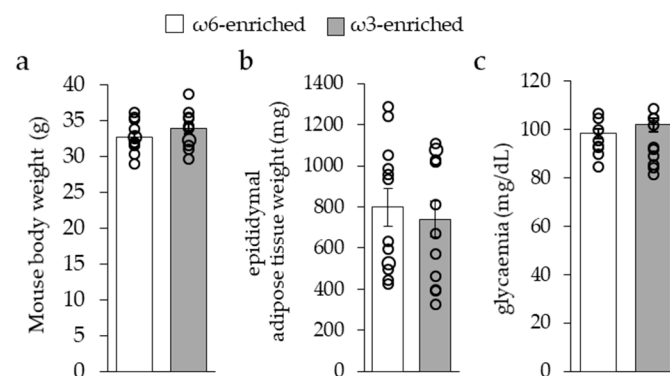


Figure 1. Mice general metabolic parameters. (a) Mouse body weight, (b) epididymal white adipose tissue weight, and (c) blood glycaemia evaluated after 12 weeks of $\omega 6$ - or $\omega 3$ -enriched diet. Results are displayed as independent mouse values (dots) and mean \pm SEM (histograms). $n = 12$.

3.1.2. Plasmatic Inflammatory Phenotype

To characterize the systemic inflammatory effect of a PUFA-enriched diet, we evaluated the blood circulating level of a panel of cytokines, see Figure 2.

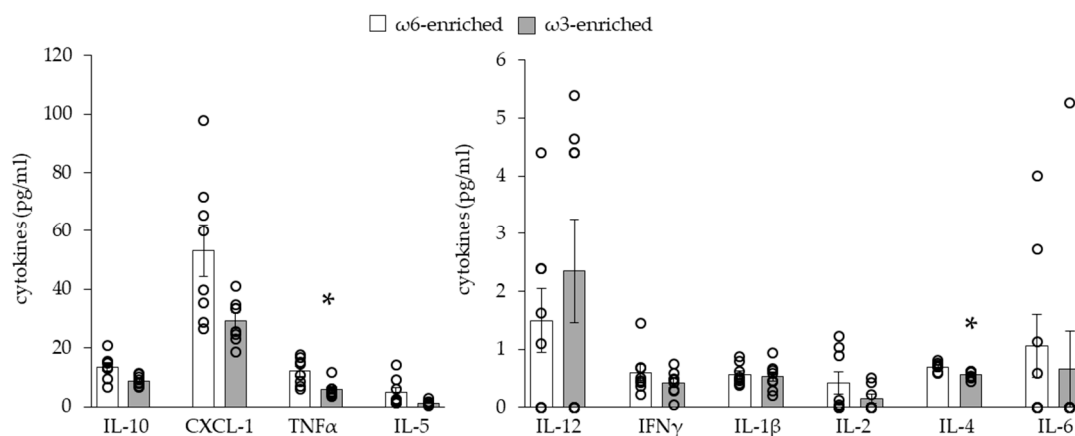


Figure 2. Analysis of blood cytokines. Results are displayed as independent mouse values (dots) and mean \pm SEM (histograms). $n = 8$. *, $p < 0.01$.

As expected, the level of most of the pro-inflammatory and anti-inflammatory cytokines was unchanged between the two groups of mice. Only TNF α (pro-inflammatory cytokine) and IL-4 (anti-inflammatory cytokine) levels slightly but significantly decreased in mice fed an ω 3-enriched diet.

3.1.3. Impact of ω 3 PUFA Supplementation on Adipose Tissue Oxylin Content

To investigate the modification induced by the two different diets within adipose tissues, we quantified the levels of 33 PUFA-metabolites within iBAT, see Figure 3, and scWAT, see Figure 4, of mice. These oxylinins were analyzed by groups following their PUFA origin, see Figures 3a and 4a, or separately, see Figures 3b and 4b. In the iBAT, ω 3 PUFA supplementation led to a significant increase of the oxylinins deriving from ω 3 PUFAs EPA (PGE3, LTB5, 18-HEPE) and DHA (RvD2, RvD1, MaR1, PDx, 17-HDoHE, 14-HDoHE), but did not affect ω 6-derived metabolites (6kPGF1a, TxB2, 11B-PGF2a, PGF2a, PGE2, PGD2, 8isoPGA2, 15dPGJ2, LxB4, LxA4, LTB4, 5,6-DiHETE, 15-HETE, 8-HETE, 12-HETE, 5-HETE, 5oxoETE, 14,15-EET, 11,12-EET, 8,9-EET, 5,6-EET derived from ARA; 13-HODE, 9-HODE derived from LA), see Figure 3a.

In scWAT, while similar results were found for ω 3 PUFA-derived and ARA-derived oxylinins, LA-derived metabolites were highly increased, as shown in Figure 4a.

LA and ω 3-PUFA derived oxylinins are considered as anti-inflammatory and pro-resolving mediators, especially through the modulation of macrophage function. Along with these oxylinins, we have found that 14- and 17-HDoHEs and 18-HEPE levels were increased in iBAT and scWAT of mice fed the ω 3-enriched diet, and 9- and 13-HODEs were increased only in scWAT, see Figures 3b and 4b. 14- and 17-HDoHE are metabolized in pro-resolving mediators as RvD1, RvD2, Mar1, PDx, and PD1, while 18-HEPE leads to RvE1 synthesis. It is interesting to note that these final metabolites were barely (PDx) or not detected within the tissue, see Figures 3b and 4b.

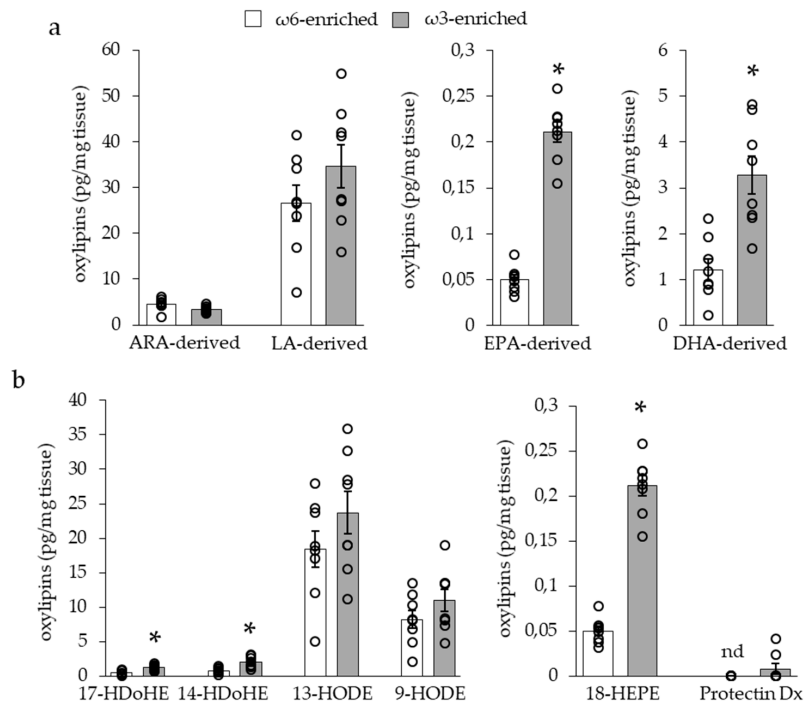


Figure 3. Quantities of oxylipins derived from dietary polyunsaturated fatty acids (PUFAs) in interscapular brown adipose tissue (iBAT). (a) Quantities of oxylipins derived from arachidonic acid (ARA) and linoleic acid (LA) ω 6 PUFAs or eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) ω 3 PUFAs. (b) Quantities of oxylipins considered as anti-inflammatory or pro-resolving mediator intermediates. Results are displayed as independent mouse values (dots) and mean \pm SEM (histograms). $n = 8$. *, $p < 0.01$.

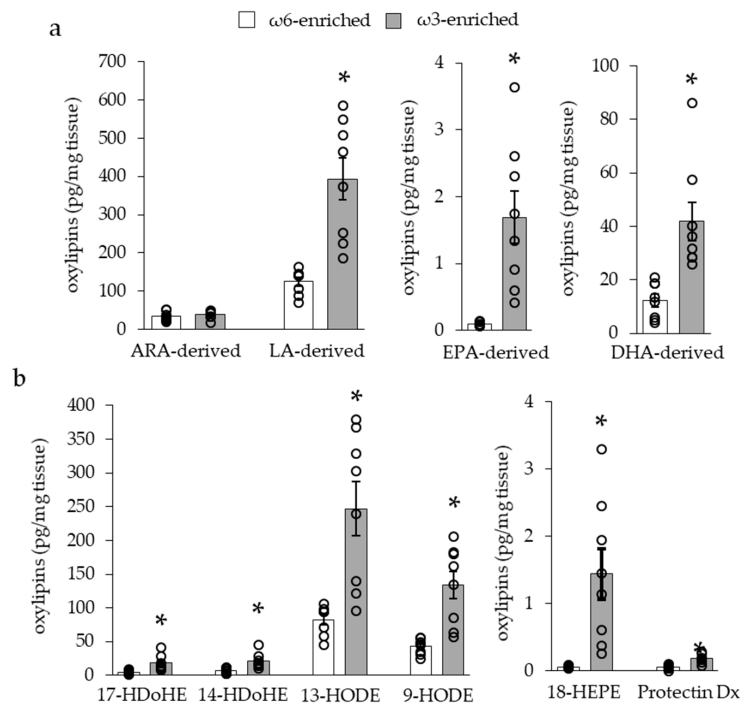


Figure 4. Quantities of oxylipins derived from dietary PUFAs in inguinal subcutaneous white adipose tissues (scWAT). (a) Quantities of oxylipins derived from ARA and LA ω 6 PUFAs or EPA and DHA ω 3 PUFAs. (b) Quantities of oxylipins considered as anti-inflammatory or pro-resolving mediator intermediates. Results are displayed as independent mouse values (dots) and mean \pm SEM (histograms). $n = 8$. *, $p < 0.01$.

3.2. Effect on Inflammatory Phenotype of Adipose Tissue

3.2.1. Histology and Cytokine Content

The histological analysis of iBAT and scWAT, see Figure 5a, revealed neither cell infiltration nor crown structure that were typical of an adipose tissue inflammatory response in both groups of mice.

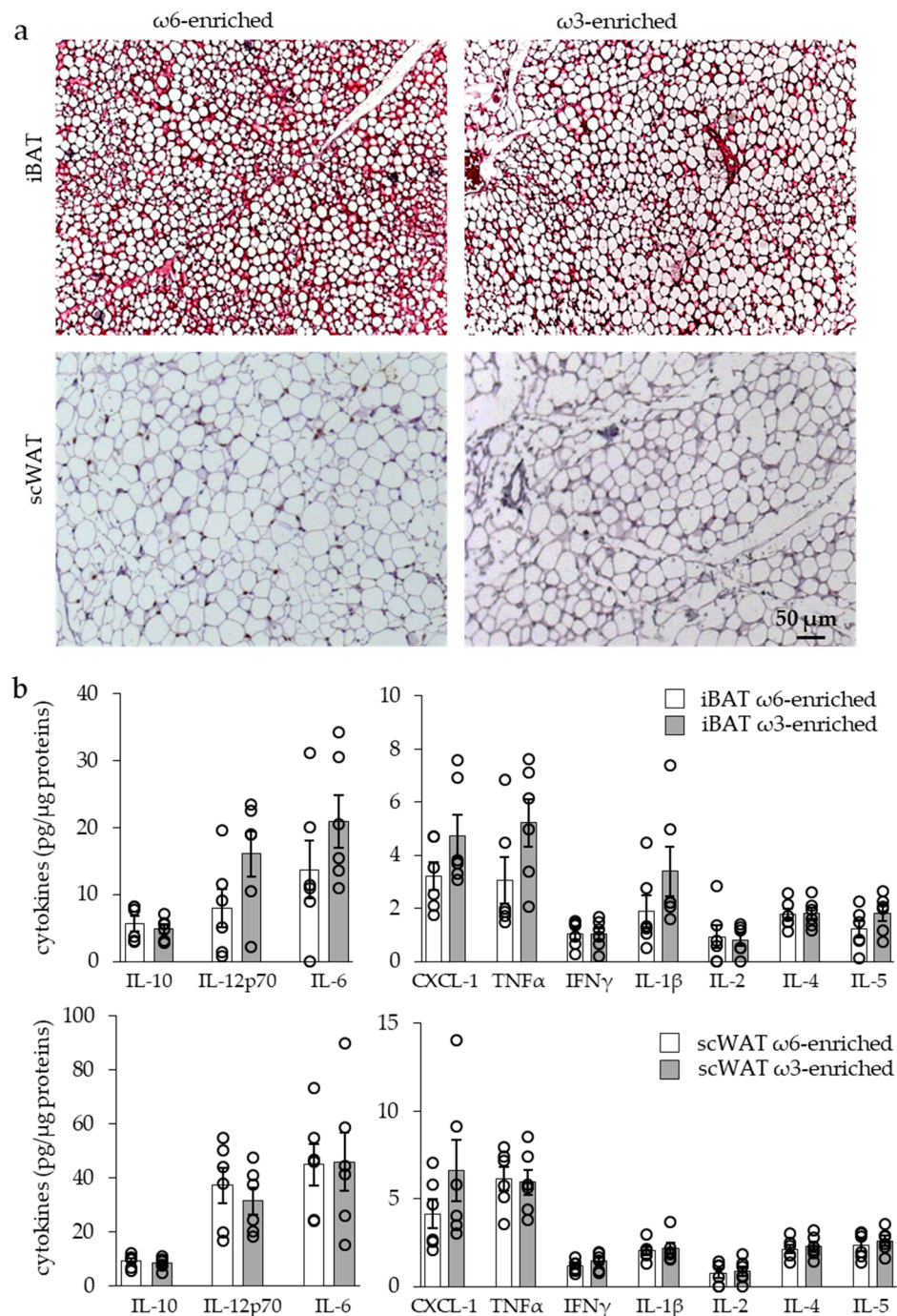


Figure 5. Inflammatory profile of iBAT and scWAT of mice submitted to ω6- or ω3-enriched diet. (a) Hematoxylin and eosin staining of tissue sections. (b) Analysis of adipose tissue cytokine levels. Results are displayed as independent mouse values (dots) and mean ± SEM (histograms). $n = 6$. *, $p < 0.01$.

In the same way, analysis of the iBAT and scWAT cytokine contents showed similar levels of both pro- and anti-inflammatory cytokines in the two groups of mice, as shown in Figure 5b.

3.2.2. Expression of Inflammatory Markers

As we did not find any modulation of cytokine levels, we analyzed marker expression of specialized macrophages to evaluate the inflammatory background of the tissue, see Figure 6.

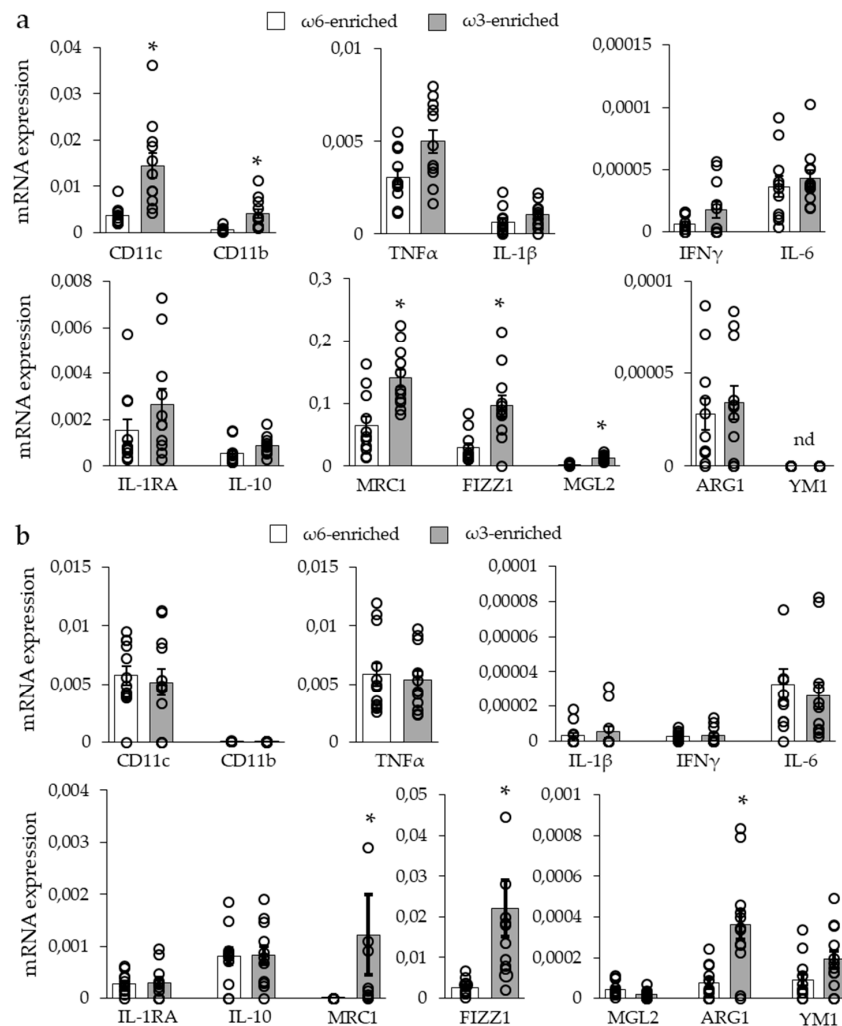


Figure 6. Macrophage marker expression in adipose tissue of mice submitted to ω 6- or ω 3-enriched diet. mRNA level analysis of general (CD11c, CD11b), M1 (TNF α , IL-1 β , IFN γ , IL-6) and M2 (IL-1RA, IL-10, MRC1, FIZZ1, MGL2, ARG1, YM1) macrophage markers in (a) iBAT and (b) scWAT. Histograms display mean \pm SEM. $n = 12$. *, $p < 0.01$.

The analysis of macrophage markers in iBAT derived from the ω 3-enriched diet group, see Figure 6a, revealed an increase in CD11b (or ITGAM, integrin α M) and CD11c (or ITGAX, integrin α X) mRNA expression, concomitantly to an increase in major M2 macrophage markers, namely MRC1 (mannose receptor 1), FIZZ1 (found in inflammatory zone 1 or RELM α), and MGL2 (macrophage galactose N-acetyl-galactosamine specific lectin 2). No change was found for other M2 macrophage markers or for M1 macrophage markers. To note, ARG1 (arginase 1) and Ym1 (chitinase 3-like 3) were either barely detected or undetected in this tissue.

The analysis of the scWAT, see Figure 6b, from ω 3-supplemented mice, showed an increased the expression of the M2 macrophage markers MRC1 and FIZZ1 (not MGL2), but no increase of CD11c (CD11b was undetected). In contrast to iBAT, our data revealed an increase of ARG1 mRNA expression

and the induction of YM1 mRNA expression. Finally, as for iBAT, no change was found for mRNA expression of M1 macrophage markers.

Altogether, these results demonstrated that an ω 3-enriched diet led to a general increase in M2 anti-inflammatory macrophage marker expression without modification in M1 pro-inflammatory markers. This was correlated and perhaps due to the increased amount of substrates for pro-resolving mediator synthesis, as well as an increased quantity of M2 polarizing oxylipins.

3.3. Effect of Potential Anti-Inflammatory Oxylipins Modified in an ω 3-Enriched Diet on THP1 Monocyte Cells

The oxylipins 9- and 13-HODEs (LA-derived oxylipins metabolized by LOX) are not known to be precursors of pro-resolving mediators but display high contents in iBAT, see Figure 3b, and scWAT, see Figure 4b, and are strongly increased in scWAT after the implementation of an ω 3-enriched diet. In order to investigate the role of 9- and 13-HODEs on macrophage polarization, we used THP-1 macrophage cell lines activated in pro-inflammatory M1 (LPS 100 ng/mL, Figure 7a) or anti-inflammatory M2-like phenotype (IL4 + IL-10 10 ng/mL each, Figure 7b). THP-1 cells were treated with 9- and 13-HODEs (9/13-HODEs, 50 nmol/L each) or with carnosic acid (CA, 10 μ M), a lipoxygenase inhibitor [39], or a combination of both.

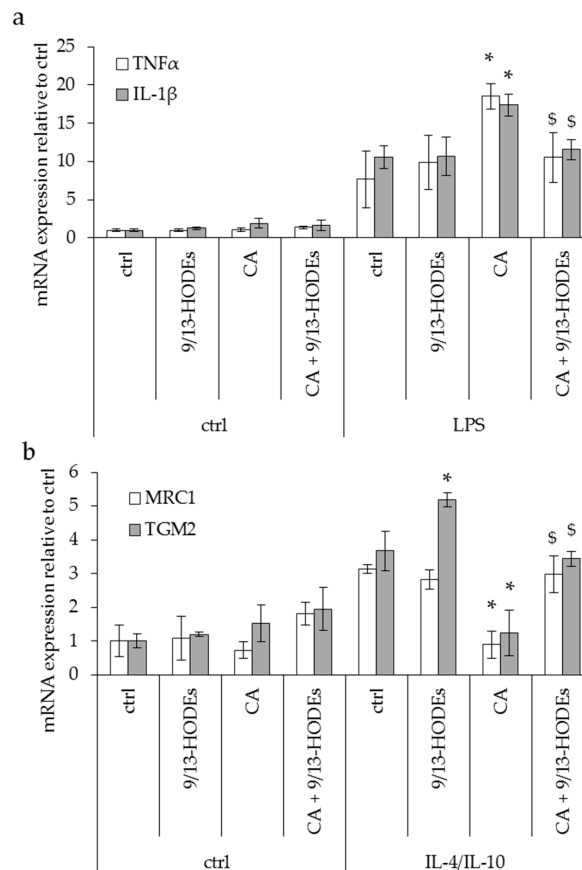


Figure 7. Macrophage marker expression in THP-1 cells under 9- and 13-HODE treatment. mRNA level analysis by RT-qPCR of M1 (TNF α , IL-1 β) and M2 (MRC1, TGM2) macrophage markers in control, lipopolysaccharides (LPS) (upper panel) or IL-4/IL-10 (lower panel) treated THP-1 macrophages. Cells were co-treated for 48 h with carnosic acid (CA, 10 μ M) and or 9- and 13-HODEs. (a) CA treatment induced opposite effects in M1- and M2-like macrophages as it increased inflammatory markers in THP-1 M1-like macrophages, and (b) decreased M2-like macrophages' markers. Histograms display mean \pm SEM. $n = 3$. *, $p < 0.01$ vs. ctrl and \$, $p < 0.01$ vs. CA.

None of the treatments modulated non-polarized THP-1, see Figure 7. Treatment with 9/13-HODEs alone showed no effect on macrophages' M1-like phenotype but increased TGM2 expression on M2-like macrophages. Remarkably, CA treatment induced opposite effects in M1- and M2-like macrophages as it increased inflammatory markers in THP-1 M1-like macrophages, see Figure 7a, and decreased M2-like macrophages' markers, see Figure 7b. Interestingly, 9/13-HODEs supplementation reversed CA effects, see Figure 7.

4. Discussion

Dietary fats are the source of essential PUFAs that are required for fetal and newborn development and trigger a variety of biological responses in adults, especially, in adipose tissue. New dietary recommendations warn against the insufficient intake of $\omega 3$ PUFAs and the excess of $\omega 6$ PUFAs which correlate with various disease developments [3,4]. In the first year of life, a high dietary $\omega 6/\omega 3$ ratio is positively associated with adiposity of infants [40–42]. In the same way, in adults, a high $\omega 6/\omega 3$ ratio can correlate to an increase of fat mass and the development of metabolic complications [6–10].

Conversely, it has been described that a low $\omega 6/\omega 3$ ratio seems to be correlated with metabolic disorder protection in different populations [43]. On a metabolic point of view, diets exhibiting a high $\omega 6/\omega 3$ ratio allow a higher ARA bioavailability for the synthesis of $\omega 6$ -derived eicosanoids due to an insufficient compensatory effect of EPA and DHA [15]. Indeed, both $\omega 6$ and $\omega 3$ PUFAs are metabolized using the same enzymatic pathways. First, LA and LNA are modified by common Δ -desaturases and elongases [44]; then, their metabolites, i.e., ARA, DGLA, EPA, and DHA, are metabolized in oxygenated derivatives also using common pathways involving cyclooxygenases, lipoxygenases, and CYP450 enzymatic reactions. Here, we provided evidence that, compared to a high $\omega 6/\omega 3$ PUFA ratio, an equilibrated ratio of four allows the synthesis of LA and EPA/DHA oxylipins instead of ARA oxylipins. As LA and LNA use a common pathway (Δ -desaturase) to be transformed, respectively, into DGLA/ARA and EPA/DHA, we hypothesize that LNA supplementation could limit LA desaturation and thus increase LA bioavailability and metabolization in oxylipins through the LOX pathways. Thus, these competitive phenomena, in addition to dietary intake, determine PUFA availability in oxylipins synthesis and, in turn, their various metabolic effects, especially for inflammatory responses [45].

It has already been described in rodents that an increase of white adipose tissue mass can be related to an $\omega 6$ PUFA-enriched high-fat diet and can be prevented by $\omega 3$ PUFA supplementation [12, 46]. It is suggested that this could only be due to a specific subset of $\omega 3$ PUFA such as EPA [47]. Moreover, eicosanoids derived from $\omega 6$ PUFA inhibit adipocyte thermogenic activity both in vitro and in vivo [11,13,48]. We and others demonstrated previously, using the same nutritional approach as in the present work, that an $\omega 3$ PUFA diet supplementation improved the thermogenic adipocyte function by promoting a more oxidative phenotype in response to β -adrenergic stimulation [14,49]. In the present study, $\omega 3$ PUFA supplementation does not induce any change in body mass, glycaemia, or white and brown adipose tissue morphologies since the mice were fed diets with normal fat content and did not receive any β -adrenergic challenge.

Most studies concerning $\omega 3$ PUFA supplementations were carried out in a context of obesity (high-fat diet) or infection (LPS treatment) and demonstrate a positive effect of $\omega 3$ PUFA supplementation on the analyzed parameters [35]. Nevertheless, other studies demonstrate the inability of $\omega 3$ PUFAs to modulate inflammation after LPS treatment [50] or in obese mice [51,52]. These discrepancies are essentially due to the differences in the experimental approaches (diet composition, mouse strain, challenge . . .) and in the analyzed parameters (cytokine concentration, mRNA expression, histology . . .). In humans, several experimental approaches have tried to link an $\omega 3$ PUFA intake to inflammatory response, again with inconsistent conclusions. For example, a one-year dietary supplementation in $\omega 3$ PUFA does not modify the circulating cytokine levels in healthy volunteers [53]. Conversely, other studies show a decrease of blood inflammatory markers after $\omega 3$ PUFA supplementation [54,55]. It is important to note that a plasma inflammatory mediator profile seems to be less representative compared to the one of adipose tissue [56]. The same discrepancy

is found for studies analyzing adipose tissue inflammation. Although one human trial (4g ω 3 PUFAs/day; 12 weeks) on insulin-resistant adults demonstrates a decrease in the crown-like structure number [57], corresponding to phagocytic activity of macrophage on adipocyte, another trial on the same type of patients (4.2g ω 3 PUFAs/day; 6 months) demonstrates no effect of ω 3 PUFA supplementation on the same parameter [58]. Moreover, a recent paper establishes that the oxylipin profile in rat adipose tissue after dietary ω 3 PUFA supplementation (ratio ω 6/ ω 3 of 0.6) is dependent of (i) the kind of ω 3 PUFA used, (ii) the kind of adipose tissue analyzed, and (iii) the sex [59].

In view of these heterogeneities, we decided to analyze the effect of PUFA intake in normal physiological conditions (thermoneutrality, no β -adrenergic challenge) using an isocaloric, isoenergetic standard diet supplemented with ethyl esters of fatty acids (instead of classic oil supplementation) and various technical approaches to characterize the inflammatory profile. With this strategy, we characterize fatty acid metabolism within subcutaneous and brown adipose tissues and the related inflammatory phenotype. Our results linking ω 3 PUFA supplementation and M2 macrophage are in line with other studies, such as a recent one demonstrating that (i) treatment of human adipose tissue explants with ω 3 PUFAs lead to an anti-inflammatory phenotype characterized by a decrease of M1 marker expression, and (ii) treatment of THP-1 cells increased expression of M2 markers [60]. In the same way, DHA supplementation in a high-fat diet context promotes mRNA expression of M2 markers within white adipose tissue without affecting the total macrophage number [61]. In this study, the authors describe the same effect for RvD1, DHA metabolites, and conclude that DHA leads to an anti-inflammatory phenotype via RvD1 synthesis. Unfortunately, they never quantify RvD1 production in vivo and thus do not link DHA supplementation to RvD1 synthesis [61]. In our study, we have not been able to detect resolvins but only their substrates. We assume that without a specific inflammatory signal, intermediates of pro-resolving mediators are synthesized but not metabolized. Indeed, these mediators are involved in the resolution of inflammation and appeared late in the process as they are not required before, differently to prostaglandins and leukotrienes which appear early [62].

In our study, we measure a defined set of oxylipins. Even if this panel includes oxylipins deriving from all pathways and PUFAs, we cannot exclude that unmeasured oxylipins triggered the anti-inflammatory effect of ω 3 PUFA supplementation found in our model. In this way, epoxide and diol metabolites derived from CYP epoxygenase/soluble epoxide hydrolase activity [63], as well as endocannabinoids, are known and interesting potential mediators of the inflammatory effect of PUFA [64]. In addition, the esterification of oxylipins, especially of eicosanoids, was described as an active and major mechanism in various cell biological responses including inflammation [65]. These esterified oxylipins can represent the majority of cell oxylipins and can be hydrolyzed from the membrane under specific stimuli [66]. In this way, it could be interesting to quantify all oxylipins (unesterified and esterified) in adipose tissue under ω 3 PUFA diet supplementation and to evaluate their hydrolysis under inflammatory conditions. Nevertheless, our unexhaustive analysis allowed a correlation between the synthesis of several oxylipins and the expression of M2 macrophage markers. We propose that 9- and 13-HODEs could drive this effect. In our in vitro results on the THP-1 cell line, we demonstrate that 9- and 13-HODEs are not enough to directly drive the polarization of THP-1 macrophage but are required to maintain the phenotype. Indeed, their supplementation restores control level expression of M1-like and M2-like markers after CA treatment. Moreover, 9- and 13-HODEs seem to play a role in the anti-inflammatory effects since they are able to increase M2 markers such as TGM2. These results are consistent with some studies describing 9- and 13-HODEs as known mediators of macrophage polarization [67] in a PPAR γ -dependent manner [68]. Of course, other oxylipins could be involved in the anti-inflammatory environment found in our mice. For example, the study of Fat-1 mouse, which is able to synthesize ω 3 PUFAs itself, displays a lowered inflammatory environment induced by obesity, correlatively to 17-HDoHE synthesis [69]. In addition to oxylipins involvement, we cannot exclude a direct action of ω 3 PUFAs on the membrane receptor. Indeed, it is shown that DHA is able to directly activate, via GPR120, an anti-inflammatory response driven by macrophage within adipose tissue [33]. This activity could be linked to the recent characterization of the DHA

inhibitory effect on NLRP3 inflammasome activity, an effect triggered by GPR40/GPR120 pathways and leading to a decreased production of mature IL-1 β [70]. As NLRP3 is activated essentially in response to an infectious environment, we do not correlate ω 3 PUFA supplementation with a decrease in IL-1 β production in our physiological context.

It is interesting to note that the ω 3 PUFA intake finely drives the kind of oxylipins synthesized. A recent study analyzed the effect of an ω 3 PUFA dietary supplementation of an already equilibrated diet (ratio ω 6/ ω 3 = 6.7) to reach an ω 6/ ω 3 ratio of 0.8. Thus, differently to our situation, LA and LNA are already desaturated equivalently, and the increase in ω 3 PUFA intake leads to a decrease of LA-derived oxylipins (9/13-HODEs) in favor of EPA and DHA derived oxylipins in the brain. Moreover, this “over”-supplementation ameliorates against an inflammatory response [71].

5. Conclusions

Previous studies have demonstrated the positive effect of ω 3 PUFA intake to counteract the adverse consequences of a high-fat diet or inflammatory situation. Herein, our study was conducted in non-obesogenic non-inflammatory conditions and also showed a beneficial influence of ω 3 PUFA dietary supplementation on the adipose tissue inflammatory phenotype. Moreover, while ω 3 PUFA metabolites have been involved in this effect, we additionally highlighted the unsuspected role of LA-derived metabolites. Finally, this already assumed beneficial outcome of ω 3 PUFA supplementation is in line with a human situation where a high ω 6/ ω 3 ratio is correlated with the development of inflammatory diseases in metabolic tissue.

Author Contributions: The author(s) have made the following declarations about their contributions: Conceived and designed the experiments: L.B., D.F.P. Performed the experiments: C.C., R.A.G., O.D., S.R., A.L., D.F.P. Analyzed the data: C.C., L.B., D.F.P. Wrote the manuscript: C.C., L.B., D.F.P.

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