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Impact of inflammation on the metabolic changes associated with obesity : dialogues between adipose tissue and the intestine

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► **To cite this version:**

Maria Pini. Impact of inflammation on the metabolic changes associated with obesity : dialogues between adipose tissue and the intestine. Tissues and Organs [q-bio.TO]. École pratique des hautes études - EPHE PARIS, 2015. English. NNT : 2015EPHE3030 . tel-02918185

HAL Id: tel-02918185

<https://theses.hal.science/tel-02918185>

Submitted on 20 Aug 2020

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École Pratique
des Hautes Études

**THESE DE DOCTORAT
DE L'ÉCOLE PRATIQUE DES HAUTES ETUDES**

Mention
Systemes Intégrés, Environnement et Biodiversité (SIEB)

Présentée par
Maria Pini

Pour obtenir le grade de
DOCTEUR DE L'EPHE

Sujet de la thèse :

**Impact de l'inflammation sur les altérations métaboliques
associées à l'obésité:
dialogues entre le tissu adipeux et l'intestin**

Soutenue publiquement le 23 juin 2015

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ACKNOWLEDGEMENTS

I address my first thanks to Professor Bernard Lacour for giving me the honor to accept to be the chair of my thesis jury, as well as Pr. Jocelyne Magre and Dr. Jean-Philippe Bastard for their availability and critical review of the manuscript. I also thank Prof. Helene Roche for having agreed to participate to the critical examination of this thesis.

I would like to thank especially Dr. Michèle Guerre-Millo, the director of the exposed projects, for having shared with me her critical approach toward qualitative scientific research and her endless love for science. I thank her very much for the confidence she granted me, her support and her mentorship over these years of scientific work together. Thanks to her I have learned also some good French expressions, that I will remember with a smile, now that I know their meaning, like “tout c’est passé comme sur des roulettes” after one of our experiments. I deeply thank also my EPHE thesis director Dr. Sophie Thenet for her kindness, availability and assistance during all the critical steps that brought me to the end of this unique itinerary.

I am sincerely grateful to Pr. Karine Clément, Director of the Institute of Cardiometabolism and Nutrition (ICAN), for allowing me to do my EPHE PhD in her team UMR S 1166, assisting the realization of this work with the necessary technical and financial supports and giving me the constant opportunity to learn from her team research interests. I extend my sincere thanks to those who contribute to the achievement of the different projects, contributing to this PhD work, making it primarily a team work. In particular I would like to thank Dr. Sébastien Andre and his PhD student Sothea Touch for their expertise in the field of immunology. A big thank to Jean-Charles Lafarge, the former PhD student of Dr. Michèle Guerre-Millo, with which I have worked on my first project in the team of K. Clément, the cathepsin S study. I thank him for his encouragement, his great sense of humor and even more for his English level, all of which helped me to pass unscathed through the transition from USA to France. A special thank to Dr. Christine Rouault for her availability, her listening and technical assistance. I thank also all the Nutromique members, past and present, in particular Dr. Daniele Lacasa for her discreet guidance and the discussions about art.

Many thanks to our collaborators from the Institute National Supérieur AgroSup in Dijon, Dr. Helene Poirier and Dr. Isabelle Niot, for their help in the study with conjugated linoleic acid. For the same study, I thank also Pr. Nathalie Delzenne and her former PhD student Celine Druart from Louvain University for their warm reception in Bruxelles and their technical competence in analyzing fatty acids.

I also want to express gratitude to Dr. Hana Koutnikova-Rousselin, Dr. Peggy Garault, Dr. Timothy Swartz, Dr. Aurélie Coutillard and Dr. Ludovic Le Chat for the interesting discussions during the preparation and finalization of Study II.

I finally want to warmly thank all the Equipe 6 members (past and present), in particular the ones working in the Centre de Recherche des Cordeliers (CRC), for sharing with me good time, but also the not so good one. A special thought to Sophie Reggio, who showed me how we can face small and big

issues in research, as in life, with the right distance. At the end, I thank all my friends near and far for their presence, support and good humor in my daily life. Last, but not least, a big thank to my Italian family and my “American one” for the unconditional love, support and guidance during the various challenges of my life, including this three year long journey.

**It's Not How Fat You Are, It's What You
Do with It that Counts.**

by Samuel Virtue and Antonio Vidal-Puig

RÉSUMÉ FRANÇAIS

A. Contexte du projet

Mon projet de thèse, intitulé « **Impact de l'inflammation sur les altérations métaboliques associées à l'obésité: dialogues entre le tissu adipeux et l'intestin** » a été effectué dans le laboratoire du Pr Karine Clément (INSERM UMRS 1166 Nutriomics Eq 6) sous la direction du Dr Michèle Guerre-Millo (INSERM) et du Dr Sophie Thenet (EPHE).

Ce projet s'inscrit dans le domaine des maladies métaboliques avec changement de masse grasse telles que l'obésité et les syndromes lipoathrophiques. Bien qu'opposées en terme de modification de la masse adipeuse, ces deux pathologies ont des co-morbidités communes, dont l'insulino-résistance et le diabète de type II. Il est maintenant reconnu que le tissu adipeux est au cœur des mécanismes impliqués dans ces co-morbidités, étant à la fois cible et acteur des dérégulations métaboliques. Par ailleurs, les altérations du microbiote et de la barrière intestinale, longtemps sous-estimées, sont actuellement sur le devant de la scène pour leur participation au développement des maladies métaboliques.

1. Les altérations du tissu adipeux dans les maladies métaboliques

Dans les pathologies chroniques s'accompagnant de modification de la masse adipeuse, la physiologie du tissu adipeux est profondément altérée. La principale fonction du tissu adipeux est de stocker l'énergie de l'organisme sous forme de triglycérides dans des cellules hautement spécialisées, les adipocytes. Les substrats précurseurs sont le glucose pour la voie de la lipogenèse *de novo* et les lipides circulants qui subissent une hydrolyse et une ré-estérification dans les adipocytes. Ces cellules possèdent également l'équipement enzymatique nécessaire pour permettre, en situation de déficit énergétique, la libération d'acides gras *via* le processus de « lipolyse ». Le stockage des lipides dans les adipocytes et leur mobilisation sont deux voies métaboliques finement régulées par l'état nutritionnel, avec un rôle primordial de l'insuline, qui stimule le stockage et inhibe la lipolyse. Si la capacité de stockage des adipocytes est dépassée, soit à cause d'un excès de substrats lipoformateurs (obésité), soit à cause d'une réduction des capacités d'expansion des cellules (lipoathrophie), les acides gras peuvent s'accumuler dans les organes périphériques, tels que les muscles squelettiques et le foie et constituer des dépôts de graisse « ectopique ». Les cellules de ces derniers tissus ne sont pas capables de stocker de grandes quantités de lipides et cette surcharge lipidique entraîne une toxicité cellulaire ou « lipotoxicité » et, à terme, l'altération des fonctions biologiques des tissus. L'une des conséquences majeures est la diminution de l'efficacité de l'insuline, qui conduit à un état d'insulino-résistance précurseur du diabète de type II.

Il est maintenant reconnu que l'inflammation contribue à la pathologie du tissu adipeux et favorise la résistance à l'insuline. Ainsi, de nombreuses études précliniques et cliniques ont établi que des cellules immunitaires sont recrutées dans le tissu adipeux de souris rendues obèses par un régime hyperlipidique et chez les sujets obèses. Localement, l'inflammation est aggravée et entretenue notamment par des macrophages pro-inflammatoires qui s'accumulent dans le tissu. Les interactions

entre adipocytes et cellules immunitaires conduisent à des changements dans la libération d'adipokines, de cytokines et d'acides gras non estérifiés contribuant à la mise en place d'un environnement inflammatoire. Il faut noter que des altérations identiques ont été décrites dans le tissu adipeux atrophique chez des souris génétiquement modifiées et chez les patients atteints de lipodystrophie associée au VIH.

En lien avec l'accumulation de cellules immunitaires, le remodelage de la matrice extra cellulaire et l'accumulation de fibrose sont considérées comme une nouvelle signature de la pathologie du tissu adipeux associée à l'obésité. Il est remarquable qu'une accumulation de fibrose est également observée dans le tissu adipeux des sujets positifs pour le VIH. L'équipe du Prof Karine Clément a été l'une des premières à mettre en évidence l'accumulation pathologique de matrice extracellulaire dans le tissu adipeux de sujets obèses. Par la suite, des études *in vitro* ont révélé le rôle crucial des interactions entre macrophages et pré-adipocytes dans le développement et le maintien de la fibrose dans le tissu adipeux. Cependant, l'importance physiopathologique de ces remaniements matriciels, plus spécifiquement en lien avec le statut glycémique et la résistance à l'insuline, n'est pas totalement élucidée.

2. Les altérations de l'intestin et du microbiote intestinal dans les maladies métaboliques

Les choix alimentaires, en particulier les régimes riches en matières grasses, conduisent à des changements du microbiote et à l'altération de la fonction de la barrière intestinale. Chez les rongeurs, l'une des conséquences d'un régime riche en graisse est l'augmentation de la perméabilité paracellulaire dans l'iléon, facilitant le passage de produits bactériens, en particulier les lipopolysaccharides (LPS), de la lumière intestinale dans la circulation. L'élévation des quantités de LPS circulantes, qualifiée d'«endotoxémie», contribue à l'inflammation et potentiellement aussi à l'insulino-résistance systémique. Chez l'homme, les liens entre régime alimentaire, microbiote intestinal et dérégulation métabolique font l'objet d'une recherche intense, essentiellement dans le cadre de l'obésité. Fait intéressant, une partie des altérations pathologiques liées au phénotype d'obésité peut être améliorée par des interventions nutritionnelles ou la chirurgie bariatrique, en relation avec une modification de la composition du microbiote vers un profil plus sain. D'autre part, des interventions nutritionnelles, telles que l'administration de probiotiques ou de prébiotiques, sont explorées en tant que nouvelles stratégies pour rétablir l'équilibre de la microflore intestinale et améliorer le statut métabolique de l'hôte. La modification de la microflore intestinale *via* l'administration de probiotiques pourrait alléger les altérations métaboliques à la lumière de plusieurs mécanismes, y compris une éventuelle augmentation de la capacité du microbiome à gérer le défi nutritionnel représenté par une diète riche en gras.

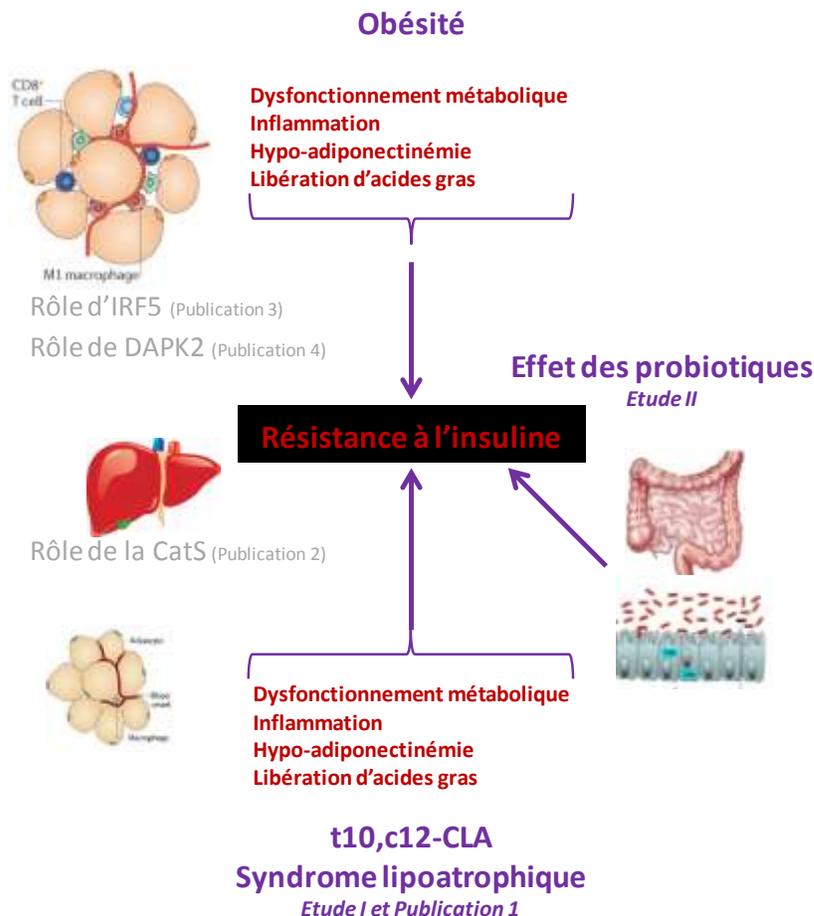
B. Objectifs et principaux résultats

Notre équipe s'intéresse aux liens entre dysfonctionnement du tissu adipeux, altérations métaboliques et microbiote intestinal, avec comme objectif global d'élaborer des stratégies thérapeutiques pour prévenir et/ou traiter les désordres métaboliques associés à l'obésité.

Mes contributions principales, basées sur l'utilisation de différents modèles de souris, sont la démonstration a) que le tissu adipeux a la capacité de s'adapter à un stress nutritionnel aigu, reposant sur le recrutement de macrophages anti-inflammatoires (Etude I et Publication 1) et b) que l'administration de probiotique n'est pas suffisante pour atténuer l'obésité et la résistance à l'insuline induite par un régime riche en graisses chez la souris (Etude II).

J'ai également contribué à trois études dans lesquelles des modèles animaux (souris génétiquement modifiées rendues obèses par un régime riche en graisse) et cellulaires (culture de d'adipocytes et de pré-adipocytes) ont été utilisés pour déterminer l'implication de nouvelles cibles moléculaires dans la régulation de la masse grasse et l'homéostasie métabolique (Publications 2, 3 et 4 présentées en annexe). Ces cibles potentielles ont été récemment identifiées dans l'équipe du Prof Karine Clément par des études d'expression génique comparant le tissu adipeux de sujets non-obèses à celui de sujet massivement obèses. Ce sont la protéase Cathepsine S, le facteur de transcription pro-inflammatoire IRF5 et DAPK2, une kinase régulatrice de l'autophagie.

Le schéma général ci-dessous place les différentes études incluses dans ce rapport dans le contexte de la contribution de différents organes (tissu adipeux, foie et intestin) à l'insulino-résistance systémique associée à l'obésité ou aux syndromes lipoatrophiques:



1. Etude I: L'adaptation du tissu adipeux à un stress métabolique induit par l'isomère *trans10, cis12* de l'acide linoléique engage des macrophages anti-inflammatoires.

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Adipose Tissue Adaptive Response to *t10,c12* Conjugated Linoleic Acid Engages alternatively activated M2 macrophages

La réponse adaptative du tissu adipeux à un stress métabolique induit par le *t10, c12*-CLA engage des macrophages M2 anti-inflammatoires

FASEB J (soumis)

Introduction

Il est largement admis que l'accumulation de macrophages pro-inflammatoires est une composante clé de la réponse du tissu adipeux à une surcharge en nutriments. Grâce à la libération de chimiokines et cytokines pro-inflammatoires les macrophages perpétuent et propagent l'inflammation dans le tissu adipeux, conduisant finalement à un état pathologique chronique. Bien qu'opposées en terme de modification de la masse grasse, les lipodystrophies sont associées à des altérations du tissu adipeux identiques à celles décrites dans l'obésité, dont l'inflammation et l'infiltration de macrophages. La présence de dépôts de matrice extracellulaire et de fibrose est une signature supplémentaire de la pathologie du tissu adipeux commune aux deux conditions. Ces altérations locales sont accompagnées d'inflammation systémique et de résistance à l'insuline, qui représentent les conséquences délétères les plus courantes de la dysfonction du tissu adipeux associée à l'obésité et aux lipodystrophies.

Le temps nécessaire à l'accumulation des macrophages dans le tissu adipeux et au développement de la résistance à l'insuline est généralement long, allant de quelques mois chez les souris nourries avec un régime riche en graisses à des années dans l'obésité humaine. Dans la situation inverse de restriction calorique, l'inflammation du tissu adipeux et le statut glycémique sont améliorés, également après de longues périodes de réduction ou de stabilisation du poids corporel. Ces observations soulignent la plasticité du tissu adipeux en réponse à des modifications à long terme du bilan énergétique, en lien avec les modifications de l'état métabolique.

Les acides linoléiques conjugués (CLA) sont un groupe de dérivés de l'acide linoléique comportant plusieurs isomères positionnels et géométriques. Parmi ceux-ci, le *trans10, cis12* CLA (*t10, c12*-CLA) exerce un effet puissant et rapide de réduction du poids corporel chez la souris, ce qui a conduit à proposer son utilisation en tant qu'agent « anti-obésité ». Mais, contrairement à la restriction calorique, l'administration de *t10, c12*-CLA n'améliore pas le statut métabolique, mais provoque au contraire une hyperinsulinémie marquée et une résistance systémique à l'insuline. Dans ce modèle, ce phénotype d'accompagne d'un recrutement de macrophages étonnamment rapide dans le tissu adipeux. La présence de cellules positives pour le marqueur macrophagique F4/80 est détectable dans le tissu adipeux après seulement 3 jours de traitement des souris par le *t10, c12*-CLA. Ces observations suggèrent que, en plus d'une plasticité à long-terme, le tissu adipeux a la capacité de produire une réponse immunitaire rapide à un stress nutritionnel.

Dans ce travail, nous avons utilisé le t10, c12-CLA comme un outil pour explorer, chez la souris, les composants et la dynamique des altérations produites par ce lipide dans le tissu adipeux d'une part, et, d'autre part, au niveau systémique et dans l'intestin. Une étude cinétique a été réalisée sur deux périodes consécutives de 7 jours d'administration de t10,c12-CLA par gavage journalier suivie de 7 jours d'interruption du traitement.

Résultats

Le t10, c12-CLA provoque des altérations métaboliques réversibles dans le tissu adipeux et au niveau systémique.

Comme attendu, le poids corporel a diminué régulièrement pendant l'administration t10, c12-CLA sans changement majeur dans la prise alimentaire. A l'arrêt du traitement, le poids corporel est revenu rapidement au niveau initial. Reflétant une lipoatrophie généralisée, le pourcentage de masse grasse est réduit après 3 jours et reste diminué après 7 jours de t10, c12-CLA. La réduction de la masse grasse a été plus prononcée dans le tissu adipeux sous-cutané (sWAT) que dans le tissu adipeux péri-utéral (pWAT). Après 7 jours sans t10, c12-CLA, la lipoatrophie et la masse du pWAT ont été normalisées, alors que sWAT est resté diminué.

L'insuline plasmatique a augmenté progressivement, sans changement significatif de la glycémie, pour atteindre des valeurs 8 fois plus élevées que chez les souris témoins après 7 jours de t10, c12, CLA. Cette situation reflète le développement rapide d'un état de résistance à l'insuline en réponse au t10, c12-CLA, en accord avec des études précédentes. A l'interruption du traitement, l'hyperinsulinémie a diminué et retrouvé des valeurs semblables à celles du groupe témoin, établissant un lien direct entre résistance à l'insuline et administration du t10, c12-CLA.

Nous montrons que le t10, c12-CLA administré par gavage journalier s'accumule dans le tissu adipeux, où les concentrations ont atteint des valeurs 8 à 20 fois plus élevées que dans l'intestin ou le foie. Cette différence souligne que le tissu adipeux est le tissu métabolique principalement ciblé par cet isomère. Après 7 jours de t10, c12-CLA, l'expression de plusieurs « adipokines » produites dans tissu adipeux est considérablement réduite et ce de manière strictement réversible. Parmi elles, l'adiponectine suit le même schéma de variation au niveau génique et dans la circulation. En revanche, les niveaux d'ARNm et plasmatiques de la leptine sont très peu modifiés. Ces données suggèrent que le t10, c12-CLA modifie le secrétome du tissu adipeux en ciblant les niveaux de transcription des facteurs sécrétés, tout en laissant intactes les voies de sécrétion.

Plusieurs gènes codant pour des enzymes clé des voies métaboliques de la lipogenèse (FAS, ACC) et de la lipolyse (HSL, ATGL, PLN1) dans le tissu adipeux étaient nettement régulés à la baisse après 1 semaine de t10, c12-CLA. Malgré ces dérégulations métaboliques majeures, la taille des adipocytes, qui reflètent le niveau de stockage des lipides dans la cellule, n'était que légèrement diminuée, ce qui indique que la majorité des adipocytes ont survécu au traitement par le t10, c12-CLA. Conformément, le niveau d'expression des gènes métaboliques est quasi normalisé à l'arrêt du traitement. Il est notable que les gènes impliqués dans le processus de « lipophagie », tels que le facteur de transcription TFEB, sa cible, la lipase acide lysosomale (Lipa), et la protéine mitochondriale UCP2, sont régulés à la hausse et de manière réversible en réponse au t10, c12-CLA. Ces observations suggèrent que les adipocytes utilisent

une voie métabolique alternative permettant l'utilisation d'une partie des lipides stockés comme substrat énergétique en réponse au stress métabolique produit par le t10, c12-CLA.

Le t10, c12-CLA provoque une accumulation réversible de macrophages dans le tissu adipeux.

Des analyses immunohistochimiques montrent que les cellules positives pour le marqueur macrophagique CD68 s'accumulent dans le tissu adipeux après 3 jours, continuent d'augmenter après 7 jours de t10, c12-CLA et chutent brusquement à l'arrêt des gavages. L'accumulation réversible de macrophages a été confirmée par l'expression du gène CD68 dans le tissu adipeux et par le nombre des cellules F4/80⁺ évalué en cytométrie, qui suivent des profils similaires. Dans ce modèle, l'abondance des macrophages est corrélée négativement avec la masse adipeuse, en opposition à la relation inverse trouvée dans l'obésité. Cependant, comme dans l'obésité, l'insulinémie est positivement associée à l'accumulation macrophagique.

Le t10, c12-CLA provoque une accumulation réversible de matrice extracellulaire dans le tissu adipeux.

Le marquage des coupes de tissu adipeux au rouge de picrosirius a révélé que t10, c12-CLA provoque des dépôts de collagène, avec un effet plus prononcé dans le sWAT que dans pWAT. A l'interruption des gavages, l'abondance du collagène a diminué dans les deux dépôts, mais n'a pas été entièrement normalisée dans le sWAT. Une analyse d'expression génique a permis d'identifier la fibronectine et le collagène 1 comme les composants de la matrice extracellulaire sélectivement augmentés dans les deux types de tissu adipeux, avec une contribution additionnelle du collagène 3 dans le sWAT. Malgré l'augmentation des dépôts de collagène, les enzymes de pontage telles que Lox, (lysyl oxidase) ou Loxl2 (lysyl oxidase-like 2) sont peu modifiées en réponse au t10, c12-CLA. De même, l'expression de plusieurs facteurs profibrotiques, tels que le TGF- β 1, l'inhibine β A et le CTGF, n'est que marginalement affectée, rendant peu probable l'induction d'un phénotype fibrotique dans les fibroblastes. Ces résultats montrent que, en réponse au t10, c12-CLA, il existe un remodelage de la matrice extracellulaire au sein du tissu adipeux, mais sans constitution d'un réseau rigide de fibrose. L'étonnante rapidité de constitution et la réversibilité de ces altérations matricielles, qui suivent temporellement l'accumulation des macrophages, révèle une plasticité structurale remarquable du tissu adipeux.

Les macrophages du tissu adipeux induit par le t10, c12-CLA ont un profil anti-inflammatoire « M2 ».

Nous avons déterminé le phénotype des macrophages induits par le t10, c12-CLA par la quantification des marqueurs de surface CD11c et CD206 dans les cellules F4/80⁺. Les cellules CD206⁺ CD11c⁻ sont la population cellulaire dominante chez les souris témoins. En réponse au t10, c12-CLA, cette population a augmenté de façon importante, ainsi qu'un sous groupe moins abondant de cellules CD206⁺ CD11c⁺, qui était pratiquement indétectable dans le groupe témoin. La régulation à la hausse d'une série de marqueurs géniques associés au phénotype anti-inflammatoire « M2 » confirme la prédominance des macrophages M2 anti-inflammatoires dans le tissu adipeux des souris traitées au t10, c12-CLA. Tous les marqueurs M2 ont fortement diminué au retrait du t10, c12-CLA. Les niveaux d'ARNm des cytokines anti-inflammatoires IL-10 et IL-1ra ont considérablement augmenté en réponse à t10, c12-CLA et démontré une réversibilité quasi-totale à l'interruption des gavages. Ces données révèlent que le

tissu adipeux réagit au t10, c12-CLA en engageant une réponse anti-inflammatoire marquée, spécifique et réversible.

Le t10, c12-CLA modifie le profil immunitaire inné et adaptatif dans le tissu adipeux.

A côté de l'abondante population de macrophages, d'autres cellules immunitaires innées, dont les cellules NK (CD3⁻ NK1.1⁺) et les cellules dendritiques (F4/80⁻ CD11c⁺) contribuent à l'infiltrat induit par le t10, c12-CLA. En outre, les cellules NKT (CD3⁺ NK1.1⁺), les cellules T et les cellules B sont également plus fréquentes dans le tissu adipeux des souris recevant le t10, c12-CLA, tout en restant moins nombreuses que les cellules immunitaires innées. Une petite population de cellules T $\gamma\delta$ a augmenté, alors que l'abondance de cellules Treg Foxp3⁺ n'a pas été significativement modifiée lors de l'administration t10, c12-CLA. Ces résultats montrent que l'administration de t10, c12-CLA provoque des modifications substantielles du profil immunitaire du tissu adipeux.

Le t10, c12-CLA modifie le profil des chimiokines dans le tissu adipeux.

L'ampleur de l'infiltrat de macrophages induit par le t10, c12-CLA soulève la question de leur origine, à partir soit d'un recrutement de précurseurs monocytaires, soit d'une prolifération *in situ* ou des deux. Les analyses d'expression génique montrent que le t10, c12-CLA induit une régulation spécifique de plusieurs chimiokines, avec une augmentation marquée de CCL7, CCL2/MCP1, de l'ostéopontine (OPN) et CXCL10. Ces données suggèrent que t10, c12-CLA génère un environnement favorisant le recrutement de cellules sanguines dans le tissu adipeux. Cependant, la prolifération *in situ* des macrophages ne peut être exclue. En effet, lorsque nous avons étudié le marqueur de prolifération cellulaire Ki-67, son niveau d'ARNm a augmenté en proportion avec celui du marqueur de macrophage CD68. Fait intéressant, l'expression de Ki-67 est restée élevée après arrêt du traitement quand l'expression du gène CD68 est normalisée. Ceci suggère que des cellules non-immunitaires pourraient proliférer dans le tissu adipeux pendant le traitement au t10, c12-CLA et également pendant la période de récupération. La prolifération de précurseurs adipocytaires pourrait contribuer à la récupération de la masse adipeuse post-t10, c12-CLA.

Résultats complémentaires

Le t10, c12-CLA modifie le profil d'expression génique dans le jéjunum.

Les effets du t10, c12-CLA au niveau du tractus intestinal ont été évalués par une étude d'expression génique dans le jéjunum et le colon des souris après 7 jours de traitement.

Contrairement aux effets produits dans le tissu adipeux, l'administration de t10, c12-CLA pendant 7 jours n'a pas entraîné d'effet majeur sur l'expression des gènes CD68 (marqueur de macrophage) et CD3 (marqueur de lymphocyte T). Bien que nous ne puissions pas exclure un effet sur d'autres types de cellules immunitaires, ces observations ne militent pas en faveur d'une modification majeure du profil immunitaire.

En revanche, le t10, c12-CLA a modifié l'expression de deux récepteurs « Toll-like », TLR4 et TLR5, qui tend à être augmentée dans le jéjunum. TLR induisent la production de cytokines pro-inflammatoires en réponse à des éléments d'origine bactérienne, comme le LPS. L'activation de ces TLR en réponse au t10, c12-CLA est suggérée par l'augmentation marquée et significative (x 5) de

l'expression du TNF α dans le jéjunum. Les ligands reconnus par les TLR sont variés. Bien que le LPS soit considéré comme le principal ligand de TLR4, l'activation par le t10, c12-CLA n'est pas exclue.

Une série de gènes impliqués dans le métabolisme des lipides au niveau intestinal a également été évaluée. Ce sont la protéine de liaison des acide gras FABP2, connue pour être exprimée exclusivement dans les entérocytes de l'intestin grêle, l'histone désacétylase 1 (HDAC1), importante pour l'homéostasie des cellules épithéliales intestinales, le récepteur SR-BI, impliqué dans l'absorption du cholestérol, ainsi que d'autres gènes du métabolisme des lipoprotéines, telles que l'apolipoprotéine B (apoB), l'apolipoprotéine A-IV (Apoa4), acétylglucosaminyltransférase mannoside 2 (MGAT2), la protéine de transfert de triglycéride microsomique (MTTP). Aucune modification majeure n'a été observée dans les niveaux d'expression de ces gènes après 7 jours de gavage par le t10, c12-CLA ni dans le jéjunum ni dans le côlon des souris traitées par le t10, c12-CLA.

Nous avons pu détecter une diminution d'environ 30 % de l'expression de gènes codant pour trois protéines des jonctions serrées (claudine, occludine et « zonula occludens-1 » ZO-1) dans le jéjunum des souris traitées par le t10, c12-CLA. Des études ultérieures devront confirmer si ces modifications d'expression génique se traduisent par une diminution des protéines et une altération de la perméabilité intestinale. Si tel était le cas, une partie des effets du t10,c12-CLA sur le tissu adipeux pourrait être relayé indirectement par des produits issus du microbiote intestinal tel que le LPS. A l'encontre de cette hypothèse, nous avons mesuré les niveaux circulants de LPS chez des souris ayant reçu du t10, c12-CLA pendant 1 mois, sans détecter de différence significative avec les souris témoins.

Le t10, c12-CLA altère le microbiote intestinal.

Les effets du t10, c12-CLA sur le microbiote intestinal a été analysé, afin de rechercher la contribution d'éventuels changements de composition de la microflore dans les perturbations métaboliques induites par ce lipide chez la souris.

Dans cette étude, les espèces microbiennes ont été organisées en utilisant le concept de « d'entérotypes », regroupant les souris suivant la prédominance des bactéries de différentes espèces dans les fèces. Trois entérotypes principaux (M1, M2 et M3) ont été trouvés dans le groupe de souris étudié :

M1 = prédominance de *Barnesiella* et *Alistipes*

M2 = prédominance de *Barnesiella*, *Alistipes*, *Clostridium* et *Lachnospiracées*

M3 = prédominance de *Bacteroides* et *Barnesiella*

Environ un tiers des souris étaient réparties dans chaque groupe au début des gavages. L'entérotype M3 enrichi en *Bacteroides* est le moins riche en variété de bactéries. Au cours des gavages, la proportion de souris avec un entérotype M3 a progressivement diminué dans le groupe traité avec le t10, c12-CLA, mais est resté stable dans le groupe contrôle. Après 7 jours d'administration du t10, c12-CLA, plus aucune souris ne présentait un entérotype M3.

Conclusion

Nos résultats montrent que t10,c12-CLA induit une dérégulation des gènes métaboliques et l'accumulation de matrice extracellulaire (ECM) dans différents dépôts de tissu adipeux sous-cutané et

profond. De plus, le profil immunologique du tissu adipeux est profondément perturbé en réponse au t10,c12-CLA, avec comme caractéristique majeure l'accumulation de macrophages M2 anti-inflammatoires. Ces altérations métaboliques, immunitaires et structurelles sont réversées après 7 jours de récupération, mais pas toujours normalisées en particulier dans le tissu adipeux sous-cutané qui reste réduit en masse. Cette réversibilité indique que la réponse du tissu adipeux est plus adaptative que pathologique. Elle reflète la capacité de ce tissu à s'adapter rapidement et efficacement à un environnement métabolique altéré, avec, cependant, une meilleure réactivité des tissus profonds par rapport aux tissus sous-cutanés. A ce stade, il est difficile d'attribuer un rôle spécifique aux différents types cellulaires dont l'abondance est modifiée par le traitement. Certaines cellules immunitaires sont considérées comme « délétères » (macrophages inflammatoires, cellules NK, B et $\gamma\delta$) et d'autres comme « protectrices » (macrophages M2, cellules NKT et Treg) pour les fonctions du tissu adipeux. La réversibilité des altérations induites par le t10, c12-CLA milite en faveur d'un effet protecteur des modifications complexes du profil immunitaire du tissu adipeux observées dans cette étude. Malgré cela, les anomalies métaboliques, cellulaires et structurales provoquées dans le tissu adipeux par le t10, c12-CLA sont temporellement associées avec le développement d'une hyperinsulinémie marquée et réversible. Ces observations renforcent l'idée que le dysfonctionnement du tissu adipeux est un facteur majeur de dérégulation de l'homéostasie glucidique.

Au niveau intestinal, l'administration par gavage du t10, c12-CLA ne semble pas modifier l'abondance des cellules de l'immunité, à l'inverse de la réponse immunitaire de grand ampleur observée dans le tissu adipeux. Ces résultats basés uniquement sur une étude d'expression génique devront être confirmés par des analyses en immunohistochimie et en cytométrie. Cependant, nos données suggèrent que le t10, c12-CLA initie une réponse inflammatoire dans le jéjunum *via* l'activation de TLR4 et la production de TNF α . Il est donc possible que des altérations du statut immunitaire interviennent dans l'intestin après une exposition de plus longue durée. De plus, il n'est pas exclu que le t10, c12-CLA modifie la perméabilité paracellulaire au niveau de l'épithélium intestinal. L'analyse du microbiote intestinal, bien que préliminaire, suggère que le t10, c12-CLA altère la composition en bactéries, avec une diminution de l'espèce bactéroïdes. Des travaux de notre équipe ont montré que cette espèce est peu abondante chez le sujet obèse et que son abondance augmente après chirurgie bariatrique et perte de poids. L'importance de ces altérations dans les complications métaboliques de l'obésité n'est pas clairement établie. Dans ce contexte, le modèle de souris traitées par le t10, c12-CLA pourrait permettre d'apporter de nouveaux éléments sur le rôle de cette bactérie commensale dans le métabolisme de l'hôte.

2. Etude II: Effet de deux souches de probiotiques sur la prise de poids et le statut glycémique chez la souris rendue obèse par un régime hyperlipidique.

L'étude II a été conçue sur la base de l'hypothèse que la modification de la microflore intestinale *via* l'administration quotidienne de probiotiques pourrait atténuer les altérations métaboliques associées à un régime obésogène chez la souris. L'effet de deux souches de probiotiques (identifiées par X et Y pour des raisons de confidentialité) a été évalué sur le poids corporel, l'adiposité, la glycémie, la sensibilité à l'insuline, la tolérance au glucose et sur des variables métaboliques. Un traitement de 12 semaines a été réalisé au cours duquel 3 groupes de souris (contrôle (C), X et Y) ont été nourries avec un

régime contenant 60 % de calories lipidiques. Les groupes X et Y ont reçu des probiotiques dans l'eau de boisson à une concentration permettant l'absorption de 10^9 bactéries vivantes par jour par souris.

Le gain de poids corporel ainsi que le pourcentage de masse grasse et l'index d'adiposité (poids des tissus adipeux/poids corporel) étaient identiques entre les groupes C et X, mais systématiquement plus élevé dans le group Y. Confirmant ce phénotype, la leptinémie était augmentée dans le groupe Y. Ce groupe a démontré une augmentation de l'insulinémie et une réponse glycémique altérée dans un test de sensibilité à l'insuline, bien que les différences n'aient pas atteint la significativité statistique. Enfin, les triglycérides hépatiques étaient augmentés dans le groupe Y et inchangés dans le groupe X par rapport au contrôle.

Ces résultats montrent qu'une des souches de probiotiques testée dans cette étude a généré un phénotype défavorable, avec gain de poids et augmentation de la masse grasse, en association avec une élévation de la leptine circulante et des triglycérides du foie et une tendance à la résistance à l'insuline. L'étude des mécanismes cellulaires et moléculaires à l'origine du phénotype observé est actuellement en cours. A terme, si des altérations dans le métabolisme des lipides dans le tractus gastro-intestinal et/ou le tissu adipeux blanc sont détectées, ces données seront informatives pour aider à détecter des effets néfastes potentiels de certaines souches de probiotique.

Pour conclure, contrairement à notre hypothèse de départ, l'administration de probiotiques dans ce contexte expérimental n'a pas atténué l'expansion du tissu adipeux ni la détérioration glycémique induite par le régime gras. Il est clair que la recherche sur les effets des probiotiques sur les paramètres métaboliques et l'homéostasie glucidique va nécessiter des études plus approfondies et sans doute des paradigmes différents d'administration des probiotiques, avec des souches spécifiques isolées, en combinaison de plusieurs souches et/ou en présence de prébiotiques.

3. Conclusion générale

En résumé, ces deux séries de travaux ont permis d'étudier les relations entre l'inflammation et le métabolisme dans le contexte d'un dialogue indirect entre le tissu adipeux et l'intestin. Compte-tenu du fait que l'ampleur des changements produits par les facteurs alimentaires est faible et cumulative, l'administration de t10, c12-CLA peut être considérée comme un stress aigu. Le régime riche en graisse, au contraire représente un stress nutritionnel chronique. En concentrant notre attention sur le tissu adipeux, nous montrons qu'en réponse à un stress nutritionnel aigu, le tissu adipeux regagne rapidement son homéostasie, grâce à la mise en oeuvre d'une réponse immunitaire appropriée. Lorsque la contrainte est plus chronique, comme dans l'obésité, la plupart des interventions, y compris l'administration de probiotiques, ne parvient pas à inverser l'état dysfonctionnel du tissu adipeux et les séquelles métaboliques qui en découlent. Le grand défi dans les années à venir reste donc de découvrir des stratégies thérapeutiques à long terme pour les maladies métaboliques.

C. Contribution à des publications présentées en annexe

1. Publication 2

Lafarge JC, Pini M, Pelloux V, Orasanu G, Hartmann G, Venteclef N, Sulpice T, Shi GP, Clément K, Guerre-Millo M.

Cathepsin S inhibition lowers blood glucose levels in mice.

L'inhibition de la cathepsine S diminue les niveaux de glucose circulant chez la souris.

Diabetologia 2014 57:1674-83

La cathepsine S appartient à une famille de protéases qui ont été impliquées dans de nombreuses pathologies, dont l'athérosclérose. Nous avons identifié la cathepsine S comme une protéine fortement surexprimée dans le tissu adipeux des personnes obèses. Après perte de poids et amélioration du statut glycémique suite à une chirurgie de gastrique, l'expression de la cathepsine S est substantiellement diminuée dans le tissu adipeux. Ces observations nous ont conduits à émettre l'hypothèse d'un rôle de cette protéase dans la pathogenèse du diabète de type 2. Cette hypothèse a été testée dans un modèle de souris déficientes pour la cathepsine S

Des souris déficientes et de type sauvage, traitées ou non avec des inhibiteurs de cathepsine S actifs par voie orale, ont été nourries en régime standard ou enrichi en matière grasse. Le phénotype métabolique des ces différents groupes de souris a été déterminé, avec un focus particulier sur l'homéostasie glucidique.

Nos résultats montrent que la délétion du gène ou l'inhibition pharmacologique de la cathepsine S produit une réduction robuste de la glycémie, qui est conservée dans l'obésité induite par le régime gras et au cours du vieillissement. Une série de tests in vivo de tolérance au glucose, de sensibilité à l'insuline et de réponse glycémique à des substrats gluconéogéniques a permis d'établir que la suppression de l'activité cathepsine S réduit la production hépatique de glucose, sans modifier la sensibilité à l'insuline. Ce phénotype a été confirmé par la régulation négative de l'expression de gènes gluconéogéniques dans le foie. De plus, une diminution de la respiration hépatocellulaire a pu être mise en évidence chez les souris déficientes en cathepsine S. D'un point de vue mécanistique, nous avons constaté que REDD1, qui est un facteur potentiellement impliqué dans la réduction de l'activité de la chaîne respiratoire, était surexprimé dans le foie de souris chez qui la cathepsine S était absente ou inhibée pharmacologiquement.

Cette étude a révélé un effet métabolique inattendu de la cathepsine S, qui pourrait contribuer à favoriser un état pré-diabétique en augmentant l'activité de la gluconéogenèse dans le foie. Des inhibiteurs de cathepsine S sont actuellement proposés pour le traitement de maladies autoimmunes. Nos résultats militent en faveur d'une extension des indications thérapeutiques aux maladies métaboliques. Ces molécules pourraient aider à réduire la production de glucose hépatique chez les personnes obèses à risque de diabète de type 2.

2. Publication 3

Dalmas E, Toubal A, Alzaid F, Blazek K, Eames HL, Lebozec K, Pini M, Hainault I, Montastier E, Denis RGP, Ancel P, Lacombe A, Ling Y, Allatif O, Cruciani-Guglielmacci C, André S, Viguierie N, Poitou C, Stich V, Torcivia A, Fougelle F, Luquet S, Langin D, Aron-Wisnewsky J, Clément K, Udalova IA and Venteclef N.

Irf5-deficiency in macrophages promotes beneficial adipose tissue expansion and insulin sensitivity during obesity in mice.

La délétion d'Irf5 dans les macrophages favorise l'expansion bénéfique du tissu adipeux et la sensibilité à l'insuline chez la souris.

Nature Medicine (NMED-A67879D).

L'accumulation de tissu adipeux viscéral est systématiquement associée à une élévation de l'inflammation et à un risque accru de maladies métaboliques. Toutefois, on connaît peu les mécanismes moléculaires qui contrôlent son expansion pathologique. Le facteur de transcription IRF5 (Interferon regulatory factor 5) a été impliqué dans la polarisation des macrophages vers un phénotype inflammatoire.

Nous démontrons dans cette étude que les souris déficientes en IRF5 ne montrent aucune différence dans la croissance de leur tissu adipeux épидидymaire, mais que leur tissu adipeux sous-cutané est plus développé que celui des souris de type sauvage en réponse à une alimentation riche en matière grasse. Le tissu adipeux épидидymaire des souris déficientes est caractérisée par l'accumulation de macrophages de type « M2 » et l'accumulation de collagène. Les souris déficientes en IRF5 présentent des adipocytes de taille plus faible dans le tissu épидидymaire et une meilleure sensibilité à l'insuline que les souris de type sauvage.

A côté de ces travaux chez la souris, nous avons réalisé une étude chez l'homme, montrant que, chez les sujets obèses, l'expression d'IRF5 est associée négativement à la sensibilité à l'insuline et à l'abondance des dépôts de collagène dans le tissu adipeux viscéral. L'analyse du génome des macrophages du tissu adipeux a permis d'identifier le gène pro-fibrotique TGFBI comme une cible directe d'IRF5.

Cette étude révèle une nouvelle fonction pour IRF5 dans le contrôle de la masse relative des différents dépôts de tissus adipeux et de la sensibilité à l'insuline. De plus, nos résultats suggèrent que l'inhibition d'IRF5 permettrait de promouvoir un état métabolique plus sain chez le sujet obèse.

3. Publication 4

Hedi Soussi^{1,2}, Sophie Reggio^{1,2}, Rohia Alili^{1,2}, Cecilia Prado², Sonia Mutel^{1,2}, Maria Pini^{1,2}, Christine Rouault^{1,2}, Karine Clément^{1,2,3}, Isabelle Dugail^{1,2}

DAPK2 down-regulation associates with attenuated adipocyte autophagic clearance in human obesity. *La baisse d'expression de la kinase DAPK2 est associée à l'atténuation de l'autophagie dans les adipocytes au cours de l'obésité humaine.*
Diabetes (en révision).

Le dysfonctionnement du tissu adipeux dans l'obésité est lié à une inflammation « bas grade » et responsable de la résistance à l'insuline. Une étude transcriptomique précédente a identifié la kinase DAPK2 comme l'un des gènes du tissu adipeux le plus fortement régulé à la baisse chez le sujet obèse. Le rôle de cette kinase est peu connu, bien que sa contribution favorisant le processus d'autophagie ait été proposée.

Dans ce travail, nous montrons que DAPK2 est exprimée principalement dans les adipocytes matures plutôt que dans les cellules du stroma-vasculaire du tissu adipeux. Le niveau d'expression de DAPK2 est bas chez les sujets obèses et ré-augmente progressivement au décours de la chirurgie bariatrique. L'expression de DAPK2 est également diminuée dans le tissu adipeux de souris rendues obèses par un régime riche en graisse. La surexpression de DAPK2 dans des adipocytes murins 3T3-L1 n'a pas affecté la taille des gouttelettes lipidiques, ni la viabilité des cellules, mais a augmenté le flux autophagique mesuré par l'augmentation des concentrations de protéine LC3-II, qui témoigne d'une accumulation d'autophagosomes. Inversement, l'inhibition DAPK2 dans des pré-adipocytes humains en culture diminue les taux d'accumulation de LC3-II. Ces résultats ont conduit à évaluer le flux autophagique dans des adipocytes isolés à partir de tissu adipeux sous-cutané des patients obèses. Nous avons pu ainsi mettre en évidence une réduction sévère de flux autophagique dans les adipocytes obèses par rapport aux témoins. Après chirurgie bariatrique, le processus autophagique est partiellement restauré dans les adipocytes, en relation avec la diminution de la taille des cellules.

En résumé, cette étude révèle l'association inverse entre les niveaux d'expression de DAPK2 et l'accumulation de lipides dans l'adipocyte. Elle montre que l'autophagie est atténuée dans les adipocytes des sujets obèses et suggère que cette inhibition est liée à une sous-expression de la kinase DPAK2.

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ABBREVIATIONS

ACC	Acetyl CoA Carboxylase
Arg1	Arginase 1
β -arr2	β -arrestin 2
CCL2/MCP-1	Chemokine (C-C) motif ligand 2, Monocyte Chemoattractant Protein 1
CCL3/MIP-1 α	Chemokine (C-C) motif ligand 3, Macrophage Inflammatory protein 1 alpha
CCL4/MIP-1 β	Chemokine (C-C) motif ligand 4, Macrophage Inflammatory protein 1 beta
CCL5/RANTES	Regulated on activation, normal T cell expressed and secreted
CCL7/MCP-3	Chemokine (C-C) motif ligand 7, Monocyte Chemoattractant Protein 1
CCR	Chemokine (C-C) Receptor
CLDN2	Claudin2
CD206	MR, Mannose Receptor
CD36	Mucosialin
CLS	Crown-Like Structure
CREB	Cyclic AMP (cAMP) response element binding
CXCL2/GRO- β /MIP2	Chemokine (C-X-C motif) ligand 2
CXCR2	Chemokine receptor 2
CXCL8/ IL-8	Chemokine (C-X-C motif) ligand 8, interleukin 8
DXA	Dual Energy Xray Absorptiometry
ECM	Extra cellular matrix
FAS	Fatty acid synthase
FFA	Free fatty acids
FoxO	Forkhead box protein O
GK	Glucokinase
G6Pase	Glucose 6 phosphatase catalytic subunit
GPR40 (FFR1)	G-protein-coupled receptor 40

GPR41 (FFR3)	G-protein-coupled receptor 41
GPR43 (FFR2)	G-protein-coupled receptor 43
GPR84	G-protein-coupled receptor 84
GPR120 (FFR4)	G-protein-coupled receptor 120
HFD	High fat diet
IGF1	Insulin-like growth factor 1
IL-1	Interleukin-1
IL1RA	Interleukin-1 receptor antagonist
IL-6	Interleukin-6
IL-8	Interleukin-8
iNOS	Inducible NO synthase
IR	Insulin resistance
LC-PUFA	Long chain polyunsaturated FA
LIPA	Lysosomal acid lipase
LPS	Lipopolysaccharide
MetS	Metabolic syndrome
MHC Class II	Major histocompatibility complex class II
MIP	Macrophage inflammatory protein
MMP-9	Matrix metalloproteinase-9, gelatinase B
NEFA	Non-esterified fatty acids
NF- κ B	Nuclear factor κ B
OCLN	Occludin
PAI-1	Plasminogen activator inhibitor-1
PECAM-1	Platelet/endothelial cell adhesion molecule-1
PEPCK	Phosphoenol pyruvate carboxykinase
PGC1 α	Peroxisome proliferator-activated receptor gamma coactivator-1

PKA	Protein kinase A
PPAR α/γ	Peroxisome proliferator-activated receptor
pWAT	peri-uterine white adipose tissue
RBP4	Retinol-binding protein 4
SAA	Serum amyloid A
SREBP-1c	Sterol regulatory element-binding protein-1c
sWAT	subcutaneous white adipose tissue
T2D	Type 2 diabetes
TGs	Triglycerides
TGF β	Transforming growth factor beta
TIMP-1	Tissue inhibitor of metalloproteinase-1
TJP1	Tight junction protein ZO-1
TNF α	Tumor necrosis factor α
TZDs	Thiazolidinediones
UCP2	Uncoupling protein-2
VEGFA	Vascular endothelial growth factor A
WAT	White adipose tissue
ZO-1	Zonula occludens-1

INTRODUCTION

A. Metabolic diseases

1. Metabolic syndrome

The term metabolic syndrome was first used in 1977 by Dr. Hermann Haller, at the time Head of the Department of Medicine in Medical Academy Dresden, Germany, to describe the interconnected relationship of obesity, diabetes, high levels of fats in blood, and other factors. Nowadays a part of scientific literature tends to neglect this concept, even if aware that those factors do not cluster together by chance. Moreover, thanks to its ability to simplify a complex health issue, the concept of the metabolic syndrome (MetS) represents a useful tool for scientific divulgation and awareness of the associated health risks for general population. The metabolic syndrome is a collection of risk factors, such as high blood pressure, high glucose and cholesterol levels, together with abdominal obesity, which associates with a two-fold increase in the risk of having a heart attack or stroke, and a five-fold increased risk of developing type 2 diabetes (Stern, Williams et al. 2004), (Reaven 2006) (Figure 1). Importantly, insulin resistance (IR) has been identified as the basis of most if not all of the features of this syndrome (Dandona, Aljada et al. 2002). Based on the International Diabetes Federation (IDF) definition, 20–25% of the world’s population has MetS (Alberti, Zimmet et al. 2006). This Federation defines MetS as central obesity (defined as waist circumference ≥ 94 cm for men and ≥ 80 cm for European women, with ethnicity specific values for other groups) plus any two of the following: elevated plasma triglyceride (TG) levels (≥ 150 mg/dl), reduced high-density lipoproteins (HDL) (< 40 mg/dl for men and < 50 mg/dl for women), increased blood pressure (≥ 130 mmHg systolic or ≥ 85 mmHg diastolic), or increased fasting plasma glucose (≥ 100 mg/dl). In 2005 the American Heart Association (AHA) and the National Heart, Lung, and Blood Institute (NHLBI) released a scientific about MetS (Grundy, Cleeman et al. 2005). Retrieved from the cited report, Table 1 intends to provide up-to-date guidance for professionals on the diagnosis and management of the MetS in adults.

Table 1: Criteria for Clinical Diagnosis of the Metabolic Syndrome (Grundy, Cleeman et al. 2005)

Measure (any 3 of 5 constitute diagnosis of metabolic syndrome)	Categorical Cutpoints
Elevated waist circumference ^{†‡}	≥ 102 cm (≥ 40 inches) in men
	≥ 88 cm (≥ 35 inches) in women
Elevated triglycerides	≥ 150 mg/dL (1.7 mmol/L)
	or On drug treatment for elevated triglycerides [‡]
Reduced HDL-C	< 40 mg/dL (1.03 mmol/L) in men
	< 50 mg/dL (1.3 mmol/L) in women or On drug treatment for reduced HDL-C [‡]
Elevated blood pressure	≥ 130 mm Hg systolic blood pressure
	or ≥ 85 mm Hg diastolic blood pressure
	or On antihypertensive drug treatment in a patient with a history of hypertension
Elevated fasting glucose	≥ 100 mg/dL
	or On drug treatment for elevated glucose

Insulin resistance/hyperinsulinemia, early identified as the principal cause of MetS (Reaven 1988), (Ferrannini, Haffner et al. 1991) once acquired, would prompt individuals with a genetic predisposition to develop all the other aspects of the disorder. Obesity, hypertension, dyslipidemia, polycystic ovarian syndrome, fatty liver disease, pre-diabetes, sleep and breathing disorder, certain cancers, and cognitive impairment represent diagnostic criteria that help to identify the syndrome. Due to its complexity, MetS has been criticized for lack of precision in the definition. However, over time, MetS has been strongly defended, especially since it is a powerful predictor for T2D and a major risk factor for cardiovascular morbidity and mortality (Isomaa, Almgren et al. 2001),(Gotto, Blackburn et al. 2006). The role of the brain in the development of MetS will not be developed in this Thesis. However it is important to mention here its critical role in modulating lipid and carbohydrate metabolism and above all in perpetuating the unhealthy behaviors, such as deregulated eating behaviors reported in pathologies as obesity (overfeeding) (Kenny 2014). The Thesis will focus on white adipose tissue, on the whole, extremely important as well in the MetS-associated alterations. This is particularly true for central visceral adiposity, now widely recognized as carrying greater health risk factors than other organs in various pathologies, including MetS.

2. Overweight and obesity

According to World Health Organization (WHO), overweight and obesity are important risks of death worldwide. Around 3.4 million adults die each year as a result of being overweight or obese. In addition, 44% of the diabetes burden, 23% of the ischemic heart disease burden and between 7% and 41% of certain cancer burdens is attributable to overweight and obesity. In 1972, in an article published by Ancel Keys in the Journal of Chronic Diseases, the new term "body mass index" (BMI) was coined as the best proxy for body fat percentage, a parameter to measure in prosperous Western societies with increasing obesity. The BMI is defined as a person's weight divided by the square of height —with the value universally being given in units of kg/m². Based on this simple formula, the worldwide prevalence of obesity has nearly doubled between 1980 and 2008, in particular within high income countries, but without exclusion of low income ones. The World Health Organization (WHO) makes these global observations using BMI to classify underweight, overweight and obesity in adults, assuming an average body composition (Table 2).

Table 2. Definition and classification of obesity based on Body Mass Index (BMI) (modified from (Flegal, Carroll et al. 1998)).

Classification	BMI
Underweight	< 18.5
Normal	18.5 to 25
Overweight	26 to 29
Obese	≥ 30
Class 1	30 to 34
Class 2	35 to 39
Class 3 (morbid obesity)	≥ 40

$$\text{BMI} = \text{weight (Kg)} / \text{height (meters)}^2$$

However BMI alone cannot differentiate between fat and fat-free body mass, so measurements of waist circumference can be used as a supplementary factor for diagnosis of obesity (obesity threshold defined as 102 cm for men and 88 cm for women). Other methods, more expensive and time consuming, such as bioelectrical impedance, densitometry, X-ray absorptiometry (DXA, dual energy Xray Absorptiometry) and imaging methods (magnetic resonance imaging (MRI) and computed tomography) can measure adipose tissue distribution. Used as a prognostic factor to evaluate potential cardiovascular and metabolic complications, fat distribution can be influenced by age, sex and ethnicity. Central obesity, the body type also known as “apple shaped” in relation to the waist circumference, is one of the key features of the MetS. Central obesity is associated with dyslipidemia, a lipid disorder that may manifest as lipoprotein overproduction or deficiency. In developed countries, most dyslipidemias are hyperlipidemias, as in obesity, and the lipoprotein pattern can help predict cardiovascular risks. Reported in Table 3 are the desirable lipid levels in adults and the values defining dyslipidemia for the US National Cholesterol Education Program III guidelines (2001).

Table 3. Reference value for Diagnostic Lipid Profile in adults (modified from ATPIII).

Lipid	Desiderable Level (mg/dL of blood)	Dyslipidemia Level (mg/dL of blood)
Total cholesterol	Less than 200 mg/dL	> or = 200 mg/dL
Low-density lipoprotein (LDL) cholesterol	Less than 100 mg/dL	> or = 130 mg/dL
High-density lipoprotein (HDL) cholesterol	More than 40 mg/dL	< 40 mg/dL
Triglycerides	Less than 150 mg/dL	> or = 150 mg/dL

3. Diabetes mellitus type II

Type 2 diabetes mellitus (T2D) is a chronic metabolic disorder, described first as a component of MetS in 1988 (Patlak 2002). About 80% of T2D patients are diagnosed with the MetS (Isomaa, Almgren et al. 2001). T2D is the most common form of diabetes characterized by hyperglycemia, insulin resistance, and relative insulin deficiency. T2D is primarily resulting from the interactions between lifestyle, environmental factors and genetics. Lack of physical activity, sedentary lifestyle, cigarette smoking and important consumption of alcohol are behaviors associated with increased risk of developing T2D (Hu, Manson et al. 2001). Overweight and obesity have been found to strongly contribute to the development of glucose intolerance (Ding, Black et al. 2014) As a consequence, dysfunctional adipose tissue and the associated ectopic fat deposition (muscle, liver and pancreas) have been receiving full attention when studying the relationship between insulin resistance and beta-cell dysfunction in T2D. In fact, in order to predict diabetes risk more than the amount of adipose tissue mass, the ability of the tissue to act as an energy buffer has clearly more importance.

4. Lipodystrophic syndromes

Lipodystrophies are rare disorders in general connected with MetS. Lipodystrophy is a clinical condition identified by an inherent failure in adipose tissue development and/or function. The loss of adipose tissue results in metabolic abnormalities that are typically more severe than those associated with obesity (Chan and Oral 2010). Individuals with lipodystrophic disorders are extremely lean, yet they often suffer from diabetes, dyslipidemia, and other metabolic complications (Virtue and Vidal-Puig 2008). The severity of the metabolic phenotype is approximately proportional to the degree of adipose tissue loss and therefore tends to be worse in generalized lipodystrophy. Two major categories classify lipodystrophies: familial and acquired. Familial lipodystrophies are congenital generalized lipodystrophy, Berardinelli-Seip syndrome, characterized by near complete lack of metabolically active adipose tissue from birth, and Dunnigan familial partial lipodystrophy, characterized by loss of subcutaneous fat from the lower part of the body during adolescence and excess fat accumulation in the face and neck. Between generalized and partial lipodystrophies the degree of metabolic abnormalities can vary. The severity of metabolic abnormalities and/or the serious co-morbidities in generalized syndromes is associated with substantial morbidity and mortality. Both varieties occur approximately three times more often in women. Acquired lipodystrophy happens, for example, in patients infected with the human immunodeficiency virus (HIV), receiving highly active retroviral therapy. These patients are subject to loss of subcutaneous fat from the extremities and face but excess fat deposition in the neck and trunk. These patients in particular present abnormal blood lipid levels (dyslipidemia) due to therapies. Other drugs or unknown mechanisms can cause localized lipodystrophies. Biological similarities between lipodystrophic and obese patients include severe insulin resistance, hyperglycemia, hypertriglyceridemia, dyslipidaemia, hepatic steatosis and low HDL-cholesterol (Huang-Doran, Sleigh et al. 2010). Diabetes frequently occurs over the course of the disease and the risk of cardiovascular complications is very high. Few treatments have proven effective for lipodystrophic syndromes, necessarily focusing on limiting hyperlipidemia and diabetic complications. Few oral antidiabetic or lipid-lowering medications have been useful, e.g. thiazolidinediones (TZDs), metformin, and fibrates (Agarwal, 2006 #903). Diabetes frequently occurs over the course of the disease and the risk of cardiovascular complications is very high. Few treatments have proven effective for lipodystrophic syndromes, necessarily focusing on limiting hyperlipidemia and diabetic complications. TZDs, peroxisome proliferator-activator receptor gamma (PPAR- γ) agonists, regulate adipocyte differentiation. In fact these compounds induce adipogenesis in cell culture models and increase weight gain in rodents and humans (Mukherjee, Hoener et al. 2000). In 2002 Rosiglitazone, another TZD, has been shown to be able to induce adipocyte differentiation in preadipocytes from congenital generalized lipodystrophic patients (Fischer, Moller et al. 2002). However when tried in patients with different types of lipodystrophies, in randomized double-blind controlled studies, this treatment, with few exception (Victoria, Cabezas-Agricola et al. 2010), was not of proven efficacy, put aside the potential negative effects (Sutinen, Hakkinen et al. 2003) (Walker, Kirschfink et al. 2003) (Sleilati, Leff et al. 2007).(Schindler, Rieger et al. 2009), (Raboud, Diong et al. 2010). More recently, depending on the form of lipodydrophy, a recombinant analogue of human leptin, Metreleptin, has been approved to treat the associated metabolic alterations (Chan, Lutz et al. 2011). This drug has been shown to decrease TG and other lipid intermediates and reduces ectopic accumulation in tissues such as liver and muscle, resulting in increased insulin sensitivity, thereby improving hypertriglyceridemia and hyperglycemia. Gastric

bypass surgery could be also taken into consideration to control metabolic alterations associated with lipodystrophies (Utzschneider and Trence 2006).

B. Metabolic diseases: the available models

In order to understand the disease process and the biological factors that participate in particular human pathologies, non-human species can be extensively studied without run into ethical and technical limitation. Moreover, animal models are important in the development process of potential treatments and cures for human diseases, despite the intrinsic limit that biological activity in a model organism does not ensure an effect in humans (Chakraborty, Hsu et al. 2009). In the past decades, humanized mouse models have been used as preclinical tool to bypass this limit and this advancement allowed for example the study of human-specific infectious agents without putting patients at risk (Brehm, Shultz et al. 2010). The mouse (*Mus musculus*) is the classic model vertebrate currently used. This animal presents several favorable characteristics to be selected over other organisms as a model: short generation time, accessibility, easy manipulation, genetics and conservation of mechanisms relatively comparable to humans and in addition easy genetic selection. Mice genetically engineered are called transgenic and it means that through gene knock-out and knock-in technologies they can be used to mimic the genetic and biological evolution of certain diseases, such as cancer, or simply to study the gene function itself.

Most of the evidence about obesity-associated health problems has been obtained from epidemiological analyses of human subjects. The use of animal models can help to explore the precise molecular mechanisms of obesity-associated deleterious conditions. Studies on body and fat mass can include: high fat or high fat/high sugar (Cafeteria) diets, bariatric surgery and dietary restriction, impact of candidate pharmaceuticals or natural compounds. The right animal model must be selected carefully, depending on the degree of fatness and the characteristics important for the planned study. Compared to human studies, using animal models we can investigate in detail the different cellular components of adipose tissue, in physiological or pathological state, i.e. mimicking the adipocyte dysfunctions driven by fat expansion in obesity. In addition though transgenic mice, the cellular responses of adipose tissue, for example, can be completed exploring the multiple molecular activities that a specific gene (inserting, removing, activating or inactivating, inducing a mutation in a time-controlled manner, over-expressing, humanizing through replacing the mouse gene by a portion or the entire human gene) can have in the whole body or in specific tissues/cells. In animal research it is important to consider gender differences. However, most studies continue to be performed only in one gender, using male mice, for complexity and cost reasons. An additional reason is linked to estrous cycle that creates a tremendous amount of variability in female mice. However, this knowledge gap in animal research reflects limits in human research that only recently has started evaluating sex differences in the obesity context.

It is important to underline here that all laboratory experiments involving living animals take into account, when planned, the recommendations of specific committee (Comité National de Réflexion Ethique sur l'Expérimentation Animale, CNREEA) and they need to be approved by the French Ministry of Agriculture and Fisheries. It is the same Institution that gives the animal experimentation authorization (renewable every 5 years) to the staff members which followed the obligatory national

training programs. Planned projects must prove the potential for benefit to human health, minimization of pain and distress, humane euthanasia, and the described protocols must follow the principles of the 3 R “*Replacement, Reduction and Refinement*”.

1. **Mouse models of obesity**

There are several rodent and non-rodent models of obesity, but we will focus our attention on the mouse models mainly used in experimental research. Two primary models are used to study obesity, the monogenic and polygenic. The most well-characterized monogenic mouse models have either the *ob* or *db* gene mutation for obesity, whereas the most commonly used polygenic obesity model is the C57BL/6 inbred mouse fed with high fat diet (diet-induced obese (DIO) mice). Despite being very useful models for obesity, they differ from the human associated complications in many different aspects. Naturally, cut-off points such as waist circumference cannot be transferred from humans to mice. In addition, MetS is characterized by a low HDL in association with an elevated triglyceride concentration. In mice, normal lipoprotein profiles have primarily atheroprotective HDL, whereas normal human lipoprotein profiles contain primarily atherogenic low-density lipoproteins (LDL). The relationship between obesity and hypertension, well established in children and adults, is not reflected in obese mice (Kennedy, Ellacott et al. 2010). On the other hand, despite the limits, mouse models reflect many of the characteristics of human obese phenotype, including hyperinsulinemia, hyperglycemia, IR, hypertriglyceridemia and hepatic steatosis, and they keep providing valuable insights into the pathophysiology of these metabolically altered conditions.

Leptin-deficient mice

The leptin-deficient or *ob/ob* mouse model reflects single gene mutation that has arisen spontaneously in rodent colonies and discovered by chance in 1949 (Ingalls, Dickie et al. 1950) The identification of the gene responsible for the obese phenotype observed took nearly 50 years (Zhang, Proenca et al. 1994). Responsible mutation gene is named as obese (*ob*) gene. The *ob/ob* mutation is recessive and mutant mice gain weight rapidly throughout their lives, reaching a weight that can arrive to be three times that of wild-type control mice. The obesity is characterized by an increase in both adipocyte number and size, associated with reduced energy expenditure (hypometabolic state). The genetic background is an extremely important factor to consider when choosing *ob/ob* mice to study MetS. Whereas leptin deficiency results in hyperphagia and extreme obesity on all backgrounds, a diabetes-like syndrome of hyperglycemia, glucose intolerance and elevated plasma insulin will appear or not depending on genetic background (Kennedy, Ellacott et al. 2010). Specifically *ob/ob* mice on the C57BL/6J background have mild and transient hyperglycemia, whereas when on the C57BL/KsJ background, *ob/ob* mice develop hyperglycemia and diabetes, with maximum weight reached at 3–4 months of age, after which they gradually lose weight and often die by 6 months of age. More recently *ob/ob* mice on the BALB/cJ background have been shown to have a 35–40% reduction in weight gain with elevations in fed insulin and plasma TG levels, whereas *ob/ob* mice on the FVB/N background have exacerbated IR compared with those on the C57BL/6J background. The model, as a whole, presents some limitations. First of all the absence of leptin, a hormone extremely important for central and neuroendocrine signaling, is influencing not only food intake and energy expenditure, but also immune system development and angiogenesis (Fantuzzi and Faggioni 2000) (Busso, So et al. 2002). Leptin-

deficient *ob/ob* mice, as leptin receptor-deficient *db/db*, display in fact a marked thymic atrophy and exhibit defective immune responses. Moreover, *ob/ob* mice short life span, and the male mice reduced fertility, make this model rather costly.

Leptin receptor-deficient mice

Leptin receptor also known as LEP-R is a protein that functions as a receptor for the fat cell-specific hormone leptin. The *db/db* mouse is a model of obesity, diabetes, and dyslipidemia wherein leptin receptor activity is deficient because the mice have a single gene mutation in the long isoform of leptin receptor gene. As for *ob/ob* mice, the obese and insulin-resistant phenotype of *db/db* mice depends upon their genetic background (Kennedy, Ellacott et al. 2010). Specifically mice on the C57BL/KsJ background develop diabetes and mice on the C57BL/6J background only become insulin resistant. At 2 weeks of age *db/db* mice already present elevated plasma insulin. After 4 weeks of age they start expressing the obese phenotype, associated with fasting hyperglycemia (Harlan Laboratories). Although a useful model to study the pathogenesis and treatment of diabetic dyslipidemia (Kobayashi, Forte et al. 2000), these mice, as *ob/ob* mice, require special care and handling because they are relatively immunocompromised. Indeed, expression of the leptin receptor in the environment has been demonstrated to be important for T cell development (Palmer, Aurrand-Lions et al. 2006). As for leptin-deficient mice, this model is also rather expensive.

High fat diet-induced obesity

Being diet one of the main environmental factors contributing to obesity and diabetes (Rolls and Shide 1992), (Swinburn and Ravussin 1993), (Marshall and Bessesen 2002), the mouse model of diet-induced obesity (DIO) can be a useful tool to study metabolic dysfunctions (Buettner, Scholmerich et al. 2007). Though chronic excessive energy intake, in particular by means of dietary fat, can gradually reproduce the development of the deleterious sequels of obesity. High fat consumption leads to an increase in body fatness in mice and in parallel it may cause IR through the excessive plasma free fatty acids (FFA) availability (Boden 2008). The HFD increases the supply of triacylglycerols (TAGs) that accumulate in the major insulin target organs (skeletal muscle and liver) and increased FFA produce the characteristic low-grade inflammation also in mouse adipose tissue, liver, muscles. Cafeteria diet, rich in sugar, salt, fat, is meant to simulate the human Western diet. However nowadays it is not the first choice for scientific research, being difficult to replicate results obtained with it (Moore 1987). High-fat diets (HFD) used in laboratory research, instead, present a well-defined purified composition that allows accurate replications for future studies. The HFD typically contain about 32 to 60% of calories from fat. Nutritionally, 60 kcal% fats is considerable extreme in human diet. However this fat percentage is commonly chosen to induce obesity, because mice tend to gain more weight more quickly (Johnston, Souter et al. 2007). The other frequent choice is 45 kcal% fat, that induced a milder obese phenotype, allowing to study the effects of external interventions (i.e. drug or probiotics) in the prevention or reversion of the deleterious alterations associated to obesity. It is important also to note that not all dietary fats have the same effects on the onset of obesity (Bell, Spencer et al. 1997). Omega-3 fatty acids-fed mice, for example, did not gain as much weight as those fed with comparable amounts of more saturated fat, such as lard or coconut oil (Ikemoto, Takahashi et al. 1996) (Wang, Storlien et al. 2002). However the health benefits reported for unsaturated fatty acids (Ikemoto, Takahashi et al. 1996) are controversial and the effects of different dietary fats are under current investigation in both humans

and mice. Finally, fatty acids and lipid mediators derived from fatty acids can strongly modulate the immune response (de Jong, Kloppenburg et al. 2014), so the diet-induced obese model can be used to evaluate the immune response during the development of different inflammatory diseases, such as rheumatoid arthritis, inflammatory bowel disease, and asthma, known to be exacerbated by obesity.

2. Lipodystrophic mouse models

There are multiple mouse models reproducing lipodystrophy features that can be used for scientific research (Kennedy, Ellacott et al. 2010). These animal models have been developed with a focus on disruption of adipocyte functions, since this cell is at the base of lipodystrophy-associated metabolic complications, including ectopic lipid accumulation and severe insulin resistance. Independently by the etiology of lipodystrophy, dysfunctional adipocytes present diminished storage capacity and altered adipokine secretion. These local alterations affect the metabolism of the organism, possibly through distinct pathways, and animal models allow focusing on single mechanisms. In lipodystrophy, as in obesity, progression of IR is linked to increased circulation of lipids, stored in insulin-sensitive organs such as the liver or muscle, and inflammatory adipokines. However, as opposed to obesity, in lipodystrophy, IR, hyperglycemia, hyperinsulinemia are provoked by low leptin due to the lack of adipose tissue (Shimomura, Matsuda et al. 2000). Low leptin in lipodystrophic patients is perceived at the brain level as a starvation signal. However the excess calories consumed due to hyperphagia cannot be stored in the dysfunctional adipocytes. It has been exactly in this field of research that the causal link between insulin sensitivity and adipose tissue function has been demonstrated using the mouse models of lipodystrophy (Shimomura, Hammer et al. 1998) (Reitman, Mason et al. 1999). The big limit in using these models is that the majority of type 2 diabetic individuals are not lipodystrophic, but obese. Indeed the question about the role of reduced or exhausted adipose tissue expansion in the development and exacerbation of metabolic alterations in an obese state remains open. To elucidate the involvement of adipose tissue expandability in the development of IR in obesity, a recent study elegantly demonstrated that when mice have a mutation equivalent to lipodystrophic patients (dominant-negative mutations in PPAR γ) on a hyperphagic ob/ob background, their adipose tissue expandability is insufficient compared to the excessive energy availability and this exacerbate IR and the metabolic complications associated with leptin deficiency (Gray, Nora et al. 2006). This study, as many other ones, proves that the use of animal models is important to decipher the different molecular mechanisms underlying regulation of body fat distribution and IR. Examples of lipodystrophic mice generated through genetic manipulation are described below.

PPAR γ -deficient mice

PPAR γ belongs to the superfamily of nuclear hormone receptors and is a primary pharmacological target in the treatment of type II diabetes, because of its role in the control of glucose homeostasis and insulin sensitivity. This gene promotes macrophage differentiation (Tontonoz, Nagy et al. 1998), inflammation, and carcinogenesis, but also adipogenesis (Tontonoz, Hu et al. 1994). Due to this important function, different kinds of mutations in PPAR γ have been shown to cause lipodystrophic phenotypes or metabolic symptoms (Ludtke, Buettner et al. 2007). Similar phenotypes have been described in mice (Barak, Nelson et al. 1999) (Wang, Mullican et al. 2013), in which the absence of PPAR γ compromises the induction of genes mediating fatty acid uptake, metabolism, and storage. It was in 2003 using the

lipodystrophic model of PPAR γ hypomorphic mice (PPAR γ hyp/hyp) that it was unequivocally demonstrated the importance of PPAR γ as the master regulator of adipogenesis (Koutnikova, Cock et al. 2003). These specific PPAR γ hyp/hyp mice also showed that glucose homeostasis can be relatively well maintained in the absence of white adipose (Koutnikova, Cock et al. 2003). Three splice variants of PPAR gamma are known: PPAR gamma1, PPAR gamma2, and PPAR-gamma3. For example PPARg2 is the nutritionally regulated isoform of PPARg and it has been proved that selective disrupting this specific variant is sufficient to impair the development and the expansion of adipose tissue (Medina-Gomez, Gray et al. 2007).

Transgenic aP2-SREBP-1c mice

The adipose-nSREBP1c transgenic mouse was created in 1998. In this mouse, SREBP1c, a gene required for glucose metabolism and fatty acid and lipid biosynthesis, is constitutively activated. These mice present adipogenesis impairment that results in a lipodystrophic animal. Mice nSREBP1c lack white adipose tissue and recapitulate the metabolic abnormalities observed in humans with lipodystrophy. Besides reduced body weight and the associated very low levels of adipokines (leptin and adiponectin), these mice present elevated plasma TG, glucose and insulin levels. It was using this model that it was demonstrated that functional adipose tissue is necessary to maintain normal carbohydrate and lipid metabolism (Shimomura, Hammer et al. 1998). However, more recently, it has been demonstrated that adipocyte fatty-acid-binding protein (aP2) is not exclusively specific to adipocytes; it can be expressed also in macrophages and in the central nervous system (Makowski, Boord et al. 2001) (Martens, Bottelbergs et al. 2010). As a consequence, results obtained targeting the promoter aP2 need to be carefully interpreted.

A-ZIP/F-1 mice

The transgenic mouse (named A-ZIP/F-1) was created in 1998 as a model for understanding human lipoatrophic diabetes and presents the same limitation as the aP2-SREBP-1c model. This mouse, express a dominant-negative protein, termed A-ZIP/F, under the control of the aP2 promoter, consequently not targeting exclusively adipocytes. However this dominant-negative protein inhibits the DNA binding and function of B-ZIP proteins in both the C/EBP and AP-1 families of transcription factors, inactivating adipocyte growth and differentiation, resulting in a mouse with no white adipose tissue and dramatically reduced amounts of brown adipose tissue, that is inactive (Moitra, Mason et al. 1998) (Reitman, Mason et al. 1999). Having no white fat tissue brings severe physiological consequences, such as diabetes, with reduced leptin (20-fold) and elevated serum glucose (3-fold), insulin (50- to 400-fold), free fatty acids (2-fold), and triglycerides (3- to 5-fold). The liver and the other organs are enlarged due to lipid accumulation. This phenotype clearly presents similarities with humans with severe lipoatrophic diabetes: IR, hyperglycemia, hyperlipidemia and fatty liver.

C. The metabolic organs

The research goal of the present Doctoral project is to use animal models to understand the signaling from three critical metabolic organs in the body, adipose tissue, liver and intestine, during metabolic altered conditions mimicking human MetS diseases. The potential interaction between gut microbiota and host metabolism is also considered, when possible (Martens, Bottelbergs et al. 2010) (Figure 1).

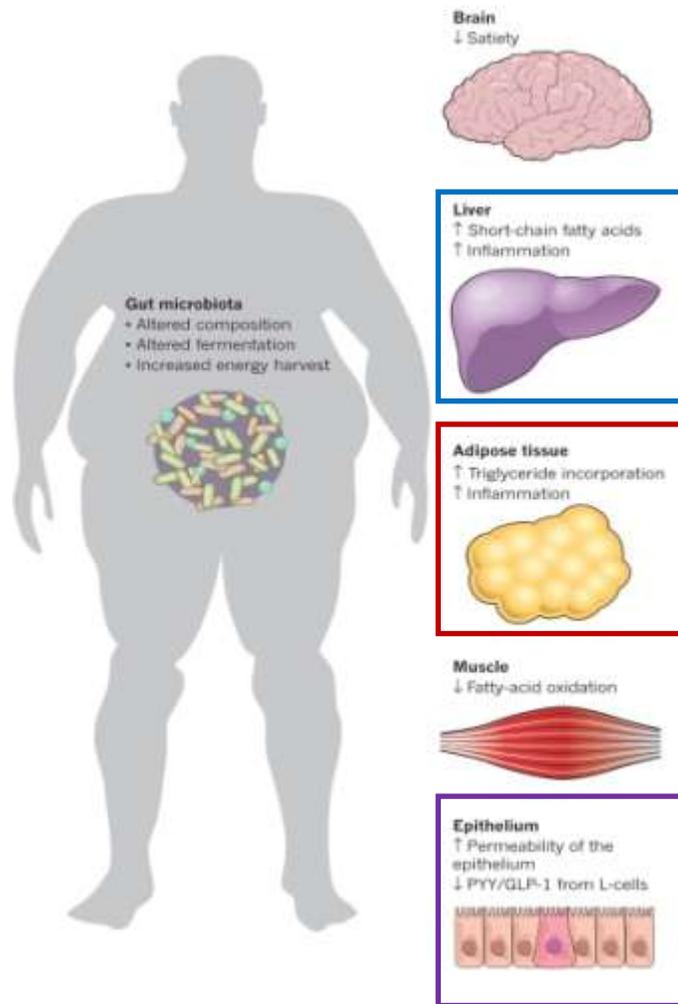


Figure 1: Features of the gut microbiota that promote obesity and insulin resistance (Tremaroli and Backhed 2012).

1. Adipose tissue: functions and dysfunctions

White adipose tissue (WAT) fills up space between organs and tissues, providing structural support, protecting them against trauma (in particular visceral depot) and functioning as a natural thermal insulator. The quantity of body fat varies widely in mammals, ranging from 2% to >50% of body mass, typically from 10% to 20% in mice and humans. Much of this variability depends on both an individual's genetic background and environmental factors, such as dietary and physical activity behaviors. In humans, adipose tissue is found in specific locations, referred to as adipose depots. Depending on the localization, recently new profiling technologies have shown that WAT displays depot-specific differences in the metabolic profiles, indicating that WAT is not a homogeneous organ. Located beneath the skin (subcutaneous fat), around internal organs (visceral fat), in bone marrow and in the breast tissue, adipose tissue is now fully recognized as an important multidepot metabolic organ. In

experimental animal models, this distinction between WAT depending on the different location is maintained. In particular in the mouse, the classical model, WAT collected under the skin around the inguinal area corresponds to the human subcutaneous fat. What in humans is visceral fat, in a mouse corresponds to WAT collected inside the peritoneal cavity, but it can have different names depending on the precise place where it is collected (perigonadal or depending on the sex of the mice, peri-uterine for females and epididymal for males = WAT located around the reproductive system; retroperitoneal = WAT behind the kidneys; mesenteric = WAT located around intestinal tractus).

The primary function of WAT is to store excess energy in the form of triglycerides and to release it as free fatty acids (FFA) or glycerol in time of fasting or starvation. The process is mediated by key lipogenic and lipolytic enzymes. Concurrently normal WAT regulates a wide array of homeostatic processes, such as glucose metabolism, whole-body insulin sensitivity and energy homeostasis, through active secretion of multiple bioactive factors called adipokines (endocrine function). However, in conditions of excess or lack of WAT, adipose tissue becomes dysfunctional, affecting other organs of the body through insulin resistance, dyslipidemia and associated metabolic complications. The excess of WAT in obesity corresponds to abnormally increased triglyceride storage as a result from a chronic, positive imbalance between energy intake and energy expenditure. In obese patients, excess lipid accumulation by large adipocytes increases basal lipolysis, a process in which triglycerides (TG) are hydrolyzed into glycerol and FFA. The net release of FFA into the bloodstream is increased and since the deregulated adipocyte cannot re-esterify them, FFA travel and accumulate to other tissues (ectopic fat deposition), such as liver, pancreas, heart and muscles. For example for skeletal muscles, the obesity-associated excessive free fatty acid production is believed to contribute to IR. In obesity, molecular mechanisms characterizing dysfunctional hypertrophic adipose tissue are abnormal production of adipokines and dysregulation of adipose tissue expandability. Similar failures in WAT functions are reflected in lipodystrophies. Metabolic complications, such as IR, diabetes mellitus, hypertriglyceridemia, and fatty liver, increase in severity with the degree of fat loss. The mechanisms that contribute to adipose tissue dysfunctions and consequently regulate altered metabolic pathways are currently under investigation in these metabolic diseases, obesity and lipodystrophy.

Metabolic and endocrine functions

Besides the efficient storage capacity, WAT has multiple metabolic roles in regulating whole-body physiology. The typical WAT cells are adipocytes that contain lipid droplets that fill the middle of the cell and are anchored by collagen fibers. The importance of adipocytes for health was clearly demonstrated with the use of a mouse model of lipodystrophic diabetes (Gavrilova, Marcus-Samuels et al. 2000). Transplantation of WAT from healthy mice into A-ZIP/F-1 mice reversed their characteristic phenotype, a severe form of lipodystrophic diabetes. Hyperglycemia and hyperlipidemia were decreased, insulin sensitivity improved, serum triacylglycerol decreased, and the amount of fat deposited in muscle and liver was decreased thanks to reconstitution with normal WAT. The complexity of endocrine hormones secreted by healthy adipose tissue has allowed increasingly recognition for this tissue as an endocrine organ. In fact, through the secretion of active molecules (adipokines), WAT influences the regulation of important physiological functions (Cao 2014). Appetite, satiety, energy expenditure, insulin sensitivity, glucose and lipid metabolism, fat distribution are some of the metabolic parameters partially or fully controlled by WAT (FFA, leptin, adiponectin, resistin, retinol binding protein-4-RBP4) (Bluher and

Mantzoros 2015) (Table 4). In recent years, pangenomic and proteomic approaches have identified new adipose tissue-secreted factors whose functions still need to be characterized (Lehr, Hartwig et al. 2012). White adipose tissue functions and how substrate utilization could regulate cell signaling attract special attention in metabolic and cardiovascular physiology, immunology, and cancer biology. Adipokines with a prominent role in auto-/paracrine crosstalk within the adipose tissue such as adiponectin and RBP4 are of particular interest in conditions of metabolic diseases in accordance with their direct effects on skeletal muscle insulin resistance, cardiovascular function and β -cell activity (Romacho, Elsen et al. 2014). The two most abundant depots, visceral and subcutaneous adipose tissues, produce unique profiles of adipokines (Samaras, Botelho et al. 2010). Adiponectin, discovered specifically expressed in WAT in 1995 (Scherer, Williams et al. 1995), at the same time of leptin (Rosen, Yamazaki et al. 1995), is more abundantly expressed in subcutaneous WAT and its main function is to directly sensitize the body to insulin and influence adipose tissue growth and function. Aside from autocrine effects on adipocytes themselves, adiponectin exerts many functional effects on peripheral tissues, such as heart, vasculature, skeletal muscle, lung, bones and not least liver (Dadson, Liu et al. 2011). Modulating a number of metabolic processes, recently, it has been proposed as a starvation gene in the course of evolution, enabling fat storage in case of adiposity loss (Kadowaki, Yamauchi et al. 2008). Systemic adiponectin, and especially adiponectin HMW levels, are paradoxically reduced in obese and type 2 diabetes mellitus patients (Dadson, Liu et al. 2011). Moreover, recent data from obese individuals confirm that visceral adiposity, more than global, influences adiponectin levels, whom production is potentially inhibited by WAT associated chronic inflammation in a self-perpetuating loop (Guenther, James et al. 2014) (Fantuzzi 2008). The decrease activity of this specific adipokine, principally caused by increased proinflammatory cytokines, in particular tumor necrosis factor α (TNF α) and monocyte chemoattractant protein-1 (MCP-1), forms a vicious adipokine network causing insulin resistance and metabolic syndrome (Kadowaki, Yamauchi et al. 2006) (Yamauchi and Kadowaki 2008). Bariatric surgery has been shown to increase circulating adiponectin, in association with improved hyperglycemia and insulin sensitivity (Sjostrom, Peltonen et al. 2012). Adiponectin concentration can be affected by pharmacological and notably dietary interventions as well (Swarbrick and Havel 2008). For example, in a recent review with meta-analysis of randomized controlled trials, it was underlined how lipid intake can change circulating adiponectin levels (von Frankenberg, Silva et al. 2014). In particular, interesting for the purpose of this project, conjugated linoleic acid supplementation showed significant adiponectin reduction compared to unsaturated fat supplementation in several studies (von Frankenberg, Silva et al. 2014). In contrast with adiponectin, circulating RBP4, a retinol transporter, synthesized in the liver and adipose tissue, is significantly increased in obesity, in association with IR in mice and humans. The profile of RBP4 in MetS patients, without cardiovascular diseases or diabetes, follows the profile of other circulating biomarkers of inflammation, known to be biologically relevant in the pathogenesis of MetS: leptin, CRP, serum amyloid A, plasminogen activator inhibitor-1, IL-1, IL-6, IL-8, and monocyte chemotactic protein-1 (MCP-1) (Bremer, Devaraj et al. 2011). However, when looking at RBP4 mRNA expression, it is important to take into consideration that the regulation of RBP4 is gender-specific, similarly to adiponectin, leptin and other AT-derived proteins. Very interesting are the recent results demonstrating the role of RBP4 in causing IR in association with the activation of adipose antigen-presenting cells, such as macrophages, and the induction of proinflammatory cytokines (Norseen, Hosooka et al. 2012) (Moraes-Vieira, Yore et al. 2014). In obesity, the role of these adipocytokines in affecting lipid metabolism/flux and thereby contributing to altered fat distribution is

fully recognized. Consequently their implication in regulating WAT expandability connects them with obesity-related IR. It is noteworthy to underline here the recognized causal link between visceral WAT expansion, macrophage accumulation and IR (Patsouris, Li et al. 2008). Visceral WAT expansion is in fact highly correlated with co-morbidities, due to the higher inflammatory potential in comparison to subcutaneous WAT in terms of increased production of pro-inflammatory adipocytokines and reduced expression of the protective adiponectin (Fantuzzi 2005).

Table 4. Tissue-specific effects of adipokines on lipid metabolism and flux (Sethi and Vidal-Puig 2007).

Adipokine	Food Intake (Energy Intake)	Fat Utilization, β -Oxidation, Thermogenesis (Energy Expenditure)	Fat Storage, Adipogenesis, Nonadipose Lipogenesis (Excess Energy Storage Capacity)	Levels in Common Forms of Obesity
Leptin	Anorexigenic	Enhances β -oxidation via AMPK activation in muscle and liver; reduces intracellular lipids; promotes thermogenesis in BAT	Antiadipogenic in vitro, promotes adipose lipolysis in vivo via the sympathetic nervous system	High (but evidence of leptin resistance)
Adiponectin	No effect on food intake, but intracerebroventricular injection leads to increased energy expenditure; present in human cerebrospinal fluid	Enhances via AMPK activation in muscle and liver; reduces intracellular lipids	Promotes lipogenesis in adipocytes in vitro	Low
TNF- α	Anorexigenic	Inhibits muscle AMPK; increased FA incorporation into diacylglycerol, and increase in intracellular ceramide levels; reduces BAT function and promotes BAT atrophy	Antiadipogenic and stimulates lipolysis via TNFR1-mediated transcriptional signals; increases hepatic de novo synthesis of fatty acid and triglycerides	High (but with autocrine/paracrine activity)
IL-6	Anorexigenic	Stimulates fat oxidation via AMPK activation	Stimulates adipose tissue lipolysis; increases hepatic de novo synthesis of fatty acid and cholesterol	High
Resistin	Potentially anorexigenic; present in human cerebrospinal fluid	Decreases fatty acid uptake and metabolism in muscle in vitro	Antiadipogenic and stimulates lipolysis in adipocytes	High
RBP4	Unknown	Unknown	Promotes liver steatosis	High
PBEF/visfatin	Unknown	Unknown	Promotes lipogenesis in adipocytes	Controversial: may be higher in visceral fat
Omentin (humans, not mice)	Unknown	Unknown	Unknown	Increased in human visceral fat

Complementary to the more classic adipokines linked to metabolic functions, WAT secretes other bioactive factors that, in an auto-/paracrine manner, regulate other tasks, like endothelial and cardiovascular function (angiotensinogen and plasminogen activator inhibitor-1, PAI-1), neuroendocrine function (sex steroids, various growth factors) and immunity (complement factors, haptoglobin). To conclude, the beneficial effects of having a “healthy” adipose tissue are receiving increasing attention, in particular in search of MetS treatment (Gunawardana 2014).

Adipose tissue plasticity

Adipose tissue plasticity can be reflected by multiple aspects that protected the functionality of this tissue during the course of evolution. It is of recent interest the great heterogeneity among the cells types that can become adipocytes, as well as the natural metabolic plasticity of mature cells. The concept of AT plasticity has been extended to two very different adipose present in mammals, different for anatomical location and functions, brown and white adipose tissue. Beige fat, as suggested by its name, is an intermediate between them. Beige adipocytes, with their newly described biology, well represents AT plasticity as well as an interesting potential for new therapies against metabolic diseases (Gunawardana 2014). Brown adipose tissue (BAT) is a highly oxidative tissue containing multilocular fat cells packed with mitochondria that contain uncoupling protein-1 (UCP1) for FFA oxidation. White adipose tissue (WAT) has been historically defined by anatomical location and the presence of parenchymal cells containing a single large lipid droplet and sparse mitochondria lacking UCP1. However, clusters of UCP1-expressing adipocytes with more oxidative capacity can develop in white adipose tissue (WAT) in response to various physiological and pharmacological conditions (Vitali, Murano et al. 2012). These adipocytes have been named beige, 'brite' (brown in white), iBAT (induced BAT), recruitable BAT and wBAT (white adipose BAT) and resemble to adipocytes in BAT for the presence of multilocular lipid droplet cells rich in mitochondria containing UCP1, but they possess a distinct gene signature (Wu, Bostrom et al. 2012). However the distinct functionality of beige cells from brown cells need to be further explored. It is unknown, for example, if beige adipocytes may secrete certain factors that affect WAT function, systemic metabolism or both. In formed WAT, mature unilocular white adipocytes transform into beige adipocytes in response to cold or β 3-adrenergic agonist (Vitali, Murano et al. 2012). However the question about transdifferentiation from pre-existing adipocytes (Himms-Hagen, Melnyk et al. 2000) or de novo differentiation (Wang, Tao et al. 2013) still needs to be answered for the different fat depots (Figure 2).

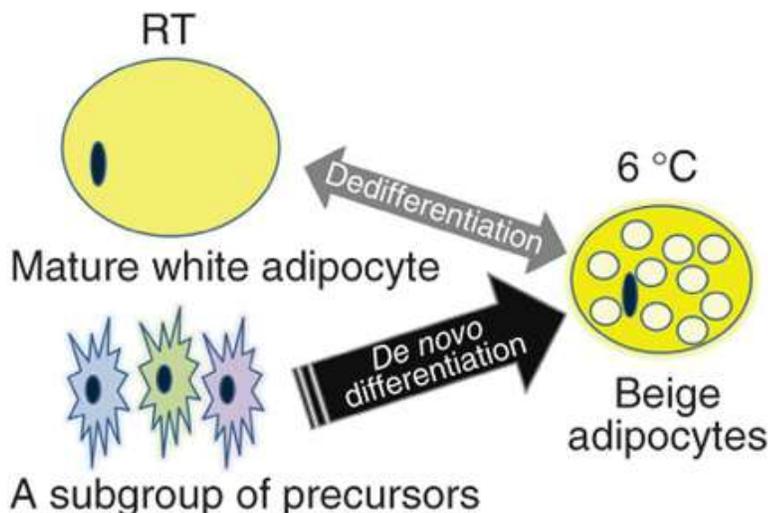


Figure 2: Generation of beige adipocytes (Wang, Tao et al. 2013).

Beige cell population arises predominantly from de novo adipogenesis rather than dedifferentiation. After cold exposure or β 3 agonist treatment of subcutaneous WAT, most beige adipocytes are induced by differentiating from precursors rather than existing mature adipocytes.

Most of these findings are related to mouse biology. Although the existence of human precursor cells capable of expressing UCP1 and other brown and/or beige characteristics in WAT is known (Elabd, Chiellini et al. 2009), the prevalence of beige adipocytes and their developmental process within different human WAT depots has not been carefully evaluated. And yet to come is a clear picture about the role of these “flexible” adipocytes in regulating metabolism, being a potential beneficial resource, especially for obese individuals, like brown adipocytes. That explains why studies on “browning” stimuli to “re-program” pre-existing adipocytes and/or to “program” fat progenitor cells have been increasing exponentially in recent years (Gesta, Tseng et al. 2007) (Esteve, Boulet et al. 2014) (Lee, Odegaard et al. 2015). In addition, adipose progenitor cells, preadipocytes, resident within WAT stromal compartment, represent an increasing area of research lately. Their potential role in influencing how adipose tissue remodels during expansion (obesity) or reduction (drastic fat mass loss) of WAT is highly studied (Jeffery, Church et al. 2015). The concept of adipose tissue hyperplasia (increased adipocyte number) versus hypertrophy (increased adipocyte size) was proposed in obesity, during the 1960s (Hirsch, Fried et al. 1989). Recent studies have shown that, during fat accumulation, preadipocytes, and their interaction with WAT immune cells, can influence the balance toward a dysfunctional hypertrophic state against a hyperplastic process in which mature adipocytes remain relatively responsive (Sorisky, Molgat et al. 2013). However, not all AT expansion is necessarily associated with pathological alterations. The adipogenic capacity of preadipocytes could influence the “healthy” AT expansion as well in physiological condition. This is when the generation of healthy functional smaller adipocytes arises from responsive and abundant preadipocytes, increasing the overall storage capacity while maintaining healthy adipose tissue function (Sun, Kusminski et al. 2011). The described hyperplastic growth clearly supports the concept of “metabolically healthy obese” state (Samocha-Bonet, Chisholm et al. 2012). The number and, even more importantly, the functionality of preadipocytes assure the constant adipocytes turnover present in adults (Spalding, Arner et al. 2008) and consequently the WAT “healthy” expansion in the context of excess caloric intake (Wernstedt Asterholm, Tao et al. 2014).

The dynamics of WAT growth definitely reflects its plasticity. In fact, adipose tissue is the only organ that has the potential of growing unlimited during the whole life. Adipogenesis is a multistep process during which fibroblast like preadipocytes developed into mature adipocytes (Kirkland, Hollenberg et al. 1990). During this process, at various levels influenced by age, gender and life style, numerous transcription factors needs to be sequentially activated, including the CCAAT/enhancer-binding protein (C/EBP) gene family and peroxisome proliferator activated receptor- γ (PPAR- γ). Several factors facilitate the maturation of preadipocytes, including insulin-like growth factor I (IGF-I), macrophage colony stimulating factor, fatty acids, prostaglandins, glucocorticoids (Ali, Hochfeld et al. 2013) and newly explored pathway, such as phosphoinositide 3-kinase (PI3K)-AKT2 (Jeffery, Church et al. 2015). Whereas, there are factors that inhibit the maturation process, such as glycoproteins, like zinc- α 2-glycoprotein (ZAG), transforming growth factor- β (TGF- β), inflammatory cytokines and growth hormones (Ali, Hochfeld et al. 2013). In response to positive energy balance, WAT responds through an increase in

number and size of adipocytes. This is when adipogenesis leads to central obesity if it occurs in the abdominal fat depot and peripheral obesity if it occurs in subcutaneous tissue. However analysis of adipocyte turnover using carbon-14 dating has demonstrated that new adipocytes form constantly to replace lost adipocytes through a dynamic and highly regulated process, particularly active in the human subcutaneous depot, where approximately 50% of adipocytes are replaced every 8 years (Spalding, Arner et al. 2008). However fat accumulation is not due only to adipogenesis, but also to a very different process, lipogenesis. The balance between fat synthesis (lipogenesis) and fat breakdown (lipolysis/fatty acid oxidation) contributes to the balance between lipid storage and mobilization and it is subjected to a complex regulation from various factors, such as glucose, insulin and leptin (Kersten 2001) (Figure 3).

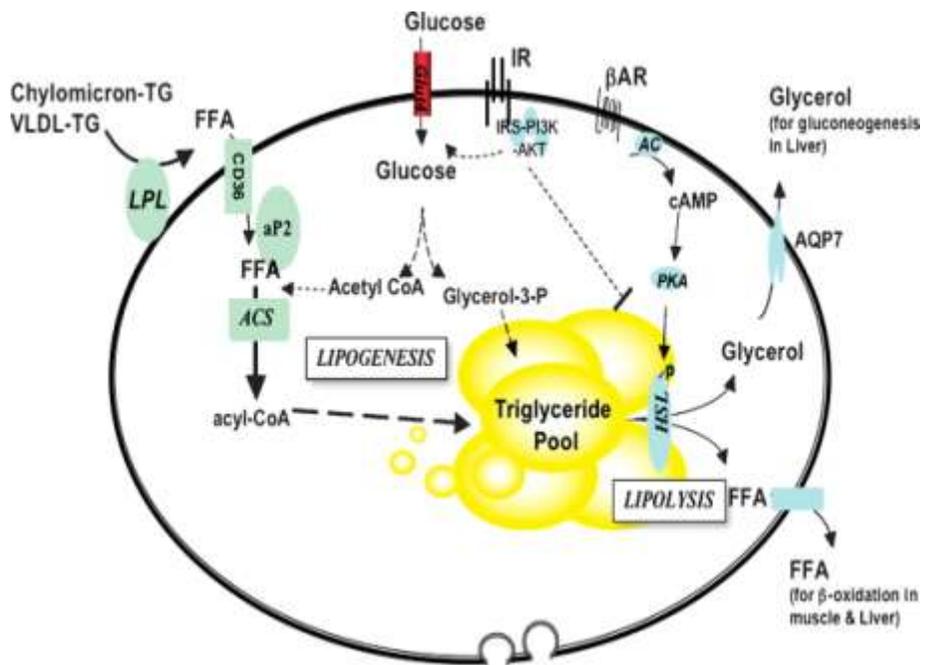


Figure 3: Homeostasis of lipid metabolism in adipocytes (Sethi and Vidal-Puig 2007).

In the last years, new data have explained different mechanisms of regulation of lipogenesis that, apart from hormones can be regulated by nutrients. For example polyunsaturated fatty acids, conjugated linoleic acid included, down-regulate expression of lipogenic genes, i.e. the peroxisome proliferator-activated receptors (PPARs), that are important lipid sensor and play a critical role in the regulation of cellular uptake, activation, and oxidation of FFA (Xu, Lambert et al. 1999) (Berger and Moller 2002). Between the three nuclear receptor isoforms, PPAR gamma, PPAR alpha, and PPAR delta, PPAR γ is the transcription factor mainly involved in the regulation of both adipogenesis and lipogenesis in WAT PPAR γ is necessary and sufficient to differentiate adipocytes (Tontonoz, Hu et al. 1994), but also important in controlling adipose tissue expansion, as it is the case for the PPAR γ 2 isoform (Medina-Gomez, Gray et al. 2007). This process, traditionally considered as passive adaptation, it is now well accepted as a dynamic nutritional adaptation. In conditions as obesity and MetS, in response to variations in energy supply, WAT gradually expands and avoids the lipotoxic side effects exerted by excess lipid and fatty acid (FA) deposition in cells other than adipocytes. Different cell types possess

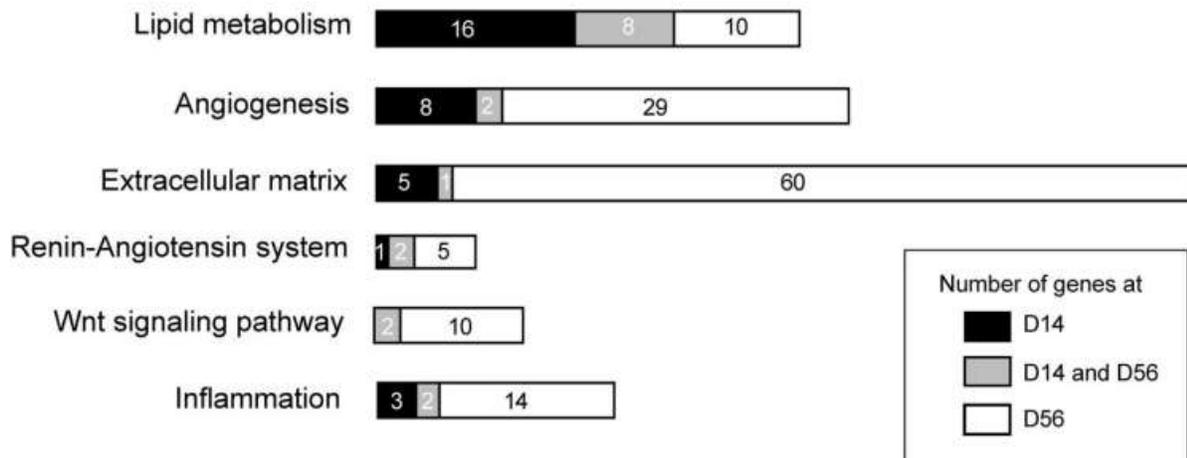


Figure 5. Evolution of the gene expression pattern in human subcutaneous adipose tissue during overfeeding (Alligier, Meugnier et al. 2012).

Bars represent the number of genes related to a given function or biological pathway after 14 days (D14, dark bars) and 56 days (D56, white bars) of overfeeding. Genes commonly regulated at D14 and D56 are indicated in gray.

It is worthwhile to notice that similar transcriptional program involving genes related to lipid metabolism and lipid synthesis is enrolled during lipodystrophies, either congenital (Liu, Jiang et al. 2014) or induced by antiretroviral therapy for treatment of HIV infection (Mallon, Unemori et al. 2005).

In 2009, the group of Prof Scherer demonstrated that during states of positive energy balance, such as obesity, the extracellular constituents are globally upregulated and this up-regulation, with a particular contribution of collagen VI, restricts further expansion of individual fat cells, increasing rigidity of adipose tissue (see Chapter e) (Khan, Muise et al. 2009). However the adipose tissue plasticity in conditions with energy deficit, such as anorexia nervosa, or rapid weight loss, such as after gastric bypass, has been less explored. Nutrient deficit could represent the other face of the coin for the effects that metabolic stress could exert on the extracellular matrix. The importance of the impact of dysfunctional WAT state, in the broadest sense, on deregulated adipose plasticity and vice versa is reflected by the current research unrest about functions like cellular turnover and autophagy.

Adipose tissue immunity and inflammation

Among the multiple bioactive factors secreted by adipocytes, there are a various range of cytokines and chemokines (Makki, Froguel et al. 2013). Anti-inflammatory adipokines such as adiponectin, transforming growth factor beta (TGFβ), interleukin (IL)-10, IL-4, IL-13, IL-1 receptor antagonist (IL-1Ra), and apelin identify the preferential secretion from “healthy and lean” adipose tissue. While “deregulated and obese” adipose tissue essentially secretes proinflammatory cytokines, such as tumor necrosis factor alpha (TNF-α), interleukin IL-1alpha, IL-1beta IL-6, IL-8, macrophage inflammatory protein (MIP), serum amyloid A (SAA), leptin, visfatin, resistin, angiotensin II, and plasminogen activator inhibitor 1 (Samaras, Botelho et al. 2010) (Ouchi, Parker et al. 2011). The inflammatory response promoted by the hypertrophic adipocytes in an obese state activates innate and adaptive immune cells resident within the tissue. The activation of these cells induces an increased recruitment of other immune cells, associated with autocrine and paracrine mechanisms of release of cytokines which

exacerbate local inflammation. These complex alterations in WAT secretion (cytokines, adipokines, and chemokines) and immune cell composition characterize the adipose tissue-related pathologies in general. In obesity, in particular, white adipocyte overexpansion carries the above described stress state that ultimately can lead to cellular death. The NLRP3 (NLR family, pyrin domain containing 3)-dependent caspase-1 activation associated to the cellular death has been hypothesized to happen through a proinflammatory programmed cell death, pyroptosis (Giordano, Murano et al. 2013). When adipocytes die, the intracellular content dispersed in the extracellular space triggers a multitude of cell and tissue changes, for example the enhanced secretion of chemokines, such as MCP-1, an insulin-resistance factor. It is in the context of insulin resistance that the concept of lipotoxicity has been introduced. When WAT expandability becomes a limiting factor as it is the case in obesity, nutrients cannot be stored safely in the adipocyte being the storage capacity exceeded. These nutrients are then transported to peripheral organs, such as muscle, liver, heart, kidney or pancreas. Despite their nutrient buffer capacity, these tissues are not designed to store large amounts of lipids and therefore a toxic state is determined by this nutritional insult. Different tissues respond differently to these potentially toxic species and different lipids can provoke different grade of toxicity, inflammation and the associated local insulin resistance (Medina-Gomez, Gray et al. 2007).

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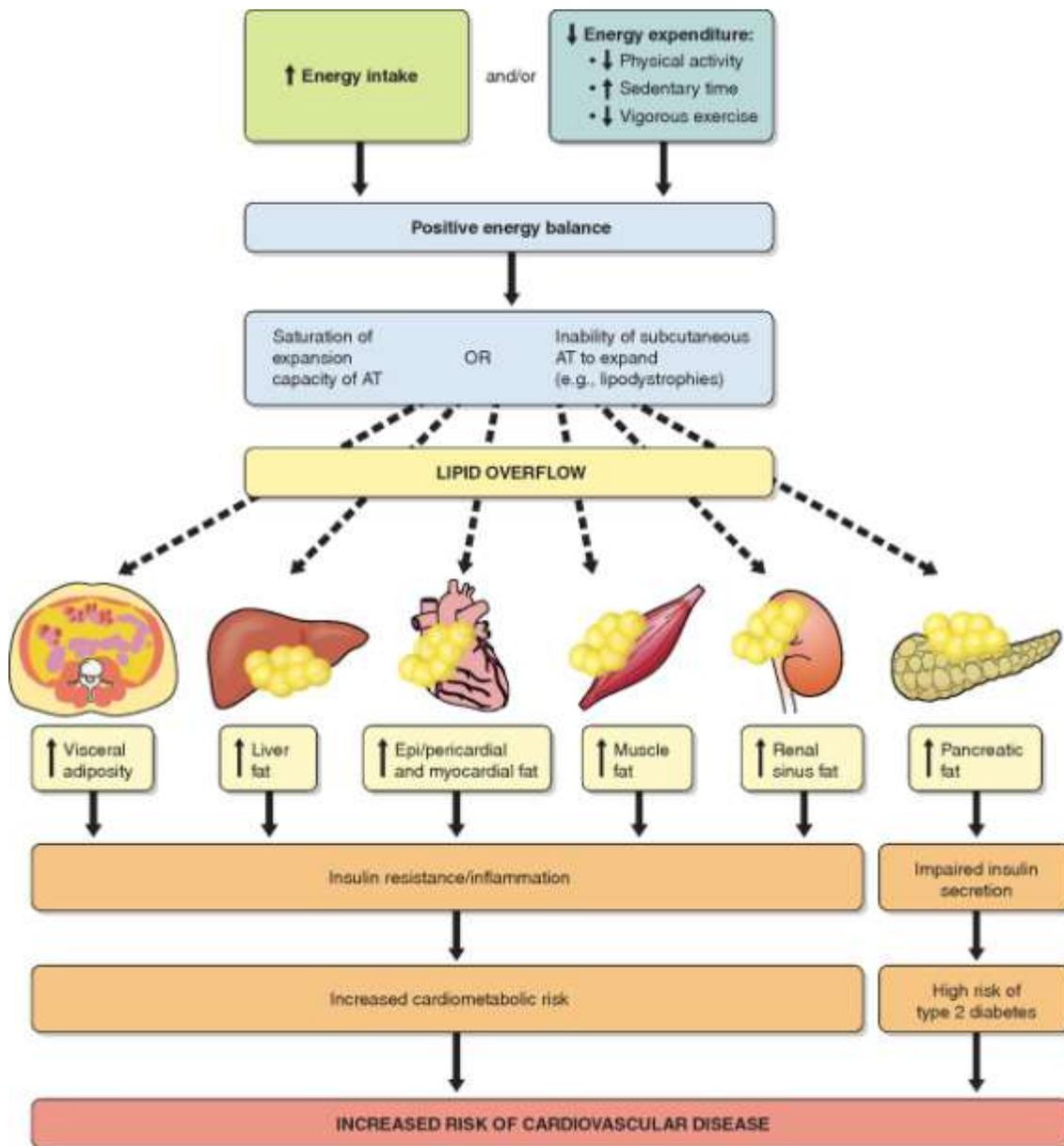


Figure 6. Adipocyte storage capacity in obesity and lipodystrophy: lipotoxicity (Tchernof and Despres 2013).

An altered metabolic state is surely represented by fat expansion, but it is now clear that fat reduction challenges the local adipocyte physiology as well (Cao 2014). Indeed, as in obesity, common inflammatory pathways are activated in lipoatrophic patients, presenting similar alterations in lipid droplet proteins (reduced expression of PLIN1, CIDEA, or CIDEA) in adipocytes (Figure 7).

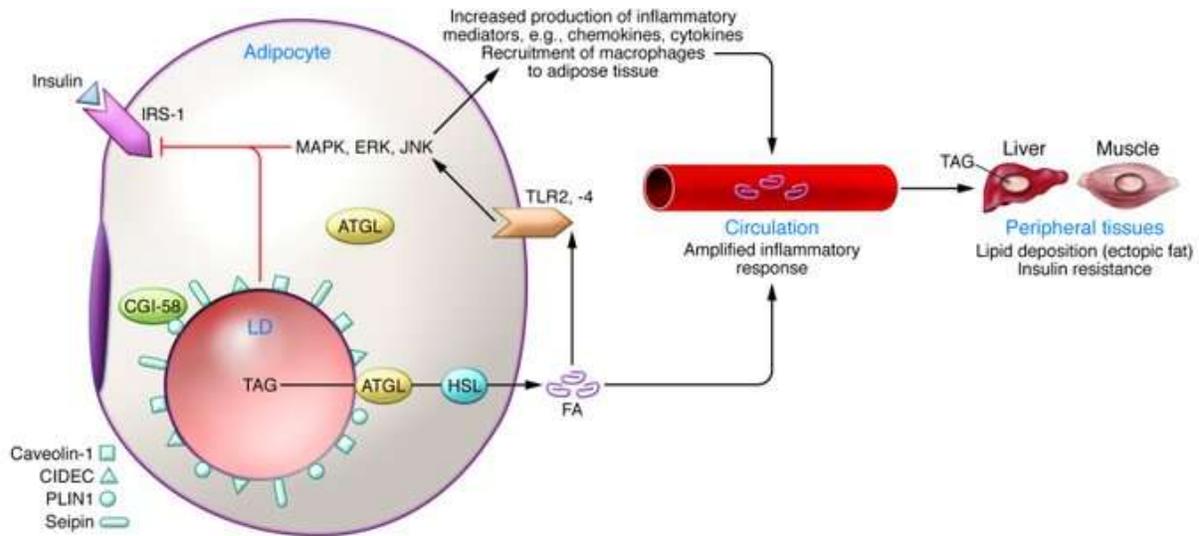


Figure 7. Alterations in lipid droplet proteins in adipocytes result in increased lipolysis and promote insulin resistance in lipotrophic and obese humans (Greenberg, Coleman et al. 2011).

The role of lipid droplets in metabolic diseases is emerging as increasingly important due to their function of “organizing centers” for triglyceride metabolism and the deleterious effects of inefficient fat storage in the promotion of local IR and inflammation (McDonough, Agustin et al. 2009) (Boden 2011). In states of metabolic diseases, the local IR through the participation of proinflammatory adipokines becomes systemic and it is maintained either directly affecting the insulin signaling pathway or indirectly via stimulation of inflammatory pathways, mostly involving TNF- α , IL-1 β , IL-6, MCP1, resistin and PAI-1 generation. These mechanisms have been demonstrated recently in vivo through the high-fat diet (HFD) model (Lee, Li et al. 2011). Adipose tissue appears to be the first tissue responding to HFD and initiate inflammation in this obesity model. In fact three days of HFD were sufficient to deregulate adipocytokine production in C57BL/6J male mice and, continuing HFD, the inability of WAT to properly accumulate TGs gives rise to lipid storage in other tissues and tissue lipotoxicity. This process is now known to lead to the recruitment and activation of immune cells in key metabolic tissues, such as liver, muscles, and pancreas. Adipose tissue is not excluded by this immune-inflammatory response. Indeed, the active immune engagement was first demonstrated through transcription profile analysis of perigonadal adipose tissue from mice in which adiposity varied due to sex, diet, and genetic background (Weisberg, McCann et al. 2003). The connection between the chronic low-grade inflammatory response of WAT and the associated immune and metabolic alterations can be illustrated by the concept of “metaflammation” (Lumeng and Saltiel 2011). Local and systemic proinflammatory and proatherogenic mediators, produced by adipocytes and endothelial cells respectively, enter the circulation to promote IR (Shoelson, Herrero et al. 2007) (Figure 8).

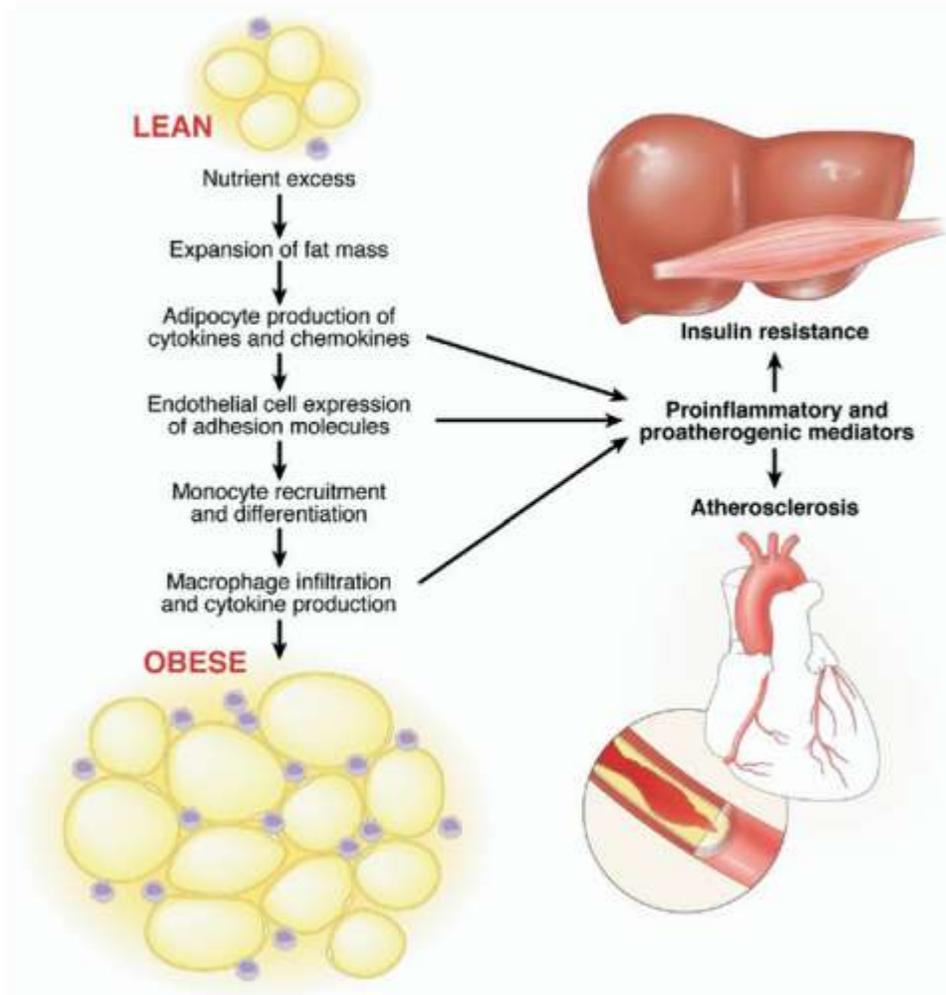


Figure 8. Potential mechanisms for obesity-induced inflammation (Shoelson, Herrero et al. 2007).

The adipocyte-, immune cell-, and endothelial cell-derived substances create an inflammatory environment that promotes IR locally and entering the circulation systemically.

However, recently the negative paradigm on WAT inflammation causing insulin resistance has been challenged by an intriguing study showing that adipocyte inflammation is essential for healthy adipose tissue expandability and consequently reduced ectopic lipid deposition (Wernstedt Asterholm, Tao et al. 2014). In conditions of excess nutrients, inflammation would then be an adaptive response that enables WAT to store correctly the lipids, positively affecting metabolic profile. Extremely important for the inflammatory responses provoked by excess of nutrients, lipids in particular, in WAT are adipocyte surface receptors, in particular toll like receptors (TLRs) and G-protein-coupled receptors, which will be developed in Chapter f.

2. Liver: functions and dysfunctions

Metabolic functions

A healthy liver has the main function of regulating blood composition for the amounts of glucose (stored as glycogen), fat and protein entering the bloodstream from digested food. Processing most of the nutrients absorbed by the intestine during digestion, but also many medicines and alcohol, the liver helps the body to use vitamins and minerals as well as to eliminate poisons and toxins from the blood (i.e. bilirubin). Essential is also its role in producing proteins that help the blood to clot.

The traditional view of nutrient processing has been that after carbohydrate ingestion, insulin is secreted by the beta cells of the pancreas, increasing portal insulin concentrations and resulting in stimulation of glucose utilization and direct suppression of endogenous glucose production. Glucose production occurs in liver and kidney as well. Gluconeogenesis is the metabolic process through which organisms are capable of producing glucose from non-carbohydrate precursors, such as pyruvate, lactate, glycerol, and glucogenic amino acids. In glycolysis, the process occurs in reverse. In both processes, hepatic glucose metabolism is highly regulated at multiple levels. Through transcription, genes like cyclic AMP (cAMP) response element binding (CREB) (Herzig, Long et al. 2001) or FoxOs (Zhang, Patil et al. 2006) (Haeusler, Kaestner et al. 2010) together with phosphoenol pyruvate carboxykinase (PEPCK) and glucose 6 phosphatase catalytic subunit (G6Pase), or on the other side glucokinase (GK)- the rate-limiting enzyme for hepatic glycolysis- contribute to the maintenance of glucose homeostasis (Figure. 9).

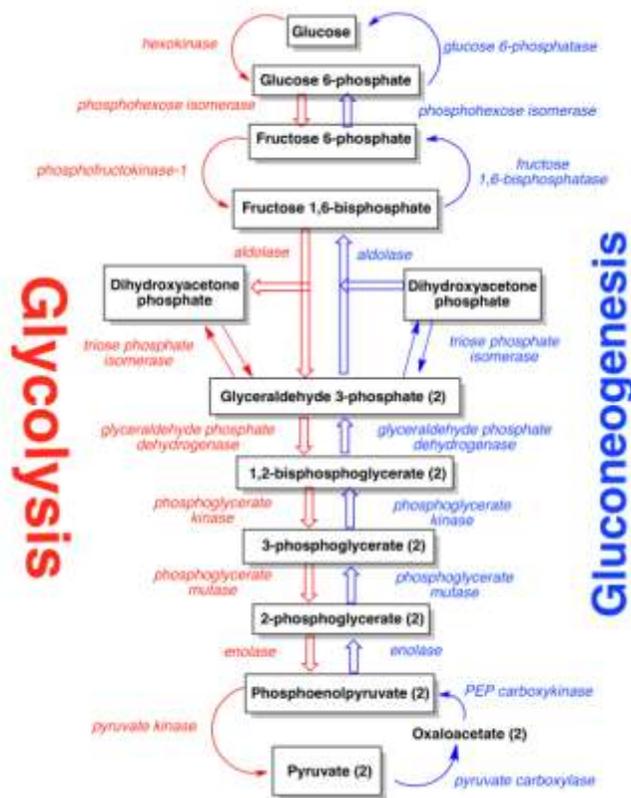


Figure 9. Diagram showing glycolytic and gluconeogenic pathways.

Since glycogen is the main storage form of glucose in the body, the process of its synthesis (glycogenesis) and breakdown (glycogenolysis) is carefully regulated as well. The fundamental form of control is the varied phosphorylation of glycogen synthase and glycogen phosphorylase by protein kinase A (PKA). In obesity and type II diabetes, defects in the regulation of glycogenolysis as well as gluconeogenesis have been shown to cause hepatic IR (Basu, Chandramouli et al. 2005). When carbohydrates are not converted into glycogen, they can be converted into fats and liver is the major organ responsible for converting excess dietary carbohydrate to lipid for storage. Lipogenesis is the process by which fatty acids, i.e. palmitate, are synthesized endogenously. Hepatocytes, under basal conditions, minimally -less than 5%- contribute to hepatic fat through the process of de novo lipogenesis (Diraison and Beylot 1998). Under metabolically altered conditions, elevated plasma glucose (hyperglycemia) combined with high insulin concentrations (hyperinsulinemia) promote lipogenesis and impairs β -oxidation, thereby contributing to the development of hepatic steatosis. Moreover the proximity of the liver to intra-abdominal fat results in greater exposure to FFA than in peripheral tissues, explaining why IR can often develop sooner in this organ. In WAT the altered activity of important lipogenesis enzymes, such as ACC (acetyl-CoA carboxylase) FAS (fatty acid synthase), participate to the increased hepatic lipogenesis, enhancing liver fat deposition up to 30% of the organ (Postic and Girard 2008). Conditions belonging to MetS, such as nonalcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) (see below), characterized by excessive lipid deposition, clearly illustrate how much de novo lipogenesis can be detrimental in liver. Transcription factors such as SREBP-1c and ChREBP, that coordinate the transcriptional regulation of enzymes directing the glycolytic end-products into lipogenesis, are of particular interest in those metabolic conditions, since they are at the interface between glucose and lipid metabolism (Postic and Girard 2008). In summary, the above mentioned set of hepatic functions presents abnormalities in patients with IR, altered lipid metabolism and deregulated adipokines (e.g. adiponectin and leptin), features of metabolic disorders included in MetS.

Non-alcoholic fatty liver disease

Nonalcoholic fatty liver disease (NAFLD) is the major hepatic disorder in patients with MetS (Watanabe, Yaginuma et al. 2008). In conditions of positive energy balance (obesity) or reduced or absent fat storage capacity (drastic weight loss or lipodystrophy), IR leads to inefficient control of lipolysis in WAT. As a consequence levels of lipids and lipoprotein variables are high in the bloodstream of those patients. Liver processes them and a deregulated triglyceride (TG) deposition is observed in insulin-sensitive tissues, such as liver and muscle, that are not intended to be organs of fuel storage other than for glycogen. In general, cells displaying an abnormally large number of lipid droplets are often impaired in function and associated with disease pathologies. Both alcoholic and non-alcoholic fatty liver diseases present this abnormality within hepatocytes (Figure 10). Different sources of fatty acids contribute to the development of fatty liver. 1) fatty acids can reach the liver as circulating FFA, when derived from lipolysis in white adipose tissue. 2) Dietary fatty acids are taken up by the liver through the uptake of intestinally derived chylomicron (CM). 3) In liver, after the esterification step, TG can then be stored as lipid droplets or secreted into the blood as VLDL.

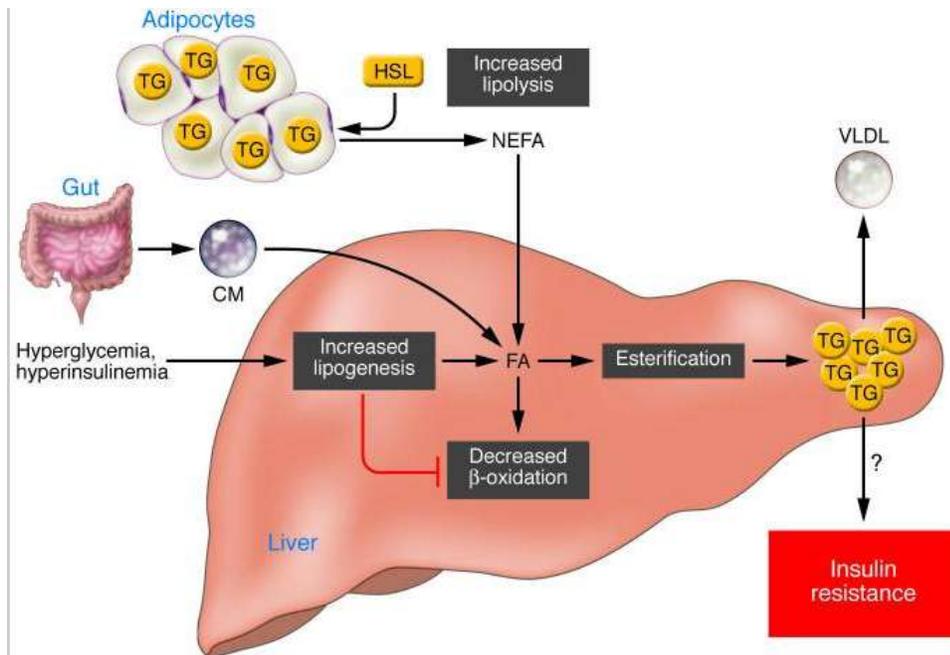


Figure 10. Contribution of metabolic alterations to hepatic steatosis (Postic and Girard 2008).

Although obesity and alcoholism are two very different health issues, their impact on the liver, on the long run, can be quite similar, with comparable complications over time, such as gastrointestinal bleeding, cirrhosis and liver failure (Marchesini, Moscatiello et al. 2008). In both contexts, the development of liver damage is critically influenced by host factors including age, gender, ethnicity, dietary choices, presence of diabetes, genetic polymorphisms and more recently by the gut microbiome (Zivkovic, German et al. 2007) (Neuman, French et al. 2014) (Browning, Szczepaniak et al. 2004) (Bellentani, Saccoccio et al. 2000). However, in the last cited Italian study, steatosis was more strongly associated with obesity than with alcohol consumption, suggesting a greater impact of body weight excess in the hepatic accumulation of fat. In fact, in developed countries the increase in Body Mass Index in the last decades goes in concert with NAFLD diagnosis. The reported prevalence of obesity with NAFLD varies between 30 and 100%, whereas the prevalence of NAFLD within Type 2 diabetes varies between 10 and 75%. Visceral adiposity, hyperglycemia, type II diabetes mellitus and hypertriglyceridemia are all metabolic risk factors that clearly identify NAFLD as the hepatic component of the metabolic syndrome. In the context of obesity, increased FFA and their metabolites, oxidative stress and altered profiles of adipocytokines follow the progression of liver pathology from steatosis alone, through the necroinflammatory disorder of non-alcoholic steatohepatitis (NASH) up to cirrhosis and liver cancer. Hepatic steatosis can be a noninflammatory condition, with no adverse consequences, for decades. NASH represents a shift from simple steatosis to an inflammatory condition characterized by marked hepatomegaly and cellular injury (Bugianesi, Moscatiello et al. 2010). The steatohepatitis can rapidly progress to cirrhosis. It is, in fact, one of the most common causes of cirrhosis in lipodystrophic patients (El-Zayadi 2008). In order to investigate the etiology, pathogenesis and potential treatments for NAFLD, it has been inevitable to study the potential of proxy markers for the disease, due to the

difficulty/impossibility of liver biopsy use. Among the richness of these biomarkers, more or less reliable, I will focus only on factors in line with the purpose of this Thesis.

NEFA concentrations have been proposed as a biochemical marker for NAFLD (Byrne, Olufadi et al. 2009), since the initial accumulation of fat in liver is critical to the disease development (Bugianesi, Moscatiello et al. 2010). Moreover, increased NEFA concentrations are associated with NAFLD independently of classic measures of insulin resistance. In fact, elevated NEFA concentrations have been associated with impaired glucose metabolism and impaired insulin secretion (Pirro, Mauriege et al. 2002). However, it is important to consider the level variation throughout the day, when using NEFA concentrations as a biochemical marker for NAFLD. Another proposed biochemical marker for NAFLD is adiponectin, since high circulating adiponectin have been reported as protective against both alcoholic fatty liver disease and NAFLD. The decreased adiponectin level in NAFLD patients may represent a pathogenic mechanism in the disease (Musso, Paschetta et al. 2013). Eventually measuring circulating triglycerides, in case of overnutrition, might be a good marker for liver status, since the degree of excess nutrient will reflect the severity of the lipotoxic insult, mediated by TNF α (Feldstein, Werneburg et al. 2004). The role of TNF α in mediating insulin resistance is long known in rodents (Hotamisligil 1999). Whereas in humans, increased TNF α (in association with increased presence of endotoxins in the blood = endotoxemia) in liver has been shown to have a role in the development of hepatic steatosis (Ruiz, Casafont et al. 2007), but the precise role of TNF in human insulin signaling still requires extensive investigation (Li, Ding et al. 2013). Knowing that IR is often associated with chronic low-grade inflammation, it is clear how numerous mediators released from immune cells and adipocytes may contribute liver damage and liver disease progression. In fact, in the whole wide spectrum of liver-damage severity presented by NAFLD patients, insulin resistance is always present and therefore is considered the pathophysiological hallmark of NAFLD (Abdelmalek and Diehl 2007). Since IR possibly causes and worsens NAFLD, thiazolidinediones, a class of oral antidiabetic drugs that improve insulin sensitivity, were suggested as a potential therapeutical option, being able to reduce hepatic and peripheral IR, decrease hepatic steatosis, and attenuate the inflammatory response. However, in human trials, TZDs still need to prove their effectiveness (Mahady, Webster et al. 2011).

3. Intestine: functions and dysfunctions

The intestine, together with the adipose tissue, is the largest endocrine organ. The intestines include the small intestine, large intestine and rectum. The small intestine is a tubular structure divided into the duodenum, jejunum, and ileum. Thanks to its large inner surface area, small intestine conducts its main function of absorbing most of the nutrients from what we eat and drink, through simple passive diffusion (i.e. lipids) or active transport (i.e. glucose) across the epithelial cells of the gut into the blood stream. It is important to note that this organ has its own defense system. Aggregates of lymphoid follicles are spread throughout the small intestine, mostly within the ileum, where they are called Peyer's patches. Located in the lamina propria layer of the mucosa, their full function has yet to be defined. However they have the important role of monitoring intestinal bacteria populations and preventing the growth of pathogenic bacteria in the intestines (Gao, Li et al. 2012).

The large intestine (colon) performs the vital functions of converting food into feces, absorbing essential vitamins produced by gut bacteria, and reclaiming water from waste. Interestingly, due to its anatomical localization, the small intestine has a serial relationship with the liver relative to the absorption. This has been clearly observed for orally administered drugs that can reach the systemic circulation in reduced amounts due to both intestinal and hepatic metabolism. The intestinal and hepatic local environments, influenced by dietary choices, weight changes, drugs, are studied with increasing attention in pharmacology as well as in pathophysiology due to their potential in affecting periphery organs and consequently systemic homeostasis.

Since the intestine has the rare property to be renewed within a few days, its capacity to adapt to its nutritional environment is currently under exploration. Research on how diet can affect the intestinal functions and as a consequence obesity-induced metabolic disorders is progressing. For example, using HFD models it is possible to study the associations between dietary lipid amounts and the magnitude of endotoxemia, inflammation and the physiological alterations developed (Mani, Hollis et al. 2013). Dietary choices, such as a high-fat meal, have been shown to increase plasma endotoxin in humans 4 hours after administration (Erridge, Attina et al. 2007), suggesting a potential contribution to inflammation through leukocyte and endothelial cell activation when consumed regularly. When a diet rich in fat is administered for several weeks in rats, it was demonstrated to be associated with gut microbiota alteration, increased activation of TLR4 in the gut wall, increased epithelial inflammation and permeability, followed by increased plasma lipopolysaccharide (LPS) (de La Serre, Ellis et al. 2010). Similarly in BALB/c mice it was demonstrated that an in situ duodenal administration of butter rich in saturated fat was sufficient to markedly increase the production of TNF- α by lamina propria macrophages (Fujiyama, Hokari et al. 2007). However, using a model of acute phase inflammation in the intestine of mice (bacterial administration, able to initiate the TLR2/4 signaling cascade) it was demonstrated that TLR and an increased immune response (DCs) could mediate an amelioration of disease (Wittmann, Bron et al. 2015). These results suggest a positive role for the activation of certain inflammatory pathways in different contexts. Indeed, it has been hypothesized that the increased expression of TLRs in patients with inflammatory bowel disease might result in counter-regulation of inflammation and serve in preventing the disease.

Several studies from the group of P. Cani have clearly shown the relevance of gut microbiota in affecting host metabolism (Cani, Amar et al. 2007; Cani, Possemiers et al. 2009; Cani, Osto et al. 2012). A recent study reported that gut microbiota changes corresponded to a metabolic adaptation to high fat diet (Serino, Luche et al. 2012). Moreover it was shown that the increased paracellular permeability in the ileum and cecum of the HFD-fed mice facilitated the local (adipose cell size and stroma vascular fraction cell number) and systemic (increased circulating adipokines) effects exerted by the different microbial community (Serino, Luche et al. 2012). However, the precise mechanism by which intestine with this low but chronic increase in plasma LPS leads to insulin resistance (Cani, Amar et al. 2007) (Naito, Yoshida et al. 2011) deserves further characterization in murine models of obesity. Whereas in humans, the connection between dietary choices, gut microbiota and host metabolism will need to be explored and validated in physiological as well as pathological conditions. In particular, in the context of lipodystrophy, the potential link between development of IR, microbial communities, pattern recognition receptors and potential bacteria-derived inflammation, waits to be uncovered.

4. Muscle: functions and dysfunctions

In skeletal muscles the excess glucose residues are converted for storage as glycogen. Glycogen/glucose levels are finely regulated by degradation (glycogen phosphorylase) and accumulation (glucose transport rate and glycogen synthase activity). Given their critical role in the control of cellular energy metabolic pathways, PGC1- α (PPARGC1A), a transcriptional coactivator of nuclear receptors regulating mitochondrial genes involved in oxidative metabolism, and peroxisome proliferator-activated receptor δ (PPAR δ)-mediated transcriptional pathway are implicated in the signaling network that controls skeletal muscles as well. Fiber-type transformation and metabolic profiles are regulated by PGC1- α and PPAR δ , that protect muscles and other tissues against IR and obesity (Finck and Kelly 2006) (Wang, Lee et al. 2003). As for liver, the chronic increase of glucose and lipid availability, in the case of obesity, for example, may result in a metabolic conflict in metabolically sensitive organs like skeletal muscle. Plasma FFA levels are elevated in most obese and lipodystrophic subjects. At the muscle level, FFA influence energy balance as well as metabolism, in particular insulin sensitivity (Boden 1997). In heart and muscle, FFA are primarily utilized as a source of energy for ATP synthesis through β -oxidation. However they can also regulate glucose utilization in skeletal muscles, in addition to insulin action (Bergman 2000). In fact, when FFA concentration increases, muscle glucose utilization can be considerably inhibited, possibly by interfering with insulin signaling and glucose transport, or phosphorylation or both (Dresner, Laurent et al. 1999).

Obese patients can be exposed to permanent muscle weakness due to severe increased muscle lipid content. However diagnosis can be difficult, because lipid metabolism disorders are inconstantly leading to a muscle lipodosis and because of subjective evaluation of lipid content by pathologists (Laforet and Vianey-Saban 2010). Considering that the average lipid fraction of the fiber volume is estimated to less than 0.2% (Di Donato S, Taroni F. Disorders of lipid metabolism. In: Engel A, editor. Myology. New York: McGraw-Hill Professional Publishing; 2004. p. 1587–615.), the magnitude of the lipotoxic insult depends on the duration of the excess of nutrients and on the oxidative capacity of the organ. The muscle is one of the primary organs affected by defects in cellular energy metabolism. Increased NEFA at the muscle level inhibit insulin sensitivity and insulin-stimulated glucose uptake. Consequently there are three main forms of metabolic myopathies linked to glycogen storage diseases, fatty acid oxidation defects, and mitochondrial disorders due to respiratory chain impairment (Volpi, Ricci et al. 2011). Patients with a diagnosis in metabolic myopathies can be subjected to cardiac complications, such as cardiomyopathy. Being heart disease is the leading cause of death in patients with diabetes, studying muscle, in general, but heart, in particular, dysfunctions in human and in animal models will allow better discerning the pathophysiologic link between diabetes and heart disease in settings of disrupted insulin signaling or lipid homeostasis (Matsuzawa, Sugiyama et al. 2013).

D. Metabolic diseases: Innate and adaptive immunity

The immunometabolism is an exciting new field emerged from the awareness of the immune system contribution to metabolic processes (Mathis and Shoelson 2011). In recent years studies on immunometabolism have been growing exponentially based on the hypothesis that obesity is a complex immune-mediated disease. The multilevel interactions between metabolism and immune system

involve the innate and adaptive immune cells in multiple metabolic organs. Although the critical role of the immune system in surveillance and modulation of metabolism in those tissues is recognized, adipose is definitely the most studied tissue (Huh, Park et al. 2014). It is in fact in the obesity context that the field of immunometabolism truly blossomed, starting from the concept of “adipose tissue inflammation” with the interaction between adipocytes and macrophages. The complexity of adipose tissue is represented by the variety of cell types that peacefully coexist in it. Next to adipocytes, preadipocytes, fibroblasts, endothelial cells and multipotent stem cells, adipose tissue contains immune cells. Details on the adipose tissue unique immune response have been emerging only recently. Resident WAT immune cells belong to both innate (neutrophils, macrophages, mast cells, eosinophils) and adaptive (regulatory T- cells, type 1 helper T- cells, CD8 T- cells, B cells) immunity (Ferrante 2013). Immune cells, able to induce lipolysis, when they need to fuel the immune response, collaborate with each other to promote immunity and control inflammation and tissue homeostasis. In adipose tissue disorders, such as those encountered in obesity and lipodystrophy, alterations in WAT distribution and function are associated with broad effects, from cytokine, chemokine, and hormone expression changes to basal systemic inflammation (Makki, Froguel et al. 2013). Clinical and preclinical studies in these two extreme and opposite ends of the adiposity spectrum, have shown that cells of the myeloid and lymphoid lineage are recruited and may play a causal role in the associated downstream complications, from local inflammation to systemic IR. The different immune-cell populations (innate, bridging and adaptive) that reside in the adipose tissue will be presented in the next pages, with a particular focus on obesity-associated alterations, the most studied condition (Figure.11).

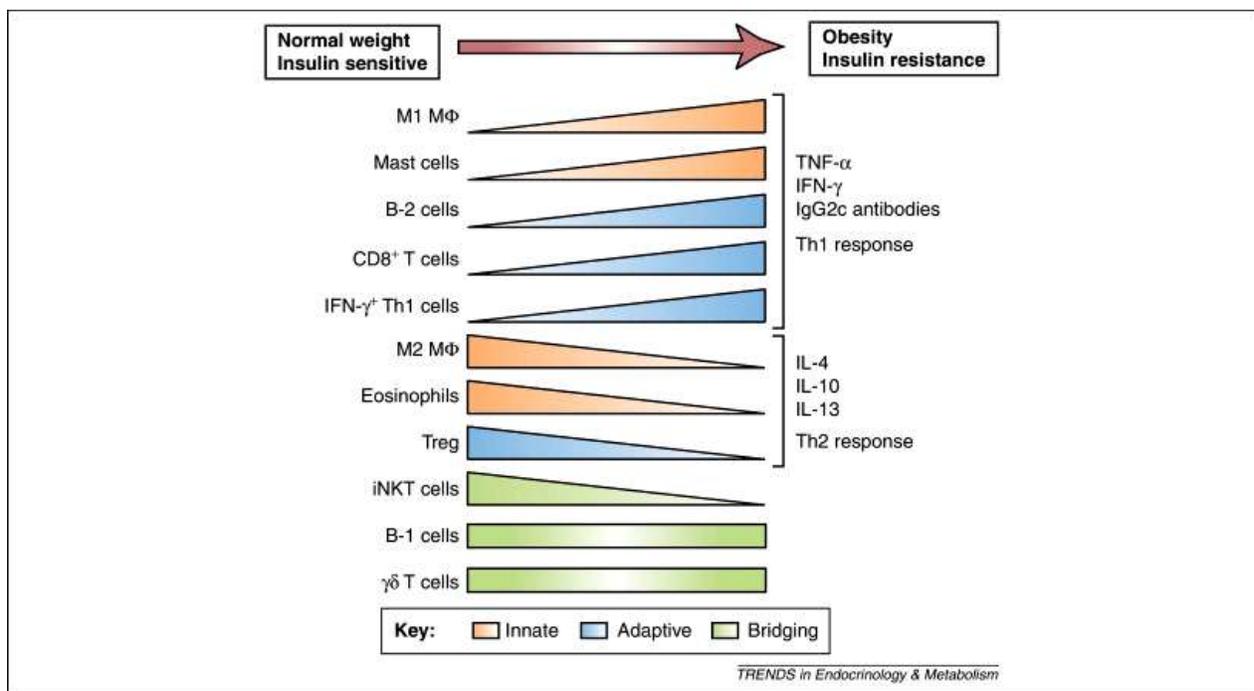


Figure 11. Immune-cell populations in adipose tissue (Schipper, Prakken et al. 2012).

1. Innate immunity

The innate immune system is composed of a diverse array of evolutionarily ancient haematopoietic cell types, including dendritic cells, monocytes, macrophages, mastocytes and granulocytes. Unlike the adaptive immune system, present only in vertebrates, innate immune system is the first line of defense in plants, fungi, insects, and primitive organisms as well, such as bacteria. The major functions of the vertebrate innate immune system include: recognition, recruitment, removal, and not least activation of the adaptive immune system (antigen presentation). In order to perform the recognition task, innate immune cells present receptors, contained on the surface or within the cell, named pattern recognition receptors (PRRs), including Toll-like receptors and cytoplasmic receptors, functionally there to recognize molecules that are broadly shared by pathogens, referred to as pathogen-associated molecular patterns (PAMPs) (Akira, Uematsu et al. 2006). In a broader view, innate immunity evolved towards discrimination of self from nonself, meaning not only molecular patterns defining infectious nonself, but also normal and abnormal self (Medzhitov and Janeway 2002) (Coers 2013). In fact innate immune cells can recognize endogenous stress signals released during cell damage, so called damage-associated molecular patterns (DAMPs). Both PAMPs and DAMPs serve as a starting signal (signal zero) in response to environmental stress, preceding the later signals that will trigger the adaptive immune response (Janeway 1989) (Figure 12). These same signals have been shown to serve to protect the cell itself in response to cellular stress. Through promotion of autophagy, a conserved lysosomal degradation pathway, the cell can control the degradation of self-constituents, i.e. cellular damage control, or the degradation of foreign invaders, called xenophagy (Tang, Kang et al. 2012). In this sense, autophagy represents an innate defense mechanism thanks to its role as earliest host defense strategies (Gutierrez, Master et al. 2004) (Tang, Kang et al. 2012).

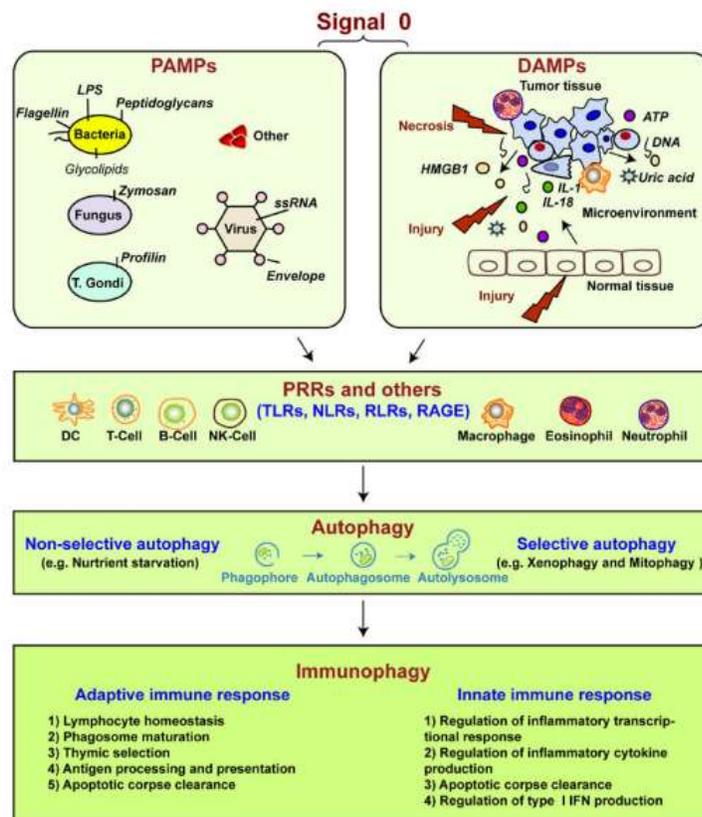


Figure 12. Initial signals play critical roles in autophagy and immunity (Tang, Kang et al. 2012).

Innate immune pathways have evolved not only to recognize pathogens, but also to assess the nutrient environment. An example of DAMPs, important for the context of this Thesis, is represented by lipids. Lipids, found in the adipocyte surrounding when an adipocyte dies (apoptosis, necrosis, pyroptosis), could be the endogenous ligands to which the immune system responds (this part will be discussed more in detail in the Chapter d). FFA can activate innate cells via the signaling pathway of TLR4 that can sense their presence (Nguyen, Favelyukis et al. 2007). Another example of nutrient sensing is represented by inflammasome that can be activated by ATP (Mariathasan, Weiss et al. 2006). Once one, or more, of similar receptors recognize a PAMP or a DAMP, the inflammatory and innate response is organized rapidly, right at the onset of an infection, burn, or injury (i.e. local lipotoxic event). All the innate immune cells can participate to inflammation, balancing a variety of potential responses, from cytokine production to tissue repair, depending by the context and the organ involved.

Mast cells

Mast cells are a type of innate immune cell that reside in connective tissue and in the mucous membranes. However they also have a role as effector cells in adaptive immunity (Galli, Nakae et al. 2005). They are intimately associated with wound healing and defense against pathogens, but are also often associated with allergy and anaphylaxis. Many inflammatory diseases involve mast cells in cross-talk with T- cells, such as atopic dermatitis, psoriasis and multiple sclerosis (Voehringer 2013). When activated, mast cells rapidly release characteristic granules, rich in histamine and heparin, along with various hormonal mediators. Tryptases and chymases are the major proteins stored and secreted by mast cells and depending on mast cell subtype, tissue, and mammal of origin can modulate inflammation, matrix destruction, and tissue remodeling (Caughey 2007). Mast cells play also a role in host defense, releasing chemotactic molecules into the environment that recruit other innate cells, such as neutrophils and macrophages (Conti, Pang et al. 1997). Mast cells can be triggered by a variety of physiological factors, including auto-secreted molecules, and environmental factors, such as stimulants of Toll-like receptors (Sandig and Bulfone-Paus 2012). Depending on the trigger, their location and stage of maturation, mast cells express different types and levels of surface antigens and are able to produce multiple effector molecules, i.g. pro- or anti-inflammatory cytokines (Bischoff, Sellge et al. 2001). For example at the intestinal level, TLRs, well known for their role in lipids sensing, could be involved in the activation of mucosal mast cells observed when an infusion of polyunsaturated fatty acid-rich lipid emulsion was administered inside the duodenal tract (Ji, Sakata et al. 2011). Possibly activated through similar pathways, these innate cells have been implicated in the pathogenesis of diet-induced metabolic disease. In this study, our group showed using genetic and pharmacologic approaches that when mast cells are absent, mice were protected from obesity-induced metabolic syndrome (Liu, Divoux et al. 2009). As for a majority of immune cells their implication in WAT inflammation was recently linked to tissue remodeling, the process characteristic of obese adipose tissue, in mice (Liu, Divoux et al. 2009) (Hirai, Ohyanne et al. 2014) and humans as well (Divoux, Tordjman et al. 2010). However, mast cells have been observed promoting fibroblast growth and collagen synthesis also in other tissues, such as liver (Armbrust, Batusic et al. 1997) (Shen 2008) and lung (Edwards, Cruz et al. 2005).

Neutrophils

Belonging to the innate immune system, neutrophils are the leukocyte subpopulation arriving first in case of acute inflammation, with the primary function of producing MCP-1 and other chemokines to promote the recruitment of inflammatory monocytes (Soehnlein, Zernecke et al. 2008). As mast cells, neutrophils also accumulate in WAT during obesity (Rouault, Pellegrinelli et al. 2013) and it is possible that they have a close modus operandi. Their transient infiltration is associated with the weight gain and IR caused by high-fat feeding (Elgazar-Carmon, Rudich et al. 2008). Neutrophils accumulate already 1 hour after a high caloric meal, rich in glucose or fat, in association with macrophages, and the expression of their specific activation markers was observed elevated (Mohanty, Hamouda et al. 2000) (van Oostrom, Rabelink et al. 2004). Neutrophils accumulate already 1 hour after a high caloric meal, rich in glucose or fat, in association with macrophages, and the expression of their specific activation markers was observed elevated (Mohanty, Ivantes et al. 2010) (Kim, Voshol et al. 2004).

In WAT and liver of diet-induced obese mice, the activity of neutrophil-specific elastase represents a good marker of the amount of infiltrated neutrophils. Notably, neutrophil elastase can degrade the insulin receptor substrate 1 and reduce insulin-induced Akt phosphorylation in adipocytes (Talukdar, Oh da et al. 2012). Through this mechanism neutrophils could participate in obesity-associated IR, in tandem with activation of the TLR-4-dependent AT inflammation. Moreover neutrophils were shown to be involved in tissue damage via the production of MMP-9 and reactive oxygen species (Mantovani, Cassatella et al. 2011). More recently, our team, using a cocultures of adipocytes and endothelial cells in a 3D Setting, proved that conditioned media from neutrophils from obese subjects have increased angiogenic capacities, possibly through the release of multiple angiogenic molecules (IL-8, CXCL4, MMP-8, MMP-9, TIMP1) (Rouault, Pellegrinelli et al. 2013). However, despite this progress, certainly more mechanisms remain to be discovered in order to elucidate the apparent contradiction of elastase deficiency in obesity (Chmelar, Chung et al. 2013).

Macrophages

Macrophages are a heterogeneous population of myeloid-derived mononuclear cells that are a critical component of the innate immune response (Gordon and Taylor 2005). They are resident in all tissues of the body, being particularly augmented in tissues that are frequently exposed to exogenous and endogenous antigens and toxins, such as the lungs and liver. Their high plasticity allows these monocyte-derived cells to adapt to the environment and the different bioactive molecules they are exposed to (Dalmas, Tordjman et al. 2011). Once abandoned circulation, macrophages can entry to normal tissues (M2-like macrophages, “resident”) or be recruited to inflammatory sites (infections, injury/cell death and exposure to toxic substances, such as FFA in obesity) returning to basal state when the inflammation is resolved (M1-like macrophages, “effector”) (Italiani and Boraschi 2014). Notably, when inflammation becomes chronic, as observed in the pathological conditions comprised in MetS, macrophages and immune system in general steadily participate in the pathophysiology of the associated metabolic abnormalities. However, it is important to consider that the definition of M1 and M2 macrophage immune activation (classic vs. alternative, also M1 and M2, respectively) originated from the pre-genomic era, when few markers could be used to establish differences and similarities in macrophage responses to stimuli (Martinez and Gordon 2014). At present, we know that precisely defining in vivo a functional phenotype of macrophages based on this dichotomy is quite hard. Knowing

that M1 and M2 stimuli do not exist alone in tissues and that, depending on micro-environmental signal complexity, macrophages assume and/or dynamically change their phenotype, the many and apparently opposed macrophage functions have to be acknowledged (Martinez and Gordon 2014). Currently, this over-simplified view of the two functionally polarized states M1 and M2 based on selected macrophage markers is still found useful to characterize stimuli effects in specific contexts (Mantovani, Sica et al. 2004) (Figure 13). For example, in the context of specific pathologies, such as autoimmunity, sepsis, cellular stress, or fibrosis, macrophages are defined M1-like, since they respond producing a panel of M1 (Th-1) proinflammatory cytokines, the prototypical ones being TNF α , IFN γ and IL1 β (Huang, Metlakunta et al. 2010).

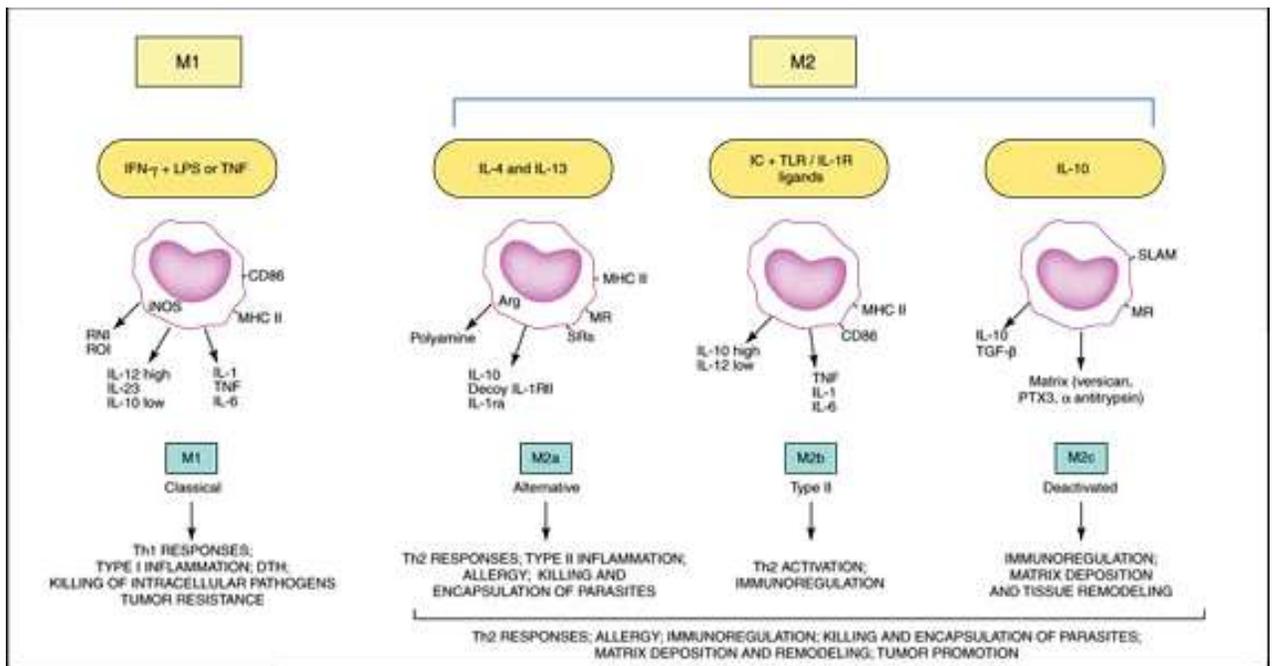


Figure 13. Inducers of macrophage polarization: M1 and M2 phenotypes (Mantovani, Sica et al. 2004).

The M1-M2 dichotomy has been used by Lumeng and colleagues to describe what happens in WAT during obesity. The proposed “phenotype switch” model represents the effects of obesity on adipose tissue macrophage (ATMs) recruitment, with increase of M1-like macrophages that shift the noninflammatory milieu maintained by M2-like resident macrophages toward a proinflammatory state (Fujisaka, Usui et al. 2009) (Lumeng, Deyoung et al. 2007) (Weisberg, Hunter et al. 2006) (Kamei, Tobe et al. 2006). The concept that obesity polarizes ATMs toward a classically activated state has been recently questioned (Xu, Grijalva et al. 2013), arguing in favor of the magnitude of macrophage accumulation in obesity. In light of these observations, for the purpose of this Thesis it is important focusing on the process of macrophage infiltration and eventually polarization in WAT in relation to fat mass changes. In states of dysfunctional lipid metabolism, such as obesity and lipodystrophy, the number and activity of macrophages in WAT are increased in rodents (Weisberg, McCann et al. 2003) (Xu, Barnes et al. 2003) (Briand, Le Lay et al. 2011) (Poirier, Shapiro et al. 2006) and humans (Cancello, Tordjman et al. 2006) (Aron-Wisniewsky, Tordjman et al. 2009). Local environment clearly influence ATMs polarizing capacity.

However in vivo the macrophage populations are much more dynamic, as demonstrated by the mixed M1/M2 phenotype observed for ATMs in obesity and in lipodystrophy, although with very different characteristics (Herrero, Shapiro et al. 2010).

Adipose tissue represents a lipid-rich environment, where macrophages develop and differentiate, most probably adapting to it. In conditions where lipid homeostasis is altered, excess of saturated fatty acids affect the adipose tissue at multiple cellular levels, in particular for macrophages affecting their number, activation state and function (Hill, Reid Bolus et al. 2014). Nevertheless, the developmental program and tissue-specific functions of ATMs, i.g. lipid metabolism, have not been deeply explored yet, either in normal or pathological settings. Cellular functions of ATMs are possibly regulated by adiposity and conversely ATMs have been shown to participate in local lipid turnover (Xu, Grijalva et al. 2013). In metabolically altered conditions, macrophage number is increased in WAT, due to the induction of a precise transcriptional program of chemokines, such as CC chemokine ligand 2 (CCL2), CCL5, and CCL3, as well as their receptors, CC chemokine receptor 2 (CCR2), and CCR5. However, scientific literature shows conflicting results about these mechanisms of recruitment (Xu, Grijalva et al. 2013). Aside from blood monocyte recruitment possibly due to adipocyte death, chemokine or FFA release, macrophage number can be influenced by recruitment-independent mechanisms such as impaired apoptosis, decreased tissue egress (migration) and increased in situ proliferation (Amano, Cohen et al. 2014).

In obesity, next to systemic adiposity, insulin sensitivity is tightly correlated with macrophage number in WAT. The role of macrophages in influencing insulin sensitivity has been demonstrated also in liver and muscles. Liver-specific macrophages, named Kupffer cells (KCs), have a role in high fat or high sucrose diet-induced alterations in hepatic lipid metabolism and insulin sensitivity. Interestingly, in vitro stimulation of KCs with saturated fatty acids alone was sufficient to induce decreases in hepatocyte fatty acid oxidation and increases in fatty acid esterification, although with no alteration of triglyceride levels (Huang, Metlakunta et al. 2010). Similarly, in a mouse model of hepatic steatosis and lipodystrophy (A-ZIP/F-1 transgenic mice), lipid accumulation in the liver leads to an increased macrophage recruitment, as a consequence of the high local and circulating level of CCL2, in association with a pro-inflammatory state (Yang, IglayReger et al. 2009). The association between circulating FFA and inflammation has been demonstrated also in skeletal muscle from obese subjects, in which an higher macrophage infiltrate was reported (Varma, Yao-Borengasser et al. 2009). Various studies have proposed a potential contribution of lipid intermediate accumulation and the associated immune-inflammatory response in muscles to the reduction of insulin sensitivity (Kraegen, Cooney et al. 2008).

Finally, though aware of the primary role of these cells, i.e. antigen presentation, it is important to follow closely recent evidence about their metabolic role. Indeed macrophages are able to substantially participate to iron homeostasis regulation in different settings and with specific functions (Recalcati, Locati et al. 2010) (Ganz 2012) (Jung, Mertens et al. 2015). Moreover macrophages could influence insulin sensitivity, behind their participation to tissue inflammation (Lee, Pamir et al. 2014)

Dendritic cells

Following over time the research that worked on defining and characterizing different populations of dendritic cells (DCs) the complexity of the DC family is evident. At the present we know that, these

sentinels of the immune system (Banchereau and Steinman 1998), whose main goal is to survey the tissues and instruct the adaptive immune system in response to peripheral cues, can be found in lymphoid organs but also in most non-lymphoid tissues (Merad, Sathe et al. 2013). An additional complexity is that DCs, contrary to macrophages, do not have phenotypic markers that can specifically identify them. Thanks to their superior ability to migrate toward lymph nodes (Forster, Braun et al. 2012), DCs are extremely effective cells for processing and presenting phagocytosed antigens to T lymphocytes. Their role in inducing both protective immunity as well as immunological tolerance is well represented in case of tissue injuries (inflamed state) and skin immunosurveillance (steady state) (Ginhoux, Collin et al. 2007). Depending on the nature of the stimuli and the tissues in which they arise, DCs phenotype will change in response to inflammation. The regulation of differentiation and function of DCs has been mostly explored in tissues such as skin, lung and intestine, whereas interest on IR resistance, concerning DCs involvement in the chronic low inflammatory grade characterizing WAT, are not that numerous. Obese mice and humans present the accumulation of these innate cells possibly contributing to insulin resistance ((Wu, Perrard et al. 2010) (Yekollu, Thomas et al. 2011). More recently, a specific set of DCs has been shown in obese WAT intervening in the complex process of obesity-associated IR (Bertola, Ciucci et al. 2012). Those DCs take part in the regulation of tissue inflammation, influencing the induction of a Th17 cell response that could substantially contribute IR (Bertola, Ciucci et al. 2012). Once again FFA, elevated in the dysfunctional WAT environment, i.e. obesity and lipodystrophy, are possibly the starting point of the local inflammation causing IR, as for macrophages (Nguyen, Favelyukis et al. 2007).

Natural killer cells

As for the other innate immune cells, natural killer cells (NK) can be found in a variety of tissues with the primary functions of controlling inflammation and maintaining immune homeostasis (Fu, Tian et al. 2014). Depending on the microenvironment, NK cells can have distinct functions, as NK(tolerant) , NK(cytotoxic) and NK(regulatory) cells, inhibiting or enhancing immune reactions. Mainly known for their regulatory proprieties in various disease contexts, such as rheumatoid arthritis joints (Pridgeon, Lennon et al. 2003) or hepatitis B therapy (Micco, Peppia et al. 2013), many aspects and functions of NK cells remain to be unveiled, especially their phenotype and function in adipose tissue (Caspar-Bauguil, Cousin et al. 2005) (O'Rourke, Gaston et al. 2013) (Chung, Markiewicz et al. 2014). The detailed study of NK cells infiltrating adipose tissue of obese subjects has revealed that these cells, known to be able to recognize lipid antigens, present a specific activated phenotype, prone to promote IR and WAT inflammation in obesity (O'Rourke, Gaston et al. 2013). And even more recently the potential mechanisms through which these cells promote visceral adipose tissue inflammation and insulin resistance in obesity were elucidated: a phenotypically distinct population of tissue-resident natural killer (NK) cells was described to be able to proliferate in situ and through interferon- γ (IFN- γ) production to induce adipose stress and pro-inflammatory macrophage differentiation in obesity (Wensveen, Jelencic et al. 2015).

2. Adaptive immunity

The adaptive immune system, in collaboration with innate cell and non-haematopoietic cell populations, has the complex role of promoting immunity and regulating tolerance at the same time. Recent findings

have clearly demonstrated the presence of adaptive lymphocytes, such as T and B cells, in adipose tissue, even if their function there has not been well established yet (Chatzigeorgiou, Karalis et al. 2012). For the purpose of this Thesis the alterations related to the state and function of adaptive cells will focus on the meta-inflammatory response to obesity, therefore on adipose tissue.

Lymphocytes

Compared to secondary lymphoid tissues adipose tissue contains few naïve T- cells and a high amount of effector/memory type CD4+ T- cells, with the main role of regulating adaptive immunity. During obesity chronic inflammation involves altered lymphocyte function, in addition to the previously described macrophage accumulation. In particular T- cells with a prevalent pro-inflammatory role accumulate in WAT, contributing to the overall inflammatory state associated to the development of metabolic abnormalities. Meanwhile resident T- cells, known for their anti-inflammatory profile, are reduced in obesity, often depleted (Chatzigeorgiou, Karalis et al. 2012). A clear example is given by regulatory T- cells, whose presence may represent a crucial factor to preserve insulin sensitivity. In abdominal WAT of normal mice an abundant population of CD4(+) Foxp3(+) T regulatory (T(reg)) cells was found, but their amount was significantly decreased in insulin-resistant models of obesity (Feuerer, Herrero et al. 2009).

All the different subpopulations of lymphocytes, such as Th1 lymphocytes (CD4+), cytotoxic lymphocytes (CD8+) or B cells have been implicated in AT inflammation (Chatzigeorgiou, Karalis et al. 2012), (Nishimura, Manabe et al. 2009). In the case specific of T- cells, using the high fat diet-induced obesity model, it has been shown that different sets of T- cells increase in number and activation state in WAT ((Xu, Barnes et al. 2003),(Wu, Ghosh et al. 2007). Obese adipose tissue has been hypothesized as a unique playground where the dominating ATMs interact with T- cells to establish a crucial network that perpetuates meta-inflammation (Cho, Morris et al. 2014). Interestingly, a T- cell accumulation, similar to the one described in obese adipose tissue, has been recently reported in a mouse model of weight cycling (Anderson, Gutierrez et al. 2013) In this study, mice were cycled between HFD and low-fat diet (LFD) for three 9-week diet periods and their metabolic and immunological profile was evaluated compared with mice that gain weight under HFD for the duration of the study (27 weeks). The increased accumulation of CD4(+) and CD8(+) in WAT of weight cycling mice demonstrate that an altered local adaptive immunity can participate to worsen metabolic dysfunction, not only during obesity but also when weight loss alternates with weight regain (weight cycling) (Anderson, Gutierrez et al. 2013). In obesity, the migration of these pro-inflammatory T- cells in WAT is coordinated by RANTES/CCL5, a chemokine specifically increased in obese WAT (Wu, Ghosh et al. 2007) and not involved in T- cells accumulation during weight cycling. The pathophysiological significance of the increased T- cells in AT in obesity is under current investigation. Ballantyne and colleagues hypothesized that T- cells reduce the ability of accumulating TG in WAT, as observed in vitro during adipocyte differentiation, and this could increase ectopic TG deposition in liver and skeletal muscle, and thus cause IR in these organs (Wu, Ghosh et al. 2007). More recently Lumeng and colleagues demonstrated the importance of MHC Class II (major histocompatibility complex class II) in activating and maintaining the loop between adipose tissue resident CD11c⁻, T- cells, and CD11c⁺ responsible for meta-inflammation (Cho, Morris et al. 2014). Finally, despite the well-established demonstration that adipokines have a role in modulating the function of immune cells, in particular T- cells (Fantuzzi, Sennello et al. 2005), the interactions between lymphocytes and adipocytes has received the full attention only recently.

Natural killer T- cells

Natural killer T- cells (NKT) are a heterogeneous group of T- cells that share properties of both T- cells and natural killer cells. They have previously been associated with obesity for their potential regulatory phenotype (Caspar-Bauguil, Cousin et al. 2005). Similarly to NK cells, NKT cells have been shown to contribute to inflammation in obesity (Sintes, Cuenca et al. 2013). In the obesity context, the abundance of self and foreign lipid antigens in AT works well with the lipid-sensitive and antigen-presentation functions carried out by these cells (lipid/CD1d complex binding). Similar mechanisms have been proposed for another family of NKT cells, invariant natural killer T (iNKT), shown to be involved in multiple metabolic diseases (Hammond, Poulton et al. 1998) (Hong, Wilson et al. 2001) (Lee, Putnam et al. 2002) (Matzinger and Kamala 2011). As for the above mentioned T-regs, iNKT cells are an example of anti-inflammatory cells greatly reduced in obesity (Lynch, Nowak et al. 2012). This evolutionarily conserved innate NK subpopulation represents a large proportion of lymphocytes in human and murine adipose tissue, and their number can be rescued after weight loss. The ability of iNKT cells to decrease body fat, circulating TG and consequently fatty liver, and improve insulin sensitivity in mice offers an interesting potential prospective for the management of human obesity (Lynch, Nowak et al. 2012).

Helper T- cells

Helper T- cells (Th) are probably the most important cells in adaptive immunity, as they are required for almost all adaptive immune responses. Through the release of T-cell cytokines, these cells help suppress or regulate immune responses of other immune cells, not only B- and T- cells, but also macrophages (at site of infection). Th effector cells stimulate an inflammatory response by recruiting more phagocytic cells into the infected site. They can secrete cytokines that act on the bone marrow to increase the production of monocytes and neutrophils, they can secrete chemokines and increase adhesion capacity of endothelial cells to allow more efficient recruitment of innate cells from the blood into the site of infection. In obesity, as regulatory T- cells, the number of Th effector cells has been shown to diminish. In HFD-fed mice CD4(+) helper cells decrease in epididymal adipose tissue (Nishimura, Manabe et al. 2009). However in humans the balance of Th1/Th2 has been shown to be modulated by leptin cells toward a Th1 phenotype and the pro-inflammatory profile of the secreted cytokines may participate in the in obesity-associated inflammation (Pacifico, Di Renzo et al. 2006).

Gamma delta T- cells

Gamma delta+ T ($\gamma\delta$ T)-cells represent a small population of overall T-lymphocytes (0.5–5%) and have variable tissue distribution in the body. The functions of $\gamma\delta$ T- cells have been studied mostly in relation to epithelial damage in obesity. When $\gamma\delta$ T- cells reside in the epidermis, they are also referred to as dendritic epidermal T- cells. These cells are impaired in number and activity in the skin of obese mice, compromising their ability to resolve tissue damage (Taylor, Mills et al. 2010). In obesity, metabolic disease, and type 2 diabetes, these epidermal cells become dysfunctional due the chronic inflammatory environment. Their negative influence on keratinocyte homeostasis is one of the mechanisms involved in the commonly associated skin pathologies (Taylor, Costanzo et al. 2011). FACS analysis of the stroma-vascular fraction of white adipose tissues demonstrated that the regulation of T cells in adipose tissue could be depot dependent in obesity (Caspar-Bauguil, Cousin et al. 2005). In fact, using a diet rich in fat, it was shown that lymphocytes in epididymal WAT display an "ancestral" immune system phenotype,

with up to 70% of natural killer (NK), $\gamma\delta$ T- and NKT cells among all lymphocytes. Moreover, obesity decreased the percentage of NK cells in epididymal fat, whereas increased $\gamma\delta$ T- cells in subcutaneous fat. However, only more recently the potential role of this particular subpopulation of adaptive cells has been investigated in the context of metabolic disorders. In 2015, research conducted on at Baylor College of Medicine in Houston (Texas) suggested that $\gamma\delta$ T- cells could be used to treat and prevent type 2 diabetes caused by obesity (Mehta, Nuotio-Antar et al. 2015). Mice lacking $\gamma\delta$ T- cells had reduced inflammation in the liver, skeletal muscle and adipose tissue, and a consequent reduced systemic IR, under a Western-type diet, rich saturated fats and sugar (Mehta, Nuotio-Antar et al. 2015). Consequently, the phenotypic and functional plasticity of $\gamma\delta$ T- cells, in particular in the cross-talk with other immune cells, is driving increasing attention not only in the field of immunology (Paul, Shilpi et al. 2015) but also immunometabolism.

B lymphocytes

Recently a specific B cell population has been described within adipose tissue, regulatory B (Breg) cells. Breg cells are identified as cells able to produce interleukin-10 constitutively in WAT (Seida, Mitri et al. 2014). In this study the role of IL10 specifically produced by Breg cells in protecting mice from the deleterious effects of high fat diet was demonstrated. Breg cells could have role in controlling the progression of adipose tissue inflammation in obesity. During onset of obesity, following reverse mechanisms, Breg cells decrease, while CD8 effector T- cells increase in number (Nishimura, Manabe et al. 2009). Whether the recruitment of CD8 T- cells to the adipose tissue occurs through antigen-dependent or -independent processes is not clear yet. However, cytotoxic CD8 (+) T- cells infiltrating the WAT may contribute to the recruitment, differentiation and activation of macrophages and consequently to WAT inflammation. For instance, B cells WAT infiltration is a very early response to high-fat feeding and can even precede the accumulation of macrophages (Nishimura, Manabe et al. 2009). Their role in affecting systemic IR and worsening glucose metabolism through modulation of T- cells and producing pathogenic antibodies has been demonstrated (Nishimura, Manabe et al. 2009) (Winer, Winer et al. 2014). Unable to produce an unique profile of pathogenic IgG autoantibody, high fat diet-induced obese mice lacking B cells are protected from IR despite weight gain (Winer, Winer et al. 2011). The vicious cycle of interactions between CD8(+) T-cells, macrophages and adipocytes clearly indicates how these interactions are critical for the initiation and propagation of adipose inflammation in obesity.

In addition to the above described cell population, adipose tissue is enriched with innate lymphoid type 2 cells (ILC2), known to support the activity of Treg and contribute to insulin sensitivity in lean individuals.

To summarize, the innate and the adaptive immune cells represent a highly dynamic system in WAT, particularly studied and characterized during the transition from a lean to an obese state (Figure 14).

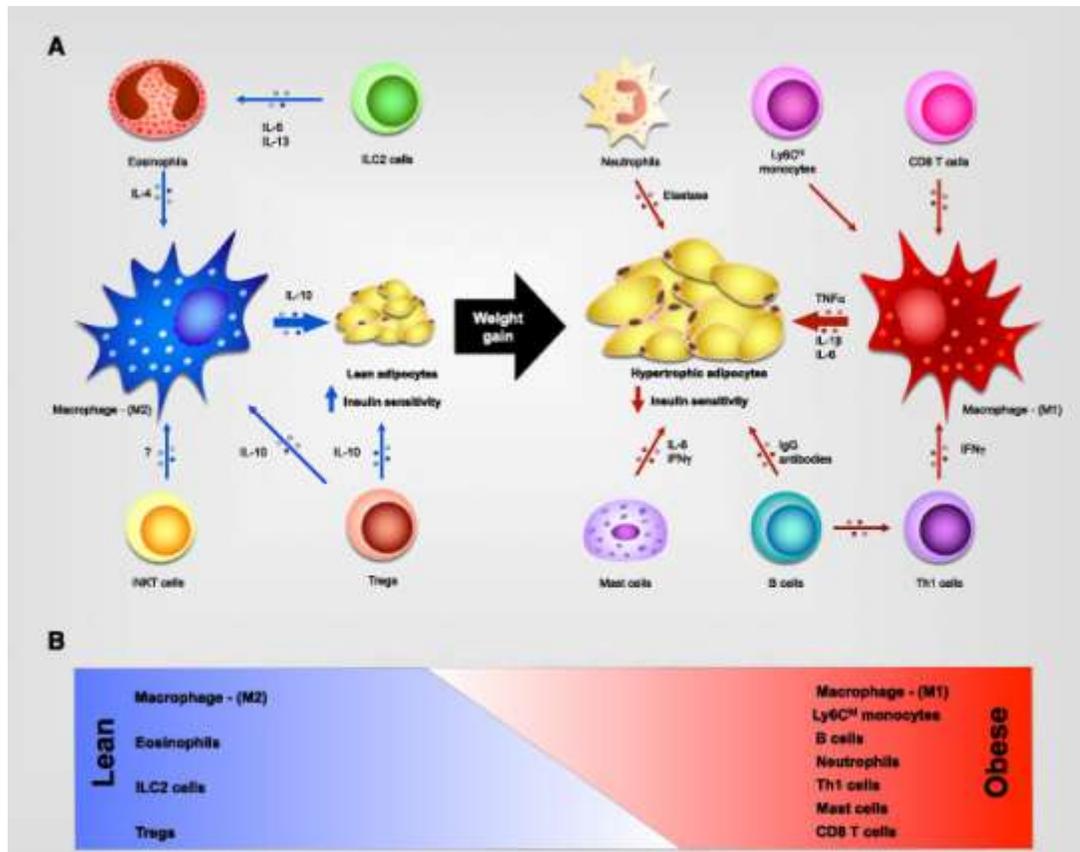


Figure 14. Leukocyte subsets in human adipose tissue (Feng, Ji et al. 2013).

3. Immunological memory

An important role of adaptive immunity is immune surveillance (Sallusto, Lenig et al. 1999). Effector (TEM) and memory (TCM) cells are T-cells that have encountered and responded to antigens (also called antigen-experienced T-cells). They are thought to be distributed to all tissues in the body. In humans, isoforms of CD45 (a phosphatase involved in cell signalling) is used to identify these cells, when analyzed in opposition to CCR7; whereas, in the mouse, the specific markers to identify memory cells are CD44 versus CD62L. To clarify with a practical example, in our experimental setting (flow cytometry on the stromal vascular fraction from WAT of linoleic and conjugated linoleic acid-treated mice), we used the above mentioned mouse markers to divide antigen-experienced T-cells in: central memory (TCM, CD44+CD62L+), effector memory (TEM, CD44+CD62L-) and naive T- (CD44+CD62L+) cells. On one hand TEM cells, as the name says, are able to migrate to inflamed tissue and display immediate effector function. On the other hand TCM cells express lymph-node homing receptors and can differentiate into effector cells upon secondary stimulation only. These cells are not restricted to lymphoid organs but can be present in significant numbers in nonlymphoid tissues. Maintaining functional memory responses is essential to respond effectively to infections and other potential endogenous and exogenous danger signals (lipids?). Obesity has been linked to numerous health problems and chronic diseases, including increased infection susceptibility; as a consequence it is critical to understand how the immune cell responsiveness can be altered by an altered microenvironment.

4. Immunity and inflammation: chemokines and other recruitment factors

The localization and dynamic distribution of the above described immune cells is controlled by various factors, first of all, chemokines and their receptors. The recruitment of leukocytes inside the tissues happens from the endothelium through sequential steps, such as integrin-dependent arrest and transmigration. This Chapter will focus on the specialized roles of chemokines during this process, looking specifically at cell infiltration inside metabolically compromised tissues. The focus will be in particular on macrophages, knowing that they have an impact on metabolism and, vice versa, tissue metabolic microenvironment affects their acquired functional phenotype (Biswas and Mantovani 2012) and also because it is the main population recruited in WAT in obesity and in the lipotrophy model we used for our experiments (Poirier, Shapiro et al. 2006). Indeed in obese subjects WAT macrophages accumulation can arrive to such a magnitude that almost half of all cells in a fat depot are macrophages (Weisberg, McCann et al. 2003). Strategies to control leukocyte mobilization and accumulation in tissues are needed today not only when thinking about potential new therapies in obesity, but also in other contexts, such as cancer immunotherapy (Vela, Aris et al. 2015) (Kufareva, Salanga et al. 2015). Modulating the activity of chemokines and/or their receptors would allow increase migration of cells critical for their high endocytic and phagocytic capacity, such as DCs or macrophages, in tissues that would require their action, such as in tumors. Alternatively, reduction of macrophage infiltrating obese WAT could be a therapeutic option conducted to improve inflammation and the associated deleterious metabolic alterations (Kanda, Tateya et al. 2006).

Adipochemokines

Chemokines are potent chemoattractants for all types of leukocytes, including hematopoietic precursors, mature leukocytes of the innate immune system, as well as naive, memory, and effector lymphocytes. Most of the inflammatory chemokines are produced by WAT macrophages and their upregulation precedes systemic IR. In particular several studies have reported the increased expression of chemokines and their receptors in obesity in mice (Xu, Barnes et al. 2003) as well as in humans (Dahlman, Kaaman et al. 2005) (Huber, Kiefer et al. 2008). The typical inflammatory chemokines linked to inflammation and obesity include: CCL2, CCL3 and CCL5, CXCL1, CXCL2 and CXCL8 (Weisberg, Hunter et al. 2006) (Xu, Barnes et al. 2003) (Curat, Wegner et al. 2006). In particular obese humans display elevation of certain specific chemokines and their coupled receptors, including CCL2/MCP-1, CCL3/MIP1 α , CCL4/MIP-1 β , CXCL2/MIP-2 α , and CXCL3/MIP-2 β in both adipocytes and PBMCs, elevation that could be possibly mediated by certain dietary choices (Munoz and Costa 2013). It is important to underline how different families of chemokines present positive feed-back mechanisms between each other, surely accelerating the progression of inflammation. Controlling chemokines has been demonstrated to have therapeutic potential. In tissues other than WAT, there are studies showing how neutralization of specific chemokines, i.e. CCL3 and CCL2/MCP1, can be effective in reducing fibrosis development (Smith, Strieter et al. 1994), (Belperio, Keane et al. 2001).

CCL2/CCR2

The most studied couple of chemokine/receptor in the context of obesity and IR is represented undoubtedly by CCL2 (MCP1/ Monocyte Chemoattractant Protein 1) and its receptor CCR2.

Despite the multiple studies showing the increased mRNA expression of CCL2 in the context of obesity (Yu et al., 2006), its role in contributing to WAT inflammation is still under debate (Kirk, Sagawa et al. 2008).

Research in diet-induced obese mice has demonstrated that CCR2 is also critical in the process of macrophage accumulation in WAT (Weisberg, McCann et al. 2003) (Ito, Suganami et al. 2008). In particular, during diet-induced obesity, CCR2 is overexpressed in recruited macrophages and not in resident tissue macrophages, indicating the important role of this receptor for the recruitment of inflammatory macrophages (Lumeng, Deyoung et al. 2007). Considering that this is a receptor common to other chemokines, including CCL7, CCL12, CCL13, it is plausible that other chemokines compensate the absence of CCL2 activity, inhibited or genetically modified, explaining the conflicting results obtained in mice.

CCL5/CCR1-3-5

CCL5 (RANTES, Regulated upon Activation, Normal T cell Expressed and Secreted) plays an important role in immune cell recruitment. Studied in particular in the pathology of atherosclerosis (Eriksson 2004), (Zernecke, Shagdarsuren et al. 2008), opposite effects of the couple CCL5/CCR5 (pro-atherogenic) and CCL5/CCR1 (atheroprotector) have been reported in mouse models, underlying complexity and specificity of functions. In HFD-fed mice this chemokine and its receptors have been observed increased at the mRNA and protein level in WAT (Wu, Ghosh et al. 2007) and their function has been related to macrophage and lymphocyte recruitment. The team of K. Clément has demonstrated that circulating CCL5 is increased in obese subjects, in line with other studies (Huber, Kiefer et al. 2008). The main cellular component that expressed CCL5 in the stroma vascular fraction from obese patients was represented by macrophages and CCL5 mRNA positively correlated with M1 markers, such as CD11b and CD11c, and protected these cells from apoptosis (Keophiphath, Rouault et al. 2010). This chemokine can be sensed by three different receptors, CCR1, CCR3 and CCR5, in common with other chemokines, such as CCL3 /MIP1 α and CCL7/MCP-3 known to be increased in WAT of obese subjects (Huber, Kiefer et al. 2008).

CXCLs/CXCR2

Other chemokines have been shown to be implicated in obesity, such as CXCL1, CXCL2, CXCL5 and CXCL8 (Nunemaker, Chung et al. 2014) (Rouault, Pellegrinelli et al. 2013) (Chavey, Lazennec et al. 2009) (Bruun, Lihn et al. 2004) that can be produced by circulating monocytes, macrophages, but also non immune cells. In common these chemokines bind to the same receptor, CXCR2, together with other CXCL- family members (Neels, Badeanlou et al. 2009). The therapeutic potential of this receptor has been evaluated in a variety of diseases, in animal models and clinical trials, and promising results have been obtained (Stadtman and Zarbock 2012). In the context of obesity, CXCR2-deficient mice challenged with HFD showed a reduced infiltration of macrophages in WAT, associated with an improvement in insulin sensitivity (Neels, Badeanlou et al. 2009). Studies of CXCR2 inhibition, with the aim of determining the efficiency in blocking immune cell recruitment showed a good tolerance of the specific inhibitor in short term time course, whereas over long term, some toxic effects were noticed (Lazaar, Sweeney et al. 2011), (Day and Link 2012). More recently blocking CXCR2 has been shown to reduce neutrophil

accumulation in pancreas, dampen the diabetogenic T-cell response taking apart the later development of the autoimmune type 1 diabetes, opening a potential therapeutic avenue for this disease as well (Diana and Lehuen 2014). Between the chemokines interacting with this receptor, CXCL1 and CXCL5 are the most studied in metabolic altered contexts. CXCL1 was recently shown to attenuate HFD-induced obesity and the associated IR (Pedersen, Olsen et al. 2012). When overexpressed in skeletal muscle CXCL1, possibly through improvement in tissue fatty acid oxidation, reduced visceral and subcutaneous fat mass of HFD-fed mice, without affecting their food intake (Pedersen, Olsen et al. 2012). Whereas CXCL1 has been shown to be important at the muscle level, CXCL5 is mostly found as an adipose tissue derived factor (Chavey, Lazennec et al. 2009). CXCL5 is increased in ob/ob and db/db and HFD-fed mice and macrophages were the main population expressing this chemokine within WAT. Inhibition of CXCL5 through antibody reduced the HFD-induced insulin resistance. However it is important to note that blocking its receptor, CXCR2, is more efficient than neutralizing one of its numerous chemokines, suggesting the compensatory role that other recruitment factors can adopt. In human obesity, the systemic level of CXCL5 is increased and recently it has been indicated as a new marker of atherosclerosis (Chen, Yang et al. 2011).

CXCL10/CXCR3

CXCL10 (IP-10 / Interferon gamma-induced protein 10) is a chemokine produced by human mature adipocytes. Its secretion positively correlates with obesity and type 2 diabetes, conditions in which can be induced by interferon gamma (IFN γ) (Herder, Hauner et al. 2007). In massively obese patients, candidate for bariatric surgery, our team has recently shown a significant increase in systemic levels of CXCL10 compared to lean subjects (Dalmás, Rouault et al. 2011). However, unlike CCL2, CXCL8 and CCL5, serum level of CXCL10 does not decrease after a drastic weight loss induced by surgery (Rouault, Pellegrinelli et al. 2013). The critical role of this chemokine in tissue inflammation has been demonstrated also in liver pathologies. Indeed due to its pathogenic role in the development of steatohepatitis in mice, CXCL10 has been proposed as potential non-invasive biomarker for NASH patients (Zhang, Shen et al. 2014).

CX3CL1/CX3CR1

By functional genomics experiments, Shah and his team have shown that CX3CL1 (fractalkine) is upregulated in human mature adipocyte during inflammation induced by LPS. Secretion of CX3CL1 is increased in obese subjects, being particularly elevated in visceral adipose tissue more than in the subcutaneous WAT. In addition, CX3CL1 favors the adherence of a cell line of monocytes (THP-1) on human adipocytes. A receptor polymorphism, CX3CR1, is associated with metabolic disorders (insulin resistance index HOMA, circulating leptin and hip circumference). Blood levels of CX3CL1 are higher among type 2 diabetic subjects than in the control subjects (Shah, Leik et al. 2010).

In conclusion, many chemokines and their receptors have been detected in adipose tissue in recent years and that their production and function, undoubtedly redundant, are influenced by obesity.

Cathepsins

However, in addition to chemokines, there are other factors important for leukocyte migration to the site of inflammation, including cathepsins. Cathepsins are proteases with different structure and actions

depending on the protein to degrade. Their chemotactic role has been mostly studied in atherosclerosis development. The expression of these proteases can be high in the cells of immune system, it is the case of Cathepsin X, and consequently they can potentially participate to the processes of inflammatory and immune responses in a variety of conditions. Cathepsin X, also known as cathepsin Z, regulates processes such as adhesion, T lymphocyte activation and phagocytosis through its interaction with beta2 integrins (Zhang, Shen et al. 2014). Contrary to other cathepsins, cathepsin X is not widely expressed in cells and tissues, but is restricted to immune system cells, mostly monocytes, macrophages and dendritic cells (Zhang, Shen et al. 2014). Interestingly, its upregulation has also been detected in the brain of patients with Alzheimer disease (Wendt, Zhu et al. 2007) and in gastric mucosa (Buhling, Peitz et al. 2004) (Krueger, Kalinski et al. 2005). Using two- and three-dimensional models that mimicked the extra cellular matrix, a recent study demonstrated that cathepsin-X-overexpressing T lymphocytes displayed an increased migratory activity and aggregation capacity (Jevnikar, Obermajer et al. 2008). Other Cathepsins are abundantly expressed in macrophages, i.e. isolated from human atheroma (Sukhova, Shi et al. 1998), or in other pathologic contexts, such as infections (Muller, Faulhaber et al. 2014), bone remodeling (Buhling, Reisenauer et al. 2001), and cancer (Shree, Olson et al. 2011), (Gocheva, Wang et al. 2010). However cathepsins can be involved not only in inflammatory responses from the innate immunity, through TLR9 signaling in particular, but also adaptive immunity, like antigen presentation (i.e. CD4+ T- cells). Recent in vitro experiments showed that Cathepsin S-deficient monocytes exhibit less migration activity through smooth muscle cell layer than wild-type monocytes. Moreover, during the last decade, these proteases have been recognized as critical in the degradation of extracellular proteins (Fonovic and Turk 2014). Macrophages, in particular, which are able to secrete these proteases, once activated, use them during the degradation process. Considered their involvement in a number of ECM-related diseases, such as osteoporosis, cancer and cardiovascular diseases, and their role in innate immune responses (mediated TLR9 in particular), further research is required also in the context of obesity.

Galectins

Galectins are a large family of animal lectins defined by their binding specificity for β -galactoside sugars. They present a broad variety of functions including mediation of cell-cell interactions, cell-matrix adhesion and transmembrane signaling. They are differentially expressed by various immune cells and their expression levels depend on cell differentiation and activation, in particular by macrophage and fibroblast. Galectins can play important roles in inflammation and immunologic responses, since they have chemotactic proprieties (Liu 2005). A total of 15 galectins have been identified. Galectin-3 (Lgal3) is the most widely studied member of this protein family. Lgal3 has been shown to actively participate to persistence of the inflammation, affecting virtually all inflammatory cells, including neutrophils and macrophages. Obese humans and mice present markedly elevated levels of Lgal3, systemically and locally (liver, subcutaneous and intra-abdominal WAT). Current data suggest that FFA and IL-6 increase Lgal3 in 3T3-L1 adipocytes, identifying one of the mechanisms possibly contributing to higher Lgal3 levels in obesity (Krautbauer, Eisinger et al. 2014). Several studies have shown upregulation of Lgals3 in association with the concomitant activation of macrophages in different contexts. In particular, in recent years, Lgals3 has gained prominence as a central regulator of chronic inflammation and fibrogenesis (In Galectins and Disease Implications for Targeted Therapeutics; Klyosov, A. et al., ACS Symposium Series; American Chemical Society: Washington, DC, 2012.). In particular,

Lgals3 has been shown involved in the development of organ fibrogenesis and scarring in kidney (Henderson, Mackinnon et al. 2008), in liver (Henderson, Mackinnon et al. 2006) and more recently in heart (Liu, D'Ambrosio et al. 2009) (Figure 15). In liver, for example, Lgal3 was demonstrated to affect tissue remodeling through macrophage activation. Macrophages in turn activate and differentiate fibroblasts into myofibroblasts, promoting collagen synthesis (Henderson, Mackinnon et al. 2006). Despite the association of Lgal3 with macrophage accumulation and fibrosis, in metabolic diseases this protein has been suggested to have a protective role against inflammation regulating adiposity and glucose homeostasis (Pejnovic, Pantic et al. 2013).

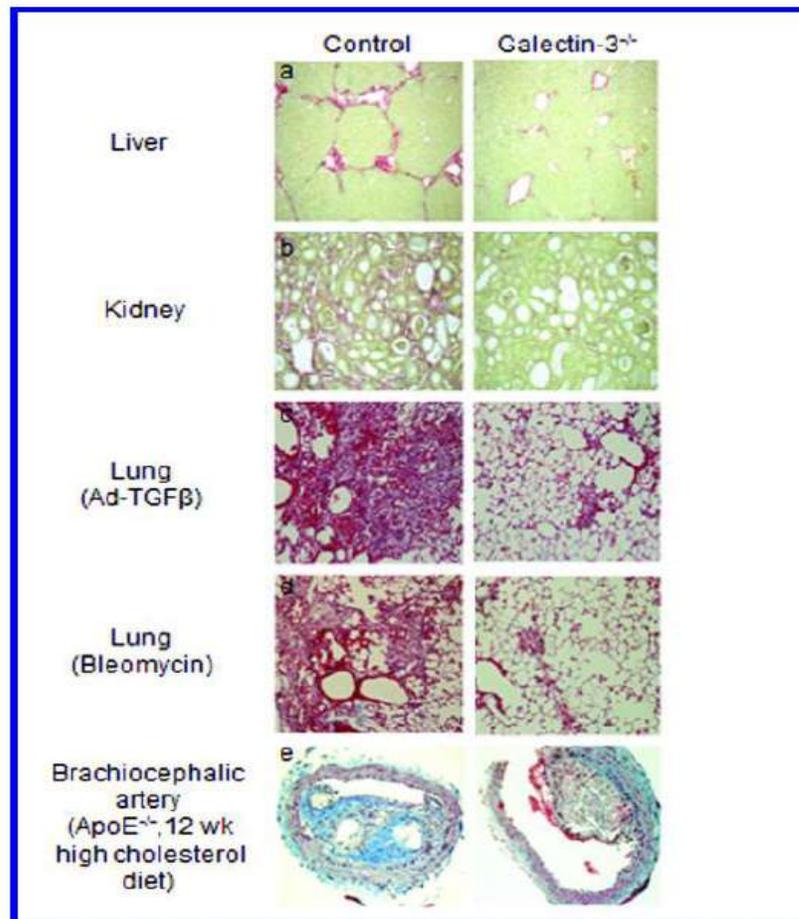


Figure 15. Galectin-3 regulates tissue fibrosis in multiple organs.

In conclusion, targeted immunomodulatory interventions on recruitment factors or specific cell subpopulations, in particular, could provide interesting therapeutic options towards amelioration of metabolic altered conditions, such as obesity.

E. Adipose tissue: cellular and structural alterations in obesity

1. Adipocytes: functions and dysfunctions

Of clear relevance for adipose tissue functionality is the diverse collection of proteins and sugars that surrounds cells in the tissue, representing the extra-cellular matrix (ECM). Only recently research started focusing on a thorough characterization of the metabolic impact of ECM on the altered adipocyte during obesity or during other metabolic conditions.

FFA are efficiently stored without harmful side effects in the form of glycerol. The resulting TGs are relatively inert and can be efficiently deposited in lipid droplets in the cytoplasm of essentially all cell types. The most efficient cellular depot of TGs in mammals is represented by the adipocyte. Adipocyte structure is adapted to store big quantities of TGs that can be hydrolyzed (mobilized) as FFA and glycerol when needed in other tissues. All oxidative tissues, but WAT itself also, can use FFA to generate energy through a process called β -oxidation. In WAT, β -oxidative capacity increases during adipocyte differentiation, as indicated by increased expression of PGC1 α , a regulator of mitochondrial biogenesis (Wilson-Fritch, Nicoloso et al. 2004). Whereas, in obesity and diabetes, adipocytes lose oxidative capacity, together with insulin sensitivity (Choo, Kim et al. 2006) (Keller and Attie 2010). Increasing the oxidative potential of the adipocyte has demonstrated efficacy in treating type 2 diabetes, as in case of thiazolidinedione drugs (Wilson-Fritch, Nicoloso et al. 2004). In response to metabolic challenging environments, adipocytes can adapt (Rutkowski, Stern et al. 2015) (Figure 19). Adipocyte highly specialized activity is regulated at the transcriptional level by a program of enzymes involved in triacylglycerol synthesis and degradation (FAS, ACC, PLIN, HSL, ATGL and more), in synthesis of adipokine (leptin, adiponectin, resistin, adiponectin), glucose transporters (GLUT4) and insulin receptors (IRS). All the mentioned components allow flexibility to the adipocyte in adapting to its nutrient environment, preserving its metabolic homeostasis (Figure 16). Local adipocyte physiology, including the WAT adipokine secretion (Cao 2014), is thus transferred at the systemic level with potentially beneficial or detrimental effects.

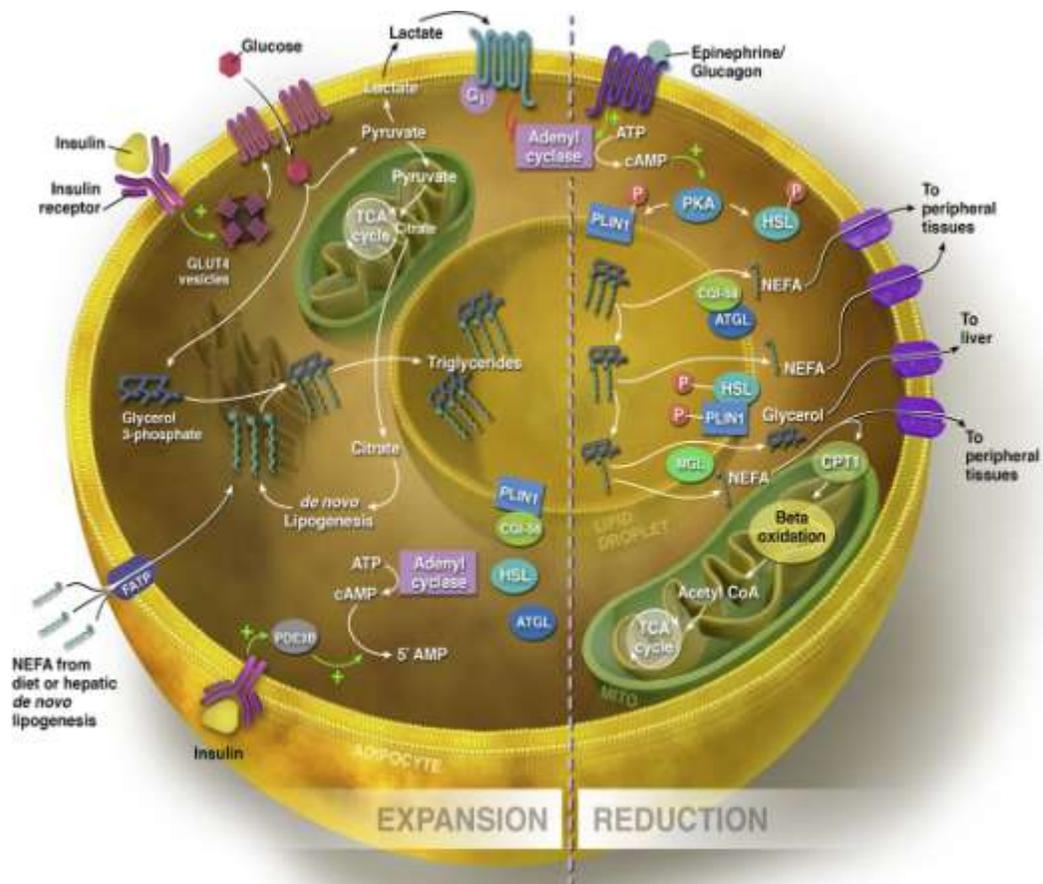


Figure 16. Adipocytes are flexible cells (Rutkowski, Stern et al. 2015).

The concept of lipotoxicity (Unger and Scherer 2010) in face of the nutritional pressures of obesity has been presented earlier in the Thesis. A nutritionally challenged microenvironment, besides mitochondrial dysfunction, inflammation and cell death, can submit adipocytes also to structural perturbations and modifications. For example it has been shown how increased concentrations of FFA the integrity of biological membranes, impairing their function and inducing endoplasmic reticulum (ER) stress. Endoplasmic reticulum, a continuous membrane extending in the cytoplasm of most types of eukaryotic cells, has been proposed to have a role in dysregulation of key metabolic tissues, including liver and adipose tissue. Its functions can vary depending on ER and cell type (Schroder and Kaufman 2005). In liver, in mice, it was suggested that ER can sense nutritional excess and translate that excess into metabolic and inflammatory responses, contributing to insulin resistance (Ozcan, Cao et al. 2004) (Boden, Song et al. 2011) (Kim, Kim et al. 2015). Other studies confirmed this link between ER stress and obesity, IR, and inflammation in animal and human tissues (Nakatani, Kaneto et al. 2005) (Boden, Duan et al. 2008). However the physiopathological role of ER in cellular adaptation to external signals, such as systemic insulin (Boden, Cheung et al. 2014) or lipids (Nivala, Reese et al. 2013) needs further investigation. In particular research on molecules participating to the signaling pathways termed the unfolded protein response, the response to ER stress, is currently revealing the complexity of the

system. Activating transcription factors (ATFs), classical sensors of ER stress, represent a good example in the context of altered metabolism (Zhang, Erbe et al. 2015) (Boden, Cheung et al. 2014) (Jang, Son et al. 2013) (Raciti, Iadicco et al. 2010) (Yamamoto, Takahara et al. 2010). In summary, nutritional challenges can transform a healthy functional adipocyte to a hypertrophic dysfunctional one. Many changes occur, such as increase in lipid storage, mitochondrial function loss, ER stress and apoptotic signaling, and they all lead, independently or not, to the inhibition of insulin signaling pathways and consequently to severe adipocyte dysfunction (Figure 17).

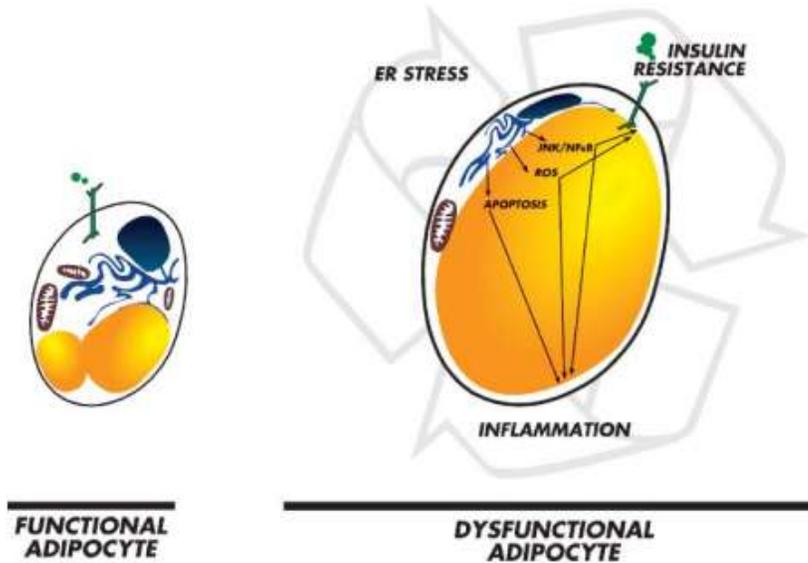


Figure 17. Adipocyte stress in the obese and insulin-resistant state (Gregor and Hotamisligil 2007).

2. Extra cellular matrix: composition and pathophysiology

The protein composition and dynamics of the extracellular matrix (ECM) is crucial for the functioning of adipocytes. Adipocytes, as endothelial cells, are surrounded by a thick ECM referred to as basal lamina (Pierleoni, Verdenelli et al. 1998). While a strong ECM is the principal function of bone and cartilage, the basal lamina in adipocytes may be more important for cell survival. ECM components of adipocytes have been studied mostly by immunohistochemistry and RT-PCR. These components are represented by different types of collagens and collagen-related proteins, such as cell surface integrins, fibronectin, elastin, laminin, proteoglycans, tenascin-C, several matrix metalloproteinases (MMP), tissue inhibitors of matrix metalloproteinases (TIMP) and some more. It is important to note that some of these proteins are not produced only by the adipocytes, but also by cells of the stromal vascular fraction containing preadipocytes, capillary endothelial cells, infiltrated monocytes/macrophages, and a population of multipotent stem cells (Mariman and Wang 2010). More than 30 collagens are known, but the most present in adipose tissue Col 1, 4 and 6 are also the best characterized (Nakajima, Yamaguchi et al. 1998) (Khan, Muise et al. 2009). Specific collagens and their regulators have a role during the physiological process of adipogenesis, when the adipocyte changes the cell shape from fibroblastic to spherical shape, changing in level and type, in parallel with level of cytoskeletal components (Pierleoni, Verdenelli et al. 1998), (Spiegelman and Ginty 1983). Obviously, ECM components intervene during the adaptive

response of adipose to cellular alterations due to expansion/reduction as well. In obesity, in both rodents and humans, expanding AT displays increases in components of the ECM, including collagen VI and thrombospondin, parallel to considerable tissue remodeling and increase in inflammatory macrophages (Varma, Yao-Borengasser et al. 2008) (Spencer, Yao-Borengasser et al. 2010) (Pasarica, Gowronska-Kozak et al. 2009) (Divoux, Tordjman et al. 2010) (Sun, Tordjman et al. 2013). ECM composition and ECM regulatory proteins, such as glycoconjugates, important in influencing the rate of assembly, size, and structure of collagen fibrils, are currently studied in conditions of broad biomedical importance from arthritis to tissue repair, from tumor invasion to cardiovascular disease (Kadler, Hill et al. 2008).

3. Extra cellular matrix: a dynamic structure

Next to protein composition of ECM, its dynamics is of crucial importance for adipocyte functioning. ECM is in fact under constant turnover. During ECM remodeling, physiological or pathological, proteins have their specific roles of mediating the balanced result between constructive and destructive enzymes together with their enhancers and inhibitors. An example of this complemented balance can be given by lumican and decorin. Despite the fact that both are collagen cross-linking proteins, these two enzymes have been found to have opposing roles in fibrosis (Pasarica, Gowronska-Kozak et al. 2009) (Khan, Muise et al. 2009). Another classical example important for ECM regulation is represented by the balance between MMPs and TIMPs. In vivo research on MMPs provides contradicting results, indicating the complexity of MMP activity that depends on relative concentrations of different MMPs, their substrate specificity, and the activity of their inhibitors (TIMPs), (TIMPs), and the fibrinolytic system comprising plasminogen/plasmin and their activators (tissue-type plasminogen activator and urokinase-type activator (Christiaens, Scroyen et al. 2008) (Demeulemeester, Collen et al. 2005) (Lijnen, Maquoi et al. 2002). Another interesting ECM component is a membrane-anchored metalloproteinase MMP14, which was proved to be important for a proper WAT development using null mice (MT1-MMP). Using null mice, MT1-MMP was proved to be important for a proper WAT development (Chun, Hotary et al. 2006). Null mice were in fact lipodystrophic and in a 3-dimensional (3-D) ECM context, during adipocyte maturation, MT1-MMP was required to modulate pericellular collagen rigidity. However, besides the physiological activity of ECM, there are pathological conditions in which ECM accumulation is deregulated, as in obese WAT. In that case the same protease, MT1-MMP, cannot properly control the formation of a rigid network of collagen fibrils and consequently contribute to restrain adipocyte expansion. Other ECM components can participate to pathological remodeling and tissue stiffening. For example lysyl oxidase (Lox) and the LOX family of secreted amine oxidases have been observed increased in many fibrotic diseases due to their role in rigid cross-linking of elastin and collagens (Sivakumar, Gupta et al. 2008) (Kagan and Li 2003) (Csiszar 2001) (Smith-Mungo and Kagan 1998). Finally, it is important to acknowledge the role of proteases in contributing to tissue remodeling through a variety of different mechanisms (Cheng, Shi et al. 2012). For example it has been shown that cathepsins can be secreted within the extracellular spaces and directly affect collagen turnover and degradation, as it is the case for cathepsin S (Shi, Munger et al. 1992). The cathepsin biology and their significance in ECM remodeling in the development and progression of various pathological conditions continue receiving scientific attention in search of successful therapeutics.

4. Adipose tissue fibrosis in obesity and after drastic weight loss

Fibrosis is the common pathological outcome of many chronic inflammatory diseases in the context of the liver, lung, kidney and several other tissues. The term fibrosis has been defined as the formation of fibrous tissue as a reparative or reactive process. In the “healthy” adipose tissue, remodeling is a complex process characterized by dynamic changes in cellular composition and function (Itoh, Suganami et al. 2011). Instead until now fibrosis in WAT has been explained as the initial response to adipocyte hypertrophy. In particular, our team demonstrated the ECM accumulation in human obesity through the method of picosirius red staining of WAT (Henegar, Tordjman et al. 2008), and also the importance of macrophage-preadipocyte interactions in WAT fibrosis development or maintenance (Keophipath, Achard et al. 2009). As the collagen content increases, the overall rigidity of adipose tissue also increase. This rigidity in WAT, as in other tissues, may impair angiogenesis, so that adipocytes surrounded by fibrotic tissue cannot be adequately oxygenated, becoming more susceptible to necrosis (Trayhurn, Wang et al. 2008). Signals emerging from hypertrophic (dysfunctional) or dying adipocytes could influence the formation of fibrous structure around the cells. Fibrosis in WAT has been presented as the hallmark response of metabolically challenged adipocytes and in particular collagen 6 has been shown to have a key role in limiting WAT expansion and consequently influencing insulin sensitivity (Pasarica, Gowronska-Kozak et al. 2009) (Khan, Muise et al. 2009). Adipose tissue inflammation and immune cell accumulation have been observed in obesity as well as during dynamic weight loss (due to caloric restriction or fasting), in relation with increased lipolytic activity (Kosteli, Sugaru et al. 2010). However, differently from obesity, adipose tissue remodeling and the potential contribution of WAT macrophages in the process has not been fully investigated after drastic weight loss (Kosteli, Sugaru et al. 2010). In 2010, our team showed that human WAT fibrosis, in particular peri-cellular fibrosis, could be a process able to limit the lipolytic activity of adipocytes, possibly a mechanism preventing an excess weight loss after bariatric surgery (Divoux, Tordjman et al. 2010). During drastic weight loss, dysfunction or death in adipocytes is also found, as suggested by perilipin-negative adipocytes engulfed in fibrosis, similarly to what has been reported in obesity for the adipocytes surrounded by crown-like structures of macrophages (Cinti, Mitchell et al. 2005). Consequently WAT fibrosis could be a response to signals emerging from adipocytes within the specific inflammatory milieu. In this view, fibrosis is considered a pathologically deregulated healing response to inflammation-induced destruction and injury of tissue, as summarized in Figure 18. During obesity, dead adipocytes as identified by perilipin-negative staining are found surrounded by macrophages in crown-like structures (Cinti, Mitchell et al. 2005). After drastic weight loss, perilipin-negative adipocytes are also found engulfed in fibrosis (Divoux, Tordjman et al. 2010). Consequently WAT fibrosis could be a response to signals emerging from dead adipocytes.

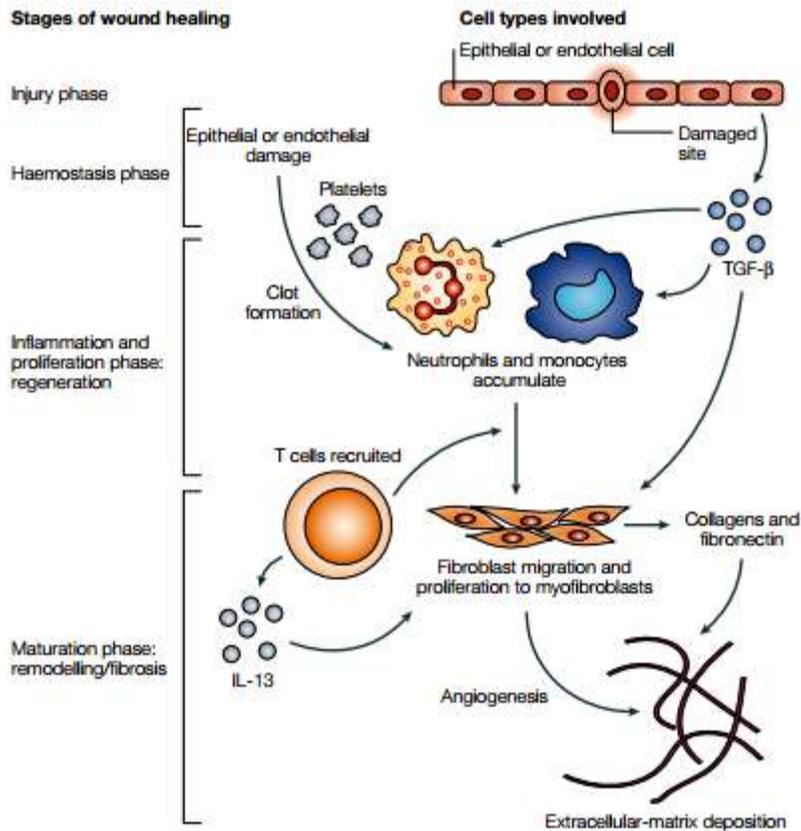


Figure 18. The pathogenesis of fibrosis (Wynn 2004).

During the fibrotic process, stimulated myofibroblasts and epithelial/endothelial cells also produce matrix metalloproteinases (MMPs) that disrupt the basement membrane, allowing the efficient recruitment of cells to sites of injury. Activated macrophages and neutrophils use their scavenger function to ‘clean-up’ tissue debris and dead cells, releasing at the same time cytokines and chemokines that recruit and activate other cells, including T- cells and fibroblasts. Failure to adequately contain or eliminate the provoking factors can exacerbate the inflammatory response, leading to chronic inflammation, excessive immune cell accumulation (as observed in obese WAT) and chronic repair that causes an excessive accumulation of ECM components. Recovering the delicate regulation between collagen synthesis and collagen degradation (MMPs and their inhibitors TIMPs) (Liu, Huang et al. 2015) is fundamental to control the process and regain homeostasis. Although ATMs accumulation is classically associated to inflammation and inflammation seems necessary for the initiation of the ECM remodeling, some observations suggest that the mechanisms promoting fibrosis can be distinct and different to those that impact inflammation. This has been shown in pulmonary, liver, kidney and intestinal (IBD) fibrosis, where molecules, classically identified as pro-inflammatory (i.e. IFN γ , IL12), were shown to reduce collagen deposition (Wynn 2004). Moreover, lasting of chronic pro-fibrotic injury may compromise tissue repair (Figure 19).

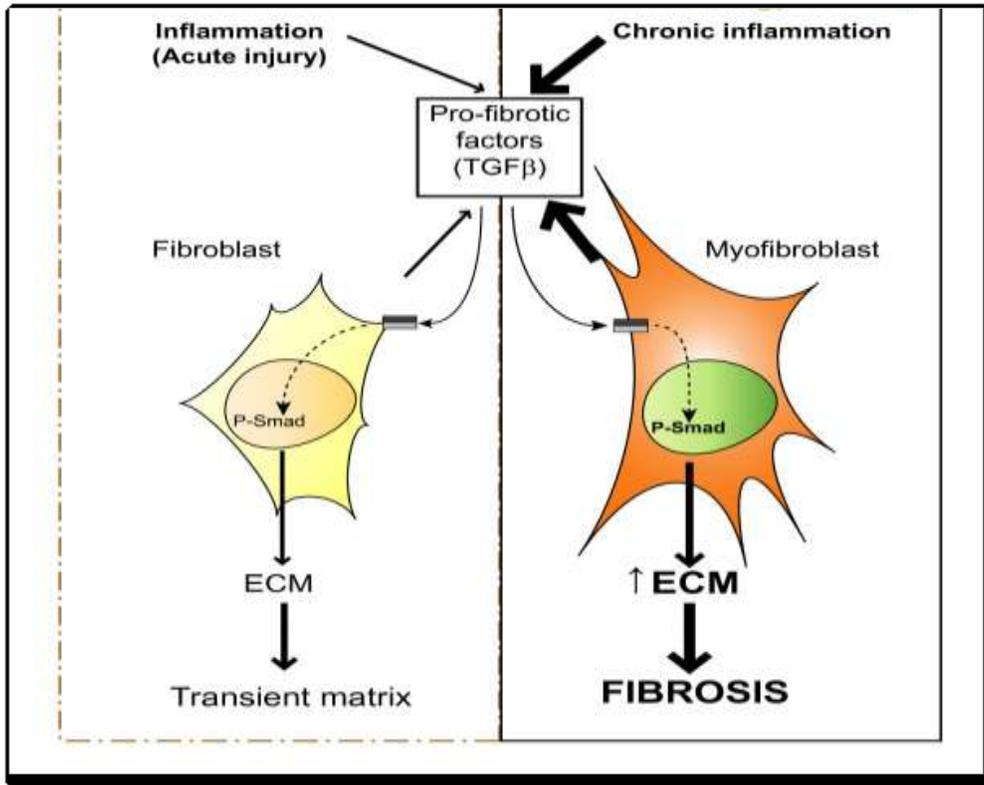


Figure 19. Acute injury versus chronic remodeling process (Mann, Perdiguerro et al. 2011).

Resident and extravasating peripheral macrophages play an important role in the early stages of muscle repair after acute injury, with pro-inflammatory (M1) macrophages first acting to clear the damage, and anti-inflammatory (M2c) macrophages and alternatively activated macrophages (M2a), implicated in the subsequent resumption of inflammation, extracellular matrix (ECM) deposition and tissue repair. M2c and M2a macrophages release anti-inflammatory cytokines and pro-fibrotic molecules such as transforming growth factor (TGF)- β , which in turn activate fibroblasts in a regulated manner to produce ECM components and ECM-remodeling factors, including autocrine production of TGF β , collagen, fibronectin, serine proteases (such as uPA/plasmin), and metalloproteinases (MMPs) and their inhibitors (TIMPs). However, during chronic tissue damage, as in muscular dystrophies, the increased and persistent presence of macrophages modify the intensity, duration and interactions of these released factors, leading to excessive ECM accumulation and replacement of muscle with fibrotic tissue. Studying adipocyte dysfunction in opposed pathological settings can further complicate the situation, since the same process could have contrasting functions. In obesity, fibrosis could serve to limit the excessive expansion of WAT, whereas in lipodystrophy, it could have the role of limiting the excessive loss of adipocyte lipid content. Research will need to further explore the regulation of these delicate mechanisms, in the context of obesity and, even less explored, weight loss, in order to possibly design new therapies.

F. A cross-road between metabolism and immunity

In our body there are “organs” considered key for their plastic endocrine functions, like the above presented adipose tissue and the more recently identified gut microbiota (Brown and Hazen 2015). Both systems are exposed to nutritional fluctuation and participate in maintaining body homeostasis converting dietary molecules into hormonal (WAT) and hormone-like (metabolites from microbiota) signals. Excess or defects of energy flux can dysregulate the delicate balance of interactions between metabolism and immunity in both “organs” (Exley, Hand et al. 2014) (Kau, Ahern et al. 2011). In the context of metabolic disorders, adipose tissue has been particularly studied, being at the junction of immune and metabolic co-regulation. WAT efficiently monitors metabolic homeostasis through the release of adipokines. These adipocyte-specific secretory proteins are now recognized participants in the regulation of immune responses through their own pro- and anti-inflammatory activities (Ouchi, Parker et al. 2011). In addition, the intrinsic presence of various immune cells, innate and adaptive, tightens up the immune-metabolic role of adipose tissue (Makki, Froguel et al. 2013). However, the interest in gut microbial communities has been rapidly increasing recently due to the impact that microbial metabolites can have in physiological and/or pathological processes. Adipose tissue and host receptors have the primary function of establishing the connection between metabolic and immune systems.

1. Nutrient-sensing and metabolism

Nutrient homeostasis is critical for living organisms, from the unicellular to the multicellular ones (Efeyan, Comb et al. 2015). Nutrient fluctuations in the environment can be directly sensed by unicellular organisms, whereas in multicellular organisms those fluctuations are internal and more limited. Energy homeostasis in the body is regulated in the different metabolic tissues by a coordinated management of cellular nutrients, such as lipids, amino acids, glucose and related sugars. Fully understanding the role of nutrient-sensing mechanisms could allow us to know how nutrients are sensed and maintained both at the intracellular and circulating levels in physiological and pathological conditions. In general, mechanisms that sense and manage nutrients integrate with immune and inflammatory pathways in a complex system evolved to protect the body. In the case of the defense system, for example, it is critical to acknowledge that the immune system efficiency strictly depends on highly energy-demanding cells, for which glucose is the main source of fuel (Wolowczuk, Verwaerde et al. 2008). This close functional and molecular integration of the immune and metabolic systems is of particular interests in metabolic diseases in which sensing mechanisms become dysfunctional (Hotamisligil and Erbay 2008).

For the interest of this project, simply lipid sensing will be developed. Lipids are a diverse set of nutrients, like for example fatty acids and cholesterol, characterized by a hydrophobic nature. For this reason, lipids are normally packaged into lipoproteins and chylomicrons or bound by albumin in the serum (Richieri and Kleinfeld 1995). Our knowledge of lipid metabolism is represented by a certain body of literature on the almost ubiquitous peroxisome proliferator-activated receptors (PPARs), with its three subtypes (α , δ , and γ), and their well-tuned role in metabolic organs, such as muscle (PPAR α and PPAR γ), liver (PPAR α) and adipose tissues (PPAR γ). Furthermore lipid-sensing mechanisms have been definitely less explored and our knowledge about them, with few exceptions, is quite limited (Efeyan,

Comb et al. 2015). In particular, an interesting area of research receiving a new wave of attention is represented by the co-evolution of metabolic and immune responses. For mounting a potent immune response to defend the host against infectious agents, it is necessary to have stored and handle energy efficiently using mechanisms for nutrient sensing that have been selected under the pressure of food deprivation. Starvation and malnutrition can impair immune function. For example, in mice, a total reduction in body fat has been shown to result in a decrease in the energy that is available for immune responses (Demas, Drazen et al. 2003). On the opposite side, energy surplus, characteristic of obesity, can also impair immune responses and induce chronic inflammation, as described above in this manuscript.

Upon immune stress, energy demands can become excessive and in tissues like liver and adipose tissue immune effector cells, such as Kupffer cells and macrophages, can represent a competitive advantage over other tissues. The local immune response is as more efficient as nutrients and soluble mediators are provided quickly. It is the case of lymph nodes embedded within adipose tissue. Focusing in particular on WAT immune cells, it is the case of the now recognized lipolytic capacity of immune cells over adipose tissue, one of the mechanisms that allow the cells to fuel a proper immune response. Nutrient-sensing is one of the multiple common signaling pathways between adipocytes (included pre-adipocytes) and macrophages which need to be further deciphered (Charriere, Cousin et al. 2003). The association between metabolism and inflammation is particularly clear in obesity, during which insulin action has a critical role. In conditions where metabolic homeostasis is altered, including lipodystrophy next to obesity, understanding what is the primary insulting factor for the induction of insulin resistance is very important (Medzhitov 2008). The intricate connections between metabolic homeostasis and inflammatory responses can converge at many levels, including at the level of cell-surface receptors, intracellular chaperones or nuclear receptors. In the present manuscript only two examples of receptors involved in the interplay between nutrient-sensing pathways, FFA in particular, and immune responses will be given: Toll like receptors (TLRs) and G-protein coupled receptors (GPRs).

In summary, the exploration of the general mechanisms that integrate nutritional status, immune response and systemic metabolic homeostasis is of future interest in order to understand and possibly therapeutically use these pathways in time of metabolic alterations, such as MetS diseases.

2. Toll like receptors (TLRs)

Toll-like receptors (TLRs) are membrane-bound pattern-recognition receptors, which can be found on immune cells, intestinal cells, and adipocytes, and are viewed as critical in ensuring bodily immune and metabolic health (Wolowczuk, Verwaerde et al. 2008).

These receptors play a central role in microbial pattern recognition in mammals. Besides this primary function, new evidence indicates that TLRs can also respond to different triggers. Of particular interest for metabolic disorders, is their capacity to sense abnormal levels of dietary lipids (Wolowczuk, Verwaerde et al. 2008). Nutrients, such as saturated fatty acids and also elevated glucose levels, have been demonstrated to activate TLRs in adipocytes and macrophages, initiating an inflammatory cascade in WAT (Shi, Kokoeva et al. 2006). The activation of TLRs can be differentially modulated depending on the types of ligands, as it is the case for dietary fatty acids (Lee, Zhao et al. 2004), but also on the cellular

context (Mamedova, Yuan et al. 2013). Similarly, after activation, the downstream signaling cascades, common or specific to single TLR (mainly represented by Toll/IL-1 receptor (TIR) domain, with the first characterized MyD88) vary depending on the contexts. The link between nutritional lipids and TLRs could reveal their potential role in the pathogenesis of obesity-associated insulin resistance, as it is the case of TLR4 (Tsukumo, Carvalho-Filho et al. 2007). Research on TLRs and saturated FFA has been mainly conducted in relation to adipocytes. However, the recent identification of TLR4 on hepatocytes (Galloway, Shin et al. 2008) (Li, Chen et al. 2011) encouraged research on potential mechanisms linking FFA to altered liver function. Using a human hepatocellular carcinoma cell line, the critical role of TLR4 in linking elevation of FFA and increased hepatic gluconeogenesis pointed to one of the mechanisms of FFA implication in the progression of obesity to type II diabetes (Collins, Xiong et al. 2006). As for adipose tissue inflammatory response, the classical signaling cascades consequent to TLRs binding are linked to activation of nuclear factor kB (NFkB), which in turn drives transcription of proinflammatory cytokines and AMPs (Ronald and Beutler 2010). Considering the role of TLRs in triggering multiple responses, based on stimuli, and macrophage activation, the contribution of specific signaling pathways needs to be evaluated to understand and possibly predict metabolic outcomes (Figure 20).

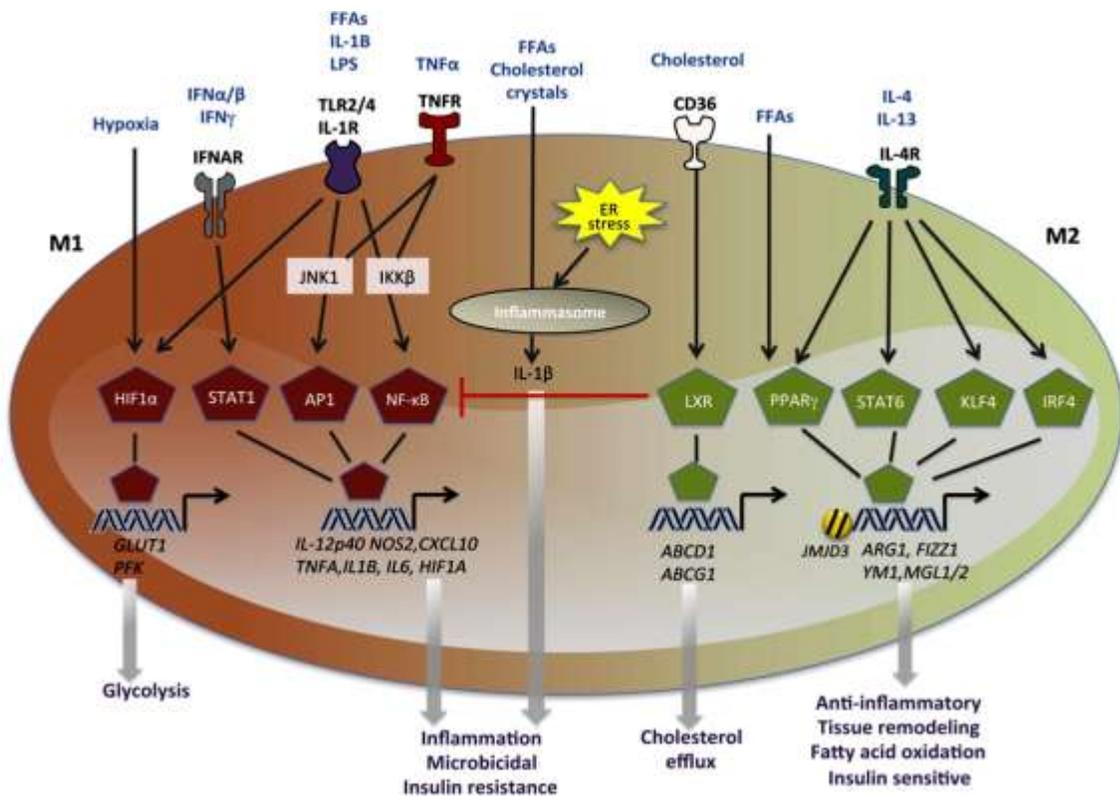


Figure 20. Signaling Pathways Connecting Macrophage Polarization to Metabolic Outcomes (Biswas and Mantovani 2012).

Toll-like receptor 4 (TLR4) is expressed on a variety of cell types, besides macrophages and adipocytes, and it is able to recognize a variety of endogenous ligands, such as lipopolysaccharide (LPS), a major constituent of the Gram-negative bacteria outer membrane, and FFA. The observation that TLR4-

deficiency protected against insulin resistance in obesity induced by a high fat diet suggested that TLR4 could be the link between diet excess and insulin resistance (Shi, Kokoeva et al. 2006). Moreover, aware of the causality link between inflammation and insulin resistance, the pathogenic role of increased TLR4 signalling in obesity-associated inflammation has also been demonstrated (Brun, Castagliuolo et al. 2007) (Cani, Amar et al. 2007) (Velloso, Folli et al. 2015). Studies using C3H/HeJ mice with a loss of function mutation in TLR4 further proved the importance of this receptor in influencing WAT inflammation (Suganami, Mieda et al. 2007) and insulin resistance (Poggi, Bastelica et al. 2007; Tsukumo, Carvalho-Filho et al. 2007). The role of a diet rich in saturated fatty acids, in particular oleate and palmitate, in inducing adipose-tissue inflammation was linked to TLR4 in monocyte/macrophage activation (Lee, Sohn et al. 2001). Co-culture experiments with peritoneal macrophages and 3T3-L1 hypertrophied adipocytes highlighted the paracrine vicious cycle that most probably augments inflammation in obese WAT. In this cellular interplay, the importance of a functional TLR4 was demonstrated in anti-inflammatory adiponectin mRNA downregulation, suggesting a mechanism for the reduced adiponectin level observed in obese patients (Suganami, Nishida et al. 2005). In obesity, increased circulating FFA, released from WAT, and also influxes of LPS, attributed to increased intestinal permeability (Brun, Castagliuolo et al. 2007) (Creely, McTernan et al. 2007), have explained some of the characteristic alterations, including infiltration of macrophages into WAT, insulin resistance and steatosis. In an elegant set of experiments, Cani et al. have shown that the chronic subcutaneous infusion of low doses of LPS in lean mice was sufficient to reproduce the dysfunctional metabolism of HFD-fed mice: higher glycemia and insulinemia, and whole-body, liver, and adipose tissue weight gain (Cani, Amar et al. 2007).

Despite all these progresses, the role of TLRs in the dialogue between adipocytes and macrophages in abnormal conditions such as obesity remains to be elucidated. In particular the physiological and pathological implication of macrophages in binding, internalizing and degrading, or transferring lipid components, including endotoxin (Hampton, Golenbock et al. 1991), to other cellular partners (i.e. adipocytes) needs to be further clarified.

In addition, TLRs are widely distributed in the body, including the brain. Glial cells (Olson and Miller 2004), neurons (Tang, Arumugam et al. 2007) and endothelial cells forming the vessels that irrigate the brain (Constantin, Cordenier et al. 2004) express functional TLRs. Better understanding the role of these receptors in the brain, in particular in regions mostly dedicated to food intake and body weight control, such as hypothalamus, will be fundamental in relation to central control of energy homeostasis in physiological and pathological conditions.

3. G-protein coupled receptors (GPCRs)

G protein-coupled receptor (GPCR) superfamily represents the largest protein family in the human genome. Identified also as GPRs, some of them are called free fatty acid receptors (FFARs) due to their role in free fatty acid sensing. Only recently, several studies have provided evidence that GPRs are dietary sensors that connect metabolic and immune systems (Ichimura, Hasegawa et al. 2014).

Nutrient sensing does not implicate only sensing extracellular nutrients (intra-organism), but also extra-organismal sensing by taste receptors, important for food source evaluation. GPRs have this dual role. In

fact, taste receptors present in the oral epithelium and at the intestinal level belong to the T1R and T2R families of G-protein coupled receptors (Wu, Rozengurt et al. 2002). In addition specific GPRs can mediate the preference for specific fatty acids, as shown in mice deficient for GPR40 and GPR120 (Cartoni, Yasumatsu et al. 2010). Moreover, next to taste FFA, in the past half decade, deorphanization of several GPCRs has revealed that GPR40, GPR41, GPR43, GPR84 and GPR120 can sense concentration of extracellular FFA with various carbon chain lengths at various levels (Yonezawa, Kurata et al. 2013). Specifically, GPR40 and GPR120 are activated by medium- and long-chain FFA. GPR84 is activated by medium-chain, but not long-chain FFA (Nagasaki, Kondo et al. 2012). GPR41 (FFR3) and GPR43 (FFR2) are activated by short-chain FFA. Our interest in these specific receptors is due to the two animal models we used during the Doctorate, since in Study 1 we used two fatty acids (linoleic acid and conjugated linoleic acids) and in Study 2 we used a model of high fat diet-induced obesity.

For all these receptors the physiological roles are multiple and they are summarized in Figure 21.

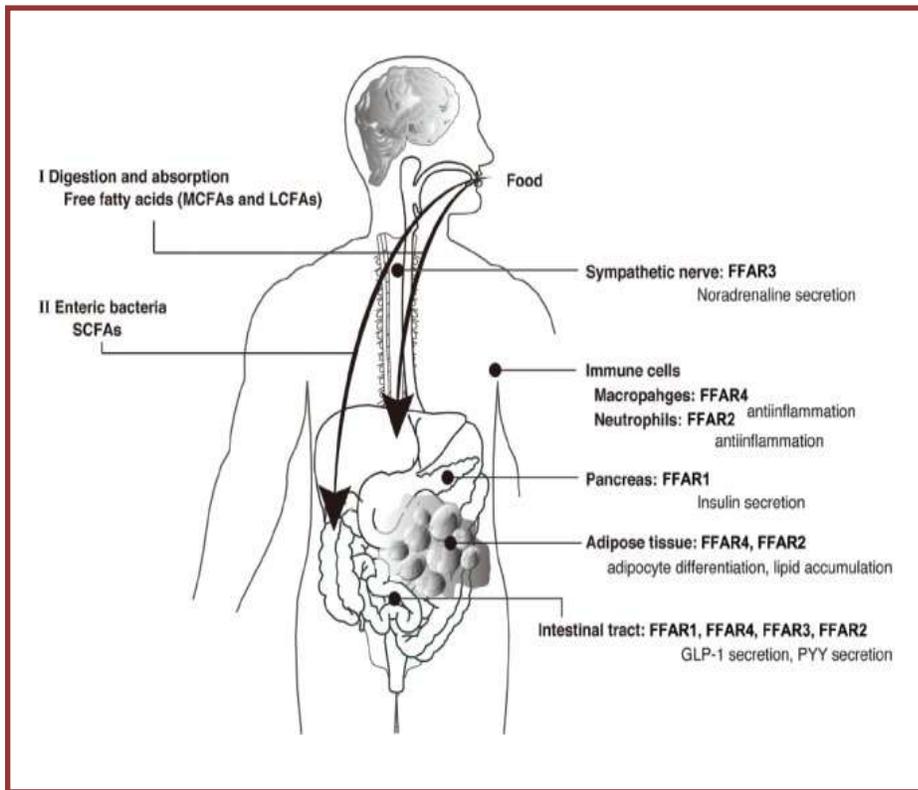


Figure 21. Physiological functions of free fatty acid receptors (FFARs) (Ichimura, Hasegawa et al. 2014).

Several GPRs are found in the intestinal tract and one of their roles is to regulate energy homeostasis. For example, GPR40 (FFR1), GPR41 (FFR3), GPR43 (FFR2) and GPR120 (FFR4) have been demonstrated expressed in glucagon-like peptide-1 (GLP-1) and peptide YY, acting as sensors for short-chain fatty acids in enteroendocrine cells. Another metabolic tissue in which these receptors are well expressed is WAT. In this tissue, mostly GPR41, but also GPR43 and GPR120, have the role of sensing short-chain fatty acids and stimulating leptin production (Xiong, Miyamoto et al. 2004), among others. However, in addition to

their role in metabolism, GPRs have been found expressed on immune cells. For example, GPR43 in leukocytes (neutrophils, B-lymphocyte) of the lamina propria, but also in peripheral blood mononuclear cells (PBMC) or GPR84 in leukocytes and monocyte/macrophage, whereas GPR41 in leukocytes, although mostly in pancreatic β -cells (Yonezawa, Kurata et al. 2013) (Ichimura, Hasegawa et al. 2014).

In general, GPR40 and GPR120 are the two main studied receptors in the process of detecting long chain unsaturated FFA. Recent studies have shown how an increase in one particular nutrient (FFA) anticipates a response to the imminent increase in another nutrient (glucose), consequently stimulating insulin release by pancreatic beta cells (Itoh, Kawamata et al. 2003) (Hirasawa, Tsumaya et al. 2005). GPR120 has been implicated in adipocyte differentiation and lipid accumulation (Gotoh, Hong et al. 2007). Indeed GPR120 is highly expressed in WAT, in particular at the plasma membrane of adipocytes more than in stromal-vascular cells (Gotoh, Hong et al. 2007). In adipocytes, it was shown to mediate insulin sensitizing effects, promoting PI3K/AKT activation (Oh, Talukdar et al. 2010). Studying its role in the context of obesity, in which GPR120 function is disrupted, this receptor has been recognized as key in the contribution to the systemic control of nutrient homeostasis and inflammation (Ichimura, Hirasawa et al. 2012), (Oh da, Walenta et al. 2014). On the opposite, GPR43 (FFAR2) involvement in adipocyte differentiation is more controversial, holding this role in vitro (3T3-L1 preadipocytes) and in vivo (mice), but possibly not in humans (Dewulf, Ge et al. 2013). In summary, due to the importance of FFA signaling in diverse metabolic processes, these dietary sensors have started to be studied in the context of inflammation and metabolic disorders.

Despite the fact that the precise mechanisms of action and regulation of GPRs in tissues are still unclear in physiological status, recent research has started to show their involvement in pathological conditions. In particular, GPR40 (FFA1) has been recently shown to be critical in the inflammatory response of microvascular endothelial cells to LPS (Lu, Li et al. 2015). Using human cardiac ECs, it was shown that palmitic acid, one of the saturated fatty acids mostly increased in T2D, increased IL-6 expression when alone and in combination with LPS, both at transcriptional and post-transcriptional level (Lu, Li et al. 2015). This same membrane receptor is activated by both CLA isomers (c9,t11- and t10,c12-CLA) in vitro; through this activation, glucose-stimulated insulin secretion is increased (Schmidt, Liebscher et al. 2011). This mechanism was used to explain the increased insulin-releasing capacity of pancreatic islets reported by Poirier et al. in CLA-fed mice (Diabetologia 2005, Biochimie 2005). However, the implication of GPR40 in the potential deleterious effects of fatty acids on β -cell function (Schmidt, Liebscher et al. 2011) was not evaluated in this study. Another example of regulation of inflammatory responses by GPRs is given by GPR43 (FFAR2). It has been demonstrated that short chain fatty acids bind the GPR43 and through those interactions profoundly affect inflammatory responses, favoring resolution in murine models of colitis, arthritis and asthma (Maslowski, Vieira et al. 2009). In particular in a model of intestinal inflammation, GPR43 was found essential to conduct an effective immune response (neutrophil recruitment) in restraining? intestinal bacterial translocation in the acute setting (Sina, Gavrilova et al. 2009), even though if in the chronic setting, GPR43 deficient mice were protected against inflammatory tissue destruction induced by DSS (Sina, Gavrilova et al. 2009). Concerning GPR120, it was suggested that this receptor has a critical role in the pathogenesis of obesity and type 2 diabetes, being highly upregulated in omental and subcutaneous adipose tissue of obese subjects (Cao, Gerhold et al. 2008). In 2010, Olefsky and his colleagues demonstrated its anti-inflammatory properties.

Upon PUFA-activation, GPR120 is able to suppress LPS-induced inflammatory response (TNF α and IL-6) through beta-arrestin 2, a downstream molecule of GPR120, involved in toll-like receptor (TLR) and TNF α pathway in macrophage, but not in adipocyte (Oh, Talukdar et al. 2010). More recently this anti-inflammatory activity of GPR120 was further explained using mouse bone marrow-derived macrophages and a differentiated human monocyte cell line. This study showed that DHA, an omega 3 FFA suppresses inflammasome activation in macrophage predominantly using GPR120 (FFAR4) (Williams-Bey, Boullaran et al. 2014). However its precise role in human pathologies needs to be further explored, as exemplified by GPR120 overexpression in correlation with TNF α in the inflamed ileal mucosa of Crohn disease patients (Tsukahara, Watanabe et al. 2015). The dysfunction of this receptor has been causally linked to obesity in humans and rodents (Ichimura, Hirasawa et al. 2012). In particular, GPR120-deficient mice fed with a high-fat diet developed obesity, impaired glucose metabolism and insulin resistance, in association with increased WAT inflammation (Ichimura, Hirasawa et al. 2012).

In summary, the role of GPRs has been most studied in the gastrointestinal tract, where the receptor activities are linked to their strategic location for sensing and transducing information to the host about nutrients and microbiome metabolic activity (Little, Isaacs et al. 2014). Instead, in WAT, their regulation and function still lack an integrative view, in particular concerning the systemic control of nutrient homeostasis in physiological and pathological conditions, such as obesity.

G. Conjugated fatty acids: the t10,c12-CLA model

Fatty acids can be divided into four general categories: saturated, monounsaturated, polyunsaturated, and trans fats. The effects of FFA have been studied mostly in relation to coronary heart disease, with saturated fatty acids and trans fats increasing the risks of CVD and monounsaturated and polyunsaturated fatty acids decreasing them. However, careful attention must be given to the fact that these associations are not consistent in the literature.

Fatty acids are long-chain hydrocarbons. In saturated fatty acids, the carbon chain has the maximum number of hydrogen atoms attached to every carbon atom, whereas in unsaturated fatty acids a pair of hydrogen atoms (mono-) or more (poly-unsaturated) is missing because of a double bond between two carbon atoms. This carbon-carbon double bond can exist in the cis or trans configuration. When the two hydrogen atoms are on opposite sides of the double bond, the configuration is called trans. When the hydrogen atoms are on the same side of the double bond, the configuration is called cis

More than 20 types of fatty acids are found in foods. They are extremely important for multiple functions, from energy production and storage to phospholipid membrane formation and signaling pathways. However due to their harmful effects in CVD, the Nutrition Committee of the American Heart Association has recommended to limit them in our diet. In particular it was recommended no more than 30 percent of a person's daily calories come from fat, with less than 7 percent of total calories from saturated fatty acids, and less than 1 percent from trans-fatty acids (Lichtenstein, Appel et al. 2006). Once ingested, dietary lipids (usually triglycerides) need to be first degraded in order to be absorbed through the intestine. Salivary, gastric and pancreatic lipases are important enzymes that have the function to break down triglycerides into FFA and to make them soluble (emulsification) to aqueous intestinal environment. In enterocytes, which are the absorptive cells of the intestinal epithelium, they

are reformed into triglycerides and packaged into chylomicrons or lipoproteins, which are released into the capillaries of the lymph system and then into the blood. Depending on their type, circulating FFA will have differential effects on the organism. This is a current area of investigation for very different aspects, such as for example how fatty acids differentially modulate the phagocytic activities of macrophages.

Only two fatty acids are known to be essential for humans: alpha-linolenic acid (an omega-3 fatty acid) and linoleic acid (an omega-6 fatty acid). This thesis will focus on the conjugated forms of Linoleic acid.

1. Conjugated fatty acids

Conjugated fatty acids are a mixture of positional and geometric isomers of polyunsaturated fatty acids with conjugated double bonds. Several studies indicate that this group of fatty acids has potent beneficial health effects, depending on different isomers (Yuan, Chen et al. 2014). More detailed evaluations of the physiological bioactivities of conjugated fatty acids isomers on lifestyle-related diseases in humans and animals continue to be of great interest.

Conjugated linoleic acid (CLA)

Conjugated linoleic acids (CLA) refer to a group of dienoic derivatives of linoleic acid (LA) produced during biological or industrial hydrogenation of linoleic acid. CLA are long chain fatty acids with 18 carbon atoms, in which at least one pair of double bonds are separated by only one single bond in 14 possible positions and with 4 geometric combinations (cis/cis, cis/trans, trans/cis and trans/trans). CLA are mainly found in ruminant meats and dairy products (Demizieux, Degrace et al. 2002), because they, are produced by microbial fermentation of linoleic acid (rumen bacteria *Butyrivibrio fibrisolvens* – (Kritchevsky 2000) in the rumen of ruminant animals and 9 desaturation of vaccenic acid in the mammary gland (Griinari, Corl et al. 2000). Up to now only 20 of these polyunsaturated fatty acids have been identified, but two in particular have been the most studied due to their biologically active properties (Pariza, Park et al. 2001): ruminic acid (cis 9, trans 11-CLA) and trans 10, cis 12-CLA, the isomer of interest for the present study (Figure 22).

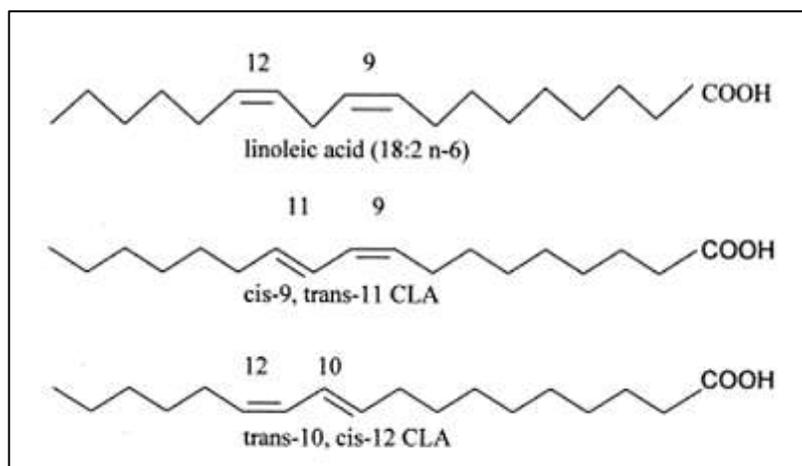


Figure 22. The chemical structure of LA, c9,t11-CLA and t10.c12-CLA.

Humans can have access to CLA from two sources, diet (essentially c9, t11-CLA – 90% in human nutrition) and synthetic dietary supplements, often on the market as weight loss supplements, which commonly contain equal proportions of the two isomers, 40% of c9t11 and 44% of t10c12 (Pariza, Park et al. 2001) (Gaullier, Berven et al. 2002) (Wang and Jones 2004), (House, Cassady et al. 2005) (Figure 22). Recent studies have reported the average of CLA consumed by non-vegetarian men and women from US and France, respectively 210 and 180 mg/day, representing less than 1% of the total energy intake (Ritzenthaler, McGuire et al. 2001).

CLA isomers: experimental effects

Different CLA isomers have been shown to exhibit a variety of unique properties such as anti-cancer (breast, lung, skin, stomach and colorectal), anti-atherogenic and immune response (Ha, Grimm et al. 1987),(Bhattacharya, Banu et al. 2006). In particular enhancing effects in controlling body fat gain were reported in animal models (Park, Albright et al. 1997) (Wang and Jones 2004) (Pariza 2004). Because technically CLA is a trans fat and there are numerous studies showing that industrial trans fats are harmful, research on the claimed health benefits of CLA has been followed carefully. Customers in general, but US customers in particular, are particularly attentive on the positive or negative press linking dietary choices to health. For example US consumers are increasingly demanding grass-fed products and also meat products designed to promote health (Young, Therkildsen et al. 2013). One of the reasons is that grass-fed beef and the derived raw dairy products have three to five times more cis-9 trans-11 CLA (seen as a potential anti-cancer and weight loss treatment) levels than those fed grain-based diet, together with lower total fat, higher minerals and vitamins.

Park et al. were the first to demonstrate that CLA modulated body composition, reducing body fat mass of 60% in both male and female mice (Park, Albright et al. 1997). The “anti-obesity” effects of CLA, have been linked to its capacity to bind and activate PPAR α , potentially affecting lipid metabolism through transcriptional events (Moya-Camarena, Vanden Heuvel et al. 1999), but a precise mechanism has yet to be defined. Despite differences in body composition changes in response to CLA, most of the animal studies report effects on food/energy intake and total body fat content reduction in mice (DeLany, Blohm et al. 1999) (Belury 2002) (Wang and Jones 2004) rats and chickens (Park, Albright et al. 2007) (Choi, Koh et al. 2007). However, conversely to fat mass, liver weight has been shown to increase, due at least in part to lipid spilling accumulation. The t10c12 isomer may be the form responsible for the effects on weight loss and body fat deposition in animals (Park, Storkson et al. 1999) (Ryder, Portocarrero et al. 2001). However, in human studies, results on CLA are controversial, with studies showing effects on fat mass, serum total lipids, decrease whole body glucose uptake (Blankson, Stakkestad et al. 2000) (Raff, Tholstrup et al. 2009) and other studies contradicting the beneficial effects (Dhurandhar, Blank et al. 1999) (Zambell, Keim et al. 2000), (Moloney, Yeow et al. 2004). The dosage, the length of the treatment and the degree of obesity are factors that can affect the consistency of clinical results (Kennedy, Martinez et al. 2010).

2. A murine model of lipoatrophic syndrome: t10, c12 CLA

In 2005, Poirier et al. demonstrated that CLA-mediated lipoatrophic syndrome is strictly dependent on the t10, c12-CLA isomer (Clement, Poirier et al. 2002). The reduction of fat mass is especially dramatic in

the mouse, in which WAT inflammation is associated with severe hyperinsulinemia, IR and massive liver steatosis in response to dietary supplementation (mixture of 50% t10,c12-CLA and 50% c9,t11-CLA) (Poirier, Niot et al. 2005) or daily gavage with t10,c12-CLA (Poirier, Shapiro et al. 2006). In Figure 23, the effects of dietary supplementation with linoleic acid, C9,t11-CLA and t10,c12-CLA for 28 days are reported for periuterine WAT, liver and circulating insulin levels. This is the animal model that we decided to use for our experiments thanks to a successful collaboration between Dr M Guerre-Millo, from the team of K Clément, and Dr H Poirier and Dr I Niot from AgroSup Dijon (Dijon, France).

	A Periuterine adipose tissue relative mass (% body mass)	B Liver relative mass (% body mass)	C Insulinemia μU/ml
Control	 1.63 ± 0.26 ns	 4.67 ± 0.12 ns	26 ± 4 ns
Linoleic acid	 1.19 ± 0.12 ns	 4.54 ± 0.18 ns	45 ± 14 ns
c9,t11 CLA	 1.47 ± 0.19 ns	 4.78 ± 0.08 ns	66 ± 18 ns
t10,c12 CLA	 0.24 ± 0.07 ***	 14.71 ± 1.14 ***	4562 ± 968 ***

Figure 23. Isomer-specific effect of CLA on adipose tissue, liver metabolic and insulinemia (Poirier, Niot et al. 2005).

In the next pages the current concepts about the effects of trans-10, cis-12 CLA (CLA) on metabolic organs will be described, from adipose tissue lipodystrophy to hyperinsulinemia and hepatic steatosis in particular. It will be a single organ review of the literature about CLA effects, although aware that the effects of CLA are clearly the result of the interconnection between the different organs.

Adipose tissue and t10, c12 CLA

Most of the studies performed in mice demonstrated that t10,c12 CLA reduces the fat mass (Bhattacharya, Banu et al. 2006). In particular the adult females C57BL6 are a hyper-sensitive model, as shown in a study in which a diet with 1% CLA (compared to body mass) was administered for four weeks caused a lipotrophic syndrome (Tsuboyama-Kasaoka, Takahashi et al. 2000). The rapid and massive changes in body composition in the mouse are not reflected only by fat store decrease, but also by an

increase in lean body mass (Park, Albright et al. 1997) (West, Delany et al. 1998). Many transcriptional factors have been involved in CLA-induced adipose tissue modifications, including SREBP-1c and PPAR γ , as shown in particular by the team of Dr McIntosh (Nord Carolina University, USA) (Kennedy, Overman et al. 2009; Kennedy, Martinez et al. 2010). In 2006, Poirier et al. demonstrated that the drastic fat reduction was associated with WAT inflammation and reduced circulating adipokines in lean mice (Poirier, Shapiro et al. 2006) and these effects were shown in obese mice as well (House, Cassady et al. 2005). In line with the increased proinflammatory cytokines (TNF α , IL6 and IL8 in particular), a drastic infiltration of macrophages was observed in WAT already after 3 days of CLA administration (daily gavage) (Poirier, Shapiro et al. 2006). The observed inflammatory response to CLA has been shown to be linked to the activation of NF-kB pathway (Liu, Purushotham et al. 2007).

The potential mechanisms explaining the effects of CLA supplementation on fat mass are multiple: CLA leads to an increase in energy expenditure (West, Delany et al. 1998) (West, Blohm et al. 2000), that could be secondary to a stimulation of sympathetic nervous activity (Ohnuki, Haramizu et al. 2001). CLA reduces lipid uptake and storage of adipocytes by inhibiting lipoprotein lipase, as demonstrated in vitro through 3T3-L1 adipocytes (Park, Albright et al. 1999). CLA reduces lipogenesis by decreasing expression of SREBP-1c and PPAR- γ and their downstream genes, ACC, FAS, and SCD1 (as for adipocytes decreased size and lipid content) (House, Cassady et al. 2005). CLA reduces adipose tissue mass through an apoptotic mechanism linked to an increase in tumor necrosis factor production (as for adipocytes decrease in number) (Kennedy, Martinez et al. 2010).

All these mechanisms contribute to the higher circulatory levels of free FA (FFA) and the consequent effects on liver. In association with the adipocyte alterations described above, only one study previously reported extracellular matrix accumulation in the mouse mammary fat pad (Russell, McGee et al. 2007). One week of t10,c12-CLA enriched diet was sufficient to induced tissue remodeling and concurrent leukocyte accumulation in this depot. In relation to the experimental setting that we chose for the Study I presented in the Research Project section of the Thesis, it is important to mention one last study that focused on adipose tissue depletion and the associated development of IR (Purushotham, Wendel et al. 2007). In this latter study the effects of CLA on hepatic steatosis and IR were observed in C57BL6 male mice after 30 days of diet containing 1.5% CLA, along with drastic reduction of epididymal adipose mass and circulating adiponectin. To my knowledge, this is the only study that reported the reversibility of the effects of CLA: body weight, adipose tissue mass, in line with adiponectin levels, and hepatic steatosis were attenuated after switching the mice to a diet without CLA for the following 30 days.

Liver and t10, c12 CLA

Liver is an important target for CLA effects. In fact another consequence of dietary CLA supplementation in mice is massive liver enlargement (Tsuboyama-Kasaoka, Takahashi et al. 2000) (DeLany, Blohm et al. 1999) (DeLany and West 2000) (West, Blohm et al. 2000), concurrent to decrease in body adiposity and development of insulin resistance. The intensity of lipid accumulation varies depending on the level of CLA in the diet, duration of feeding, physiological condition, and animal species.

Our collaborators in Dijon demonstrated that fatty liver and hyperinsulinemia, were associated to lipoatrophy specifically induced by the t10,c12- isomer of CLA in mice, whereas 4 weeks of a diet enriched in in c9,t11- CLA and LA had no significant effect (Poirier, Niot et al. 2005). This observation

suggests that hepatic lipid accumulation is the result of mobilized fatty acids from adipose tissue. One of the mechanisms suggested is that liver in this condition is probably not able to increase FFA oxidation and export of synthesized triglycerides sufficiently (Vyas, Kadegowda et al. 2012). Adipocyte changes are certainly linked to steatosis. In fact re-establishing the levels of leptin through external supplementation or adiponectin maintenance using rosiglitazone is sufficient to attenuate hepatic steatotic condition and normalize the insulin levels in CLA-fed mice (Tsuboyama-Kasaoka, Takahashi et al. 2000) (Tsuboyama-Kasaoka, Miyazaki et al. 2003) (Wendel, Purushotham et al. 2008). However it is interesting to underline that leptin has no effect when infused into A-ZIP/F-1 fatless mice, suggesting that leptin insufficiency alone is not the cause of IR provoked by CLA (Gavrilova et al 2000). Not only re-establishing adipokine level, but also dietary FA or oil supplementation with higher n-3 and n-6 PUFA administered along with CLA were proved to ameliorate liver steatosis (Oikawa, Tsuyama et al. 2009), demonstrating that changes in hepatic FA composition could play an important role in progression of hepatic steatosis (Vyas, Kadegowda et al. 2012). Another hypothesis proposed to explain CLA-induced liver steatosis is that it is the result of increased hepatic lipogenesis stimulated by high insulin levels. However, noteworthy is one study that showed CLA-induced hepatic steatosis in streptozotocin (STZ)-injected mice, with low insulin levels, indicating that next to hepatic lipogenesis induced by hyperinsulinemia, other regulatory mechanisms affect hepatic lipid accumulation (Jourdan, Djaouti et al. 2009).

Several transcription factors, including PPAR α , liver-X-receptors (LXRs) and sterol responsive element-binding protein 1 (SREBP1), have been evaluated for their role in hepatic lipid metabolism in an attempt to explain the mechanisms behind the massive hepatic steatosis observed in response to CLA (Roche, Noone et al. 2002) (Vyas, Kadegowda et al. 2012). For example, in mice, during hepatic steatosis, dietary CLA was associated with upregulation of genes associated with fatty acid uptake, such as FAT/CD36 and fat transporters (FATP5, FABP-1, FABP-4, and FABP-5). Finally, experiments in vitro, with Hep G2 cells, also contributed to clarify mechanisms of action of conjugated linoleic acids. For example, t10,c12 CLA in vitro reduces triglyceride secretion (Lin, Schuurbiens et al. 2001) and acts on stearyl-CoA desaturase-I regulation (Choi, Park et al. 2001).

Intestine and t10, c12 CLA

Due to its properties, conjugated linoleic acids have been proposed as important pharmaco-nutrients for modulating mucosal immunity and therapeutic responses in patients with inflammatory bowel disease (IBD)(Bassaganya-Riera and Hontecillas 2006). In this context, a mixture of 50:50 CLA isomers (c9,t11 and t10,c12-CLA) has been shown to contribute to delaying the onset of experimental IBD (Evans, Misyak et al. 2010). Moreover, a mixture of the two conjugated linoleic acid isomers appeared promising as a chemopreventive agent against colorectal cancer in mice, in part through a PPAR γ -dependent mechanism (Borniquel, Jadert et al. 2012). However this proposition has been undermined by other studies reporting different effects. When used in vitro on human enterocyte-like Caco-2 cells, CLA altered global gene expression in an isomer-specific manner and increase paracellular epithelial permeability (Roche, Terres et al. 2001), as indicated by an altered distribution of occluding and ZO-1, structural components of the tight junctions regulating epithelium integrity. More recently, a microarray-based study further underlined the exclusive effect of t10,c12-CLA over c9,t11-CLA, in the intestinal model of Caco-2 cells (Murphy, Hooiveld et al. 2007). In the specific study, t10,c12-CLA modulates processes inherently related to carcinogenesis, such as cell cycle, cell proliferation, and DNA

metabolism, and transepithelial Ca transport (including claudin 2 and 4). However, the authors pointed out how the exact role of the individual claudin proteins within the functionality of the tight junction and consequently the physiological meaning of their alteration by t10,c12-CLA has still to be elucidated (Murphy, Hooiveld et al. 2007). Finally, it is important to consider that CLA is introduced in our body through the diet, but also it can be produced and/or converted by bacteria inside the gastro-intestinal tract (Lee, Park et al. 2006), (Murphy, Hooiveld et al. 2007), (Druart, Neyrinck et al. 2014). Although most probably confined at the intestinal level, the biological role of the endogenously produced CLA could have effects on the intestinal epithelial barrier, as it has been recently demonstrated for a gut microbial metabolite of linoleic acid (Miyamoto, Mizukure et al. 2015).

Since in obesity intestinal permeability is increased (Brun, Castagliuolo et al. 2007) and has been linked to endotoxemia and WAT inflammation, it is surprising that CLA-induced WAT inflammation has not been carefully explored yet in relationship to an organ so critical for metainflammation.

Pancreas and t10, c12 CLA

In humans the association between increased FFA concentration (from approximately 50 to approximately 750 microM) and increased IR was demonstrated using healthy volunteers during euglycemic-hyperinsulinemic clamping (Boden, Chen et al. 1994). In several studies the use of CLA was associated with hyperinsulinemia, with no hypoglycemia either in fed or fasted state. No alteration of blood glucose concentration were found after oral glucose tolerance test, whereas insulin sensitivity was markedly compromised in C57Bl/6J females fed for several weeks with a mixture of CLA isomers (Tsuboyama-Kasaoka, Takahashi et al. 2000). In 2005, Poirier et al demonstrated that CLA *in vivo* administration provokes changes in the secretory capacities of pancreas (Poirier, Rouault et al. 2005). They studied the islets of Langerhans from healthy C57BL6 mice fed for 4 weeks with a CLA mixture and stimulated with different dose of glucose (2.8mM, basal dose and 16.7mM, high dose) and they observed non only that CLA increased insulin secretion, but also the size of islets was significantly increased (hyperplasia)(Poirier, Rouault et al. 2005). The precise mechanisms that are at the origin of such massive hyperinsulinemia in response to CLA are yet to be identified, even if the drastic reduction of adipokines, in particular adiponectin, has been called into question possibly as initial trigger.

Muscle and t10, c12 CLA

Up to now, only few studies have investigated the impact of CLA (mixture or single isomers) on insulin sensitivity of the muscle. In one study, in which CLA was supplemented 28 days, a slight up-regulation of Glut4 (1.5 fold) was found in gastrocnemius, contrasting with a massive down regulation in WAT (Tsuboyama-Kasaoka, Takahashi et al. 2000). In 2003, He et al showed that mice invalidated for PPAR γ specifically in WAT presented lipoatrophy and insulin resistance in adipose tissue and liver, but not in muscles (He, Barak et al. 2003). More recently another study reported that CLA-fed mice do not accumulate excess lipid in their muscle, but preferentially in liver (Kanosky, Ippagunta et al. 2013), suggesting that muscle probably remain insulin-sensitive during treatment. Indeed CLA does not appear to be a good model for lipid accumulation in muscles in this last study (Scalerandi, Gonzalez et al. 2014). Lipid metabolism in muscles was decreased by CLA (mix-CLA) in growing mice, compared to mice fed with diets containing olive, maize and rapeseed oils supplemented with ruminic acid (c9,t11-CLA) for 30 days (Scalerandi, Gonzalez et al. 2014).

Immunity and t10,c12-CLA

Shortly after CLA administration a massive amount of macrophages infiltrate WAT of mice (Poirier, Shapiro et al. 2006) (Liu, Purushotham et al. 2007). CLA was also reported to induce mast cell recruitment in adipose tissue of murine mammary gland, in a study on CLA as a dietary chemopreventive agent in cancer; the effect was specific to the t10,c12 isomer (Russell, McGee et al. 2007).

Few human studies have reported a significant effect of CLA on leukocyte number (Gaulhier, Halse et al. 2004) (Gaulhier, Halse et al. 2005) (Larsen, Toubro et al. 2006); in one study no effects were observed (Whigham, O'Shea et al. 2004). The significant increase observed at the circulatory level in humans has not been further explored by flow cytometry, so details about different subpopulations and tissue specific-infiltration are missing. In completely different experimental settings, CLA has been shown to affect monocyte migration (McClelland, Cox et al. 2010), to increase chemotaxis of PMNs (Paek, Kang et al. 2010) or to modulate macrophage phagocytic activity and phenotype (Stachowska, Baskiewicz-Masiuk et al. 2007) (Song, Kang et al. 2007) (de Gaetano, Alghamdi et al. 2015).

In metabolic disorders, macrophages in particular uniquely possess a dual functionality, regulating and sustaining the chronic inflammatory response and regulating lipid accumulation and metabolism (Chinetti-Gbaguidi and Staels 2011). For these reasons, the effects of CLA on function and activity of this particular immune population deserve further investigations in vivo and in vitro.

Finally, conjugated linoleic acids have been and continue to be used as an interesting research tool (Reynolds and Roche 2010). However the relevance of their systemic and local effects has yet to be clarified within the context of human health.

H. Intestinal microflora and metabolic diseases

1. Microbiota composition: functions and dysfunctions

In human adults, the gut contains between 1,000 and 1,500 bacterial species (commensal and symbiotic microbes), of which at least 160 species shared among individuals, forming a real organ with its proper metabolism and functions (Qin, Li et al. 2010). Each person has a different microbiota in terms of type and quantity of species and of functional composition (Arumugam, Raes et al. 2011) and its composition changes with age and diet, among other factors. In healthy humans, most of the bacterial cells belong to 4 phyla namely Firmicutes (65%), Bacteroidetes (16%), Proteobacteria (9%) and Actinobacteria (5%) (Eckburg, Bik et al. 2005). The figure below (Figure 26) shows slightly differences for humans, since it has been adapted from a more recent study (Arumugam, Raes et al. 2011), together with studies for the other organisms (Brinkman, Hildebrand et al. 2011) (Chandler, Lang et al. 2011) (Roeselers, Mittge et al. 2011) (Figure 24). Different phyla are represented by different color and the relative abundance of the lower taxonomic levels is indicated by font size.

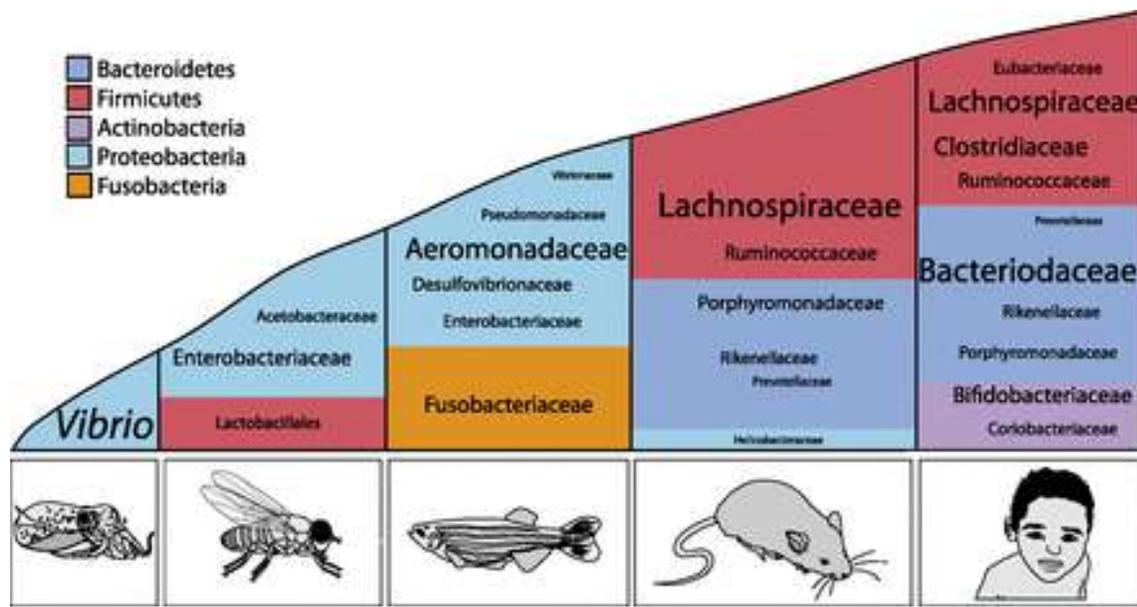


Figure 24. The composition of the microbiota across species (Kostic, Howitt et al. 2013).

Looking at the phylum level, it is clear that mouse and human microbiota are similar, but at the genus level they differ – 99% of wild-type mouse bacterial cells belong to two divisions only: Firmicutes and Bacteroidetes (Ley, Backhed et al. 2005). Moreover rodents for their anatomy (i.e. gastrointestinal tract compartmentalization) and behavior (e.g., coprophagia) clearly differ significantly from humans. However, despite the differences, mice can still be a fundamental tool for unraveling mechanisms of host–microbiota interactions relevant to humans. Environmental factors can affect this immensely diverse ecosystem. Diet is considered the strongest determining factor. For example our gastrointestinal tract is colonized differently in early life, depending on dietary inputs. Human milk favors colonization by bifidobacteria (Firmicutes), whereas Bacteroides are dominant in formula-fed infants. Or another example is given by humans, who eat plenty of protein and animal fats typical of Western diet, displaying predominantly Bacteroides species in their gut, while for those who consume more carbohydrates, especially fibers, the Prevotella species dominate (Wu, Chen et al. 2011). Or another example can be the gut microbiota of vegetarians and vegans, who are unable to metabolize L-carnitine, a nutrient present in red meat (Koeth, Wang et al. 2013). Interestingly the combined genomes of the gut microbiota complement the human genome with 150-fold more genes (Qin, Li et al. 2010) and these genes contribute significantly to the host physiology and metabolism (Sommer and Backhed 2013), (Tremaroli and Backhed 2012). In contrast to humans, gene richness of microbiota is much more plastic and alterations in gene presence and/or composition have been associated with several diseases. This association has been demonstrated in digestive diseases, such as inflammatory bowel diseases (IBD), but interestingly also in systemic diseases, including cancer and Parkinson. Metabolic diseases are obviously not excluded. An accumulating body of literature suggests that gut microbiota is an additional contributing factor to the pathophysiology of diseases such as obesity, anorexia and type II diabetes (Turnbaugh, Ley et al. 2006) (Rosser, Oleinika et al. 2014). This is a rapidly-evolving field, where gut microbiota composition and gene richness are studied in relation to the altered metabolism of the host

in order to find potential therapeutic. In 2007, it was demonstrated that consumption of a high-fat diet induces changes in the gut microbiota (Erridge, Attina et al. 2007), whereas in 2008 the other side of the coin was revealed, showing that modulation of gut microbiota could be sufficient to control obesity-associated metabolic alterations (Cani, Possemiers et al. 2009). Right after, a key study from the group of JI Gordon presented an effective model to test the effects of environmental and genetic factors on the gut microbiota and host physiology, the humanized gnotobiotic mice (Turnbaugh, Ridaura et al. 2009). In these mice, fresh or frozen adult human fecal microbial communities were transplanted into germ-free C57BL/6J mice, overcoming possible confounding factors like human environmental exposures, microbial ecology and genotype. This study not only demonstrated how gut microbiota adapt in composition and function in relation to dietary changes in short time, but also that a specific human metabolic phenotype (metabotype) can be transferred via the gut microbiota (Turnbaugh, Ridaura et al. 2009). This same approach helped exploring the potential causal relationship between altered gut microbiota and obesity in a study where microbiota from a human female twin discordant for the phenotype of obesity was transferred in germ free mice (Walker and Parkhill 2013) as illustrated in Figure 25. Germ-free mice inoculated with microbiota from the obese twin (red outline) had an increase in adiposity, whereas those receiving the lean microbiota (blue outline) remained lean (Walker and Parkhill 2013). This elegant study proved for the first time that the microbiota from lean or obese humans induces similar phenotypes in mice. And even more remarkably this study substantiated that, when microbiota from lean donors was inoculated in the obese-recipient mice, the lean phenotype is transmissible and modifiable if the diet is appropriate (obese mice showed reduce adiposity gain).

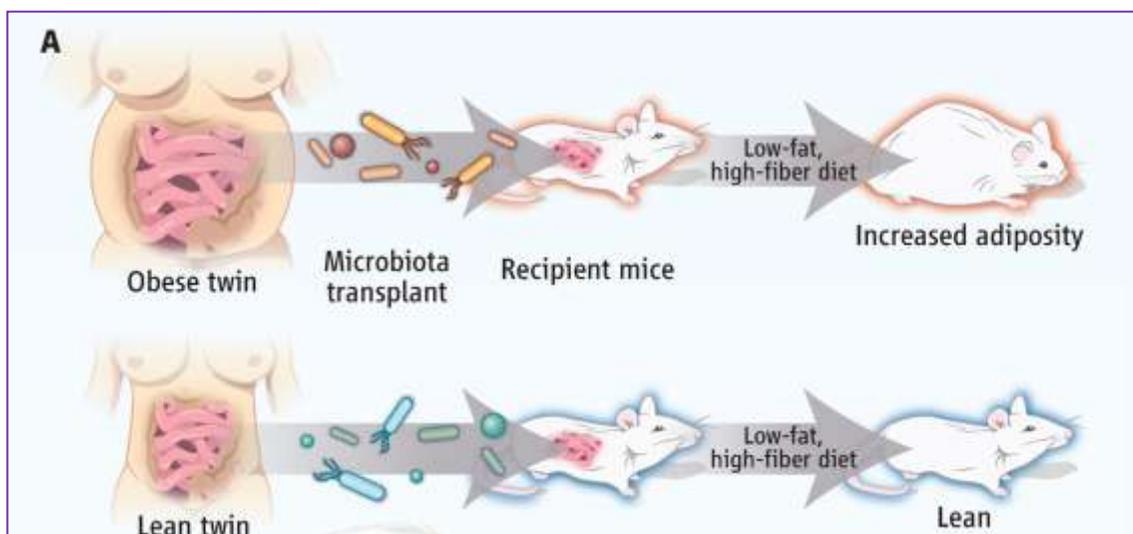


Figure 25. Human microbiota transfer into mice induces transfer of phenotypic characteristics (Walker and Parkhill 2013).

Moreover, recently, the temporal relationships between food intake, gut microbiota and metabolic and inflammatory phenotypes were investigated by our team in diet-induced weight-loss and weight-stabilization interventions (Cotillard, Kennedy et al. 2013). This approach revealed that obese people are more likely to have a low microbial gene richness (on average 379,436 genes - well below the threshold of 480,000 genes) that is associated with a greater gain of weight resistance and more marked

inflammatory phenotype in general (increased serum leptin and triglycerides, decreased serum adiponectin and HDL-cholesterol) than healthy people (Cotillard, Kennedy et al. 2013). The reduced microbial gene richness (40%) in association with these deleterious characteristics could bring obese individuals to increased risk of comorbidities such as pre-diabetes, type-2-diabetes and cardiovascular disorders (Le Chatelier, Nielsen et al. 2013). This study, together with a concomitant one (Kong, Tap et al. 2013), remarkably observed that part of the pathological alterations linked to the obese phenotype can be rescued by dietary interventions or bariatric surgery, including the modification of microbiota composition toward a more healthy profile. However, despite the recent progress, more research is needed to clarify the precise mechanisms by which the gut microbiota modulates obesity. In fact, little is known apart from a potential role in energy harvest from the diet and lipid metabolism (Backhed, Ding et al. 2004), (Turnbaugh, Ley et al. 2006) (Backhed, Manchester et al. 2007) (Tremaroli and Backhed 2012). Moreover colonized mice consume less food than their germ-free counterparts and so increased food intake cannot explain the obese phenotype in colonized mice (Backhed, Ding et al. 2004). The use of genetically modified germ-free mice may facilitate to delineate the molecular mechanisms by which specific microbes or communities affect host metabolism.

2. Symbiosis between the host and the microbiota

Since each individual has his own microbiota, it is hard to evaluate the role and the involvement of the different bacterial strains at the local and systemic level. It was in 2011 that the concept of enterotypes was introduced (Arumugam, Raes et al. 2011) as a way to stratify human individuals based on their gut microbiome. This study published by the MetaHIT consortium combined 22 newly sequenced faecal metagenomes of individuals from four countries with previously published data sets and identified three robust clusters (referred to as enterotypes) that are not nation or continent specific (Arumugam, Raes et al. 2011). Precise computational procedures revealed that gut microbiota variation generally stratified, indicating the existence of a limited number of well-balanced host–microbial symbiotic states that might respond differently to diet and drug intake (Arumugam, Raes et al. 2011). At the core of this classification there is the symbiotic relationship between host and microbiota, a relationship in which both entities benefit. In fact the host provides an ecologic niche and nutrient source for the microbiota, and, in turn, the commensal organisms help maintaining a healthy homeostasis (Kostic, Howitt et al. 2013),(Chow, Lee et al. 2010). The gut microbiota has the capacity to contribute to host physiology within and outside the gut. Within the gut, microbiota is essential for normal development and homeostasis of the immune system in the gut, protects against pathogenic bacteria, and modulates proper development of villus architecture and angiogenesis within the intestine (Sommer and Backhed 2013) (Tremaroli and Backhed 2012). Outside the gut, microbiota affects bone mineral density, several host metabolic functions, even behavior (Greiner, Hyotylainen et al. 2014) (Sommer and Backhed 2013) (Tremaroli and Backhed 2012), and potentially systemic immunity (Surana and Kasper 2014). All the potential effects of gut microbiota on the host are the result of the delicate harmony of the symbiotic relationship. If, however, this delicately balanced system is perturbed, a state termed “dysbiosis” may result and consequently an expansion of harmful (“proinflammatory” pathobionts) bacteria could occur (Round and Mazmanian 2009) (Figure 26).

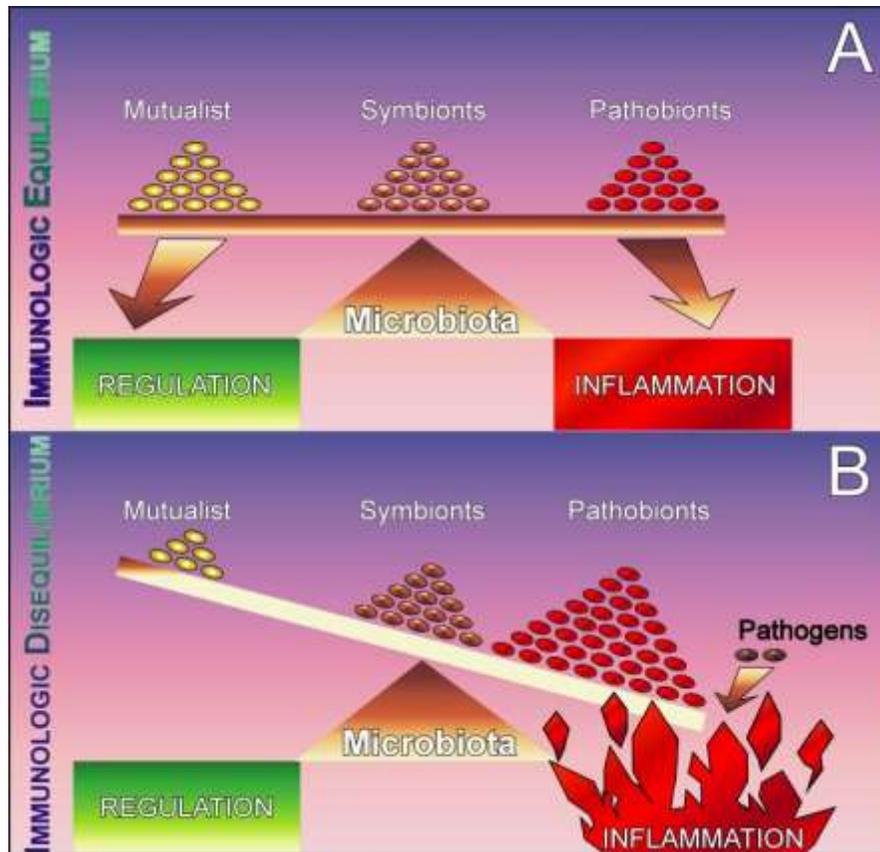


Figure 26. Gut dysbiosis: immunological disequilibrium (Round and Mazmanian 2009).

The causes for this deregulation are not completely clear, but genetic predisposition and civilization effects have been suggested. A perfect example is given by inflammatory bowel disease in link with the “leaky gut” syndrome (due to the resulting increase in the intestinal permeability) (Tamboli, Neut et al. 2004) (Paoella, Mandato et al. 2014). In this case microflora role has been studied with more interest in Europe compared to US (Kaur, Chen et al. 2011). In IBD, but also in the metabolic syndrome context, the associations between specific bacterial populations and the disease process (independently of the studied pathology) are typically correlative as they examine differences in the microbiota of patients with or without a given disease (case-control approach). Consequently, in order to find specific candidate organisms that prevent or exacerbate inflammatory conditions and/or metabolic alterations, broad prospective investigations will be surely welcomed in the next future.

3. Microflora modulation: potential therapeutics

Very few therapeutic solutions are available for patients at early stages of obesity; drug treatments or/and lifestyle interventions are their main options. These solutions are yet limited in terms of long term health improvements, resulting ineffective for subjects at advanced stage of obesity (BMI > 35). Through selective criteria, patients who can medically benefit from bariatric surgery are identified, with the necessary careful evaluation of the potential complications. Effective alternatives are urgently

needed and interventions to maintain or repair the dysregulated equilibrium of intestinal microflora in relation to the host health status could be promising options.

Improvement of human health by modifying the intestinal flora was proposed long ago (Metchnikoff E. 1908. In "The Prolongation Of Life: Optimistic Studies"). More recently there has been renewed interest in the use of fecal transplants for various disease states (i.e. colitis), based on the idea that the healthier transplanted flora will replace the disease-promoting microbiota (Pamer 2014). Another therapeutic option that has shown potential in improving the host inflammatory and consequently metabolic status could be oral intake of probiotics to modify the metabolically unfavorable ecosystem (Naito, Yoshida et al. 2011) (Yadav, Lee et al. 2013) (Yoo, Kim et al. 2013) (Stenman, Waget et al. 2014) (Chen, Guo et al. 2014) (Wang, Tang et al. 2015). The clinical world in general, but customers in particular, are now aware that in the late 2000s the benefic effects of probiotics have been claimed by the industry without being based on high quality scientific research (Francisca Joly Gomez, Edition Marabout 2014). Besides the missing approved health claims for some probiotic-containing food, the term has been clearly misused, for example, on products such as shampoos, disinfectants and aftershave, for which maintenance of viability and efficacy of the microbes used are not established. Moreover, even when rigorous scientific results prove probiotic value in a specific setting, further investigations are needed before presenting any conclusion on the effectiveness of one single administered organism. This is the case for *Faecalibacterium prausnitzii* in the context of colitis (Sokol, Pigneur et al. 2008) or other potentially beneficial external interventions to modify gut microbiota and impact human health (Surana and Kasper 2014). Finally, going back to the concept of enterotype, future research will most probably need to look into patient stratification and the degree of success in microflora modulation interventions.

4. Effectiveness of probiotic and prebiotic interventions

When considering interventions, it is necessary to make a distinction between prebiotic and probiotic. Based on the World Health Organization, the definition of probiotic is (any) "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (May 2002, FAO/WHO Guidelines for the evaluation of probiotics in food) (Reid 2005). This vague definition opens interpretations and misuses of probiotic term. Last year, an Expert Meeting was organized by the International Scientific Association for Probiotics and Prebiotics (ISAPP) in order to refine precisely the term probiotic. All the stakeholders involved in the probiotic field worked together performing their respective duties with the final goal to benefit society from scientific advancement in the field (Hill, Guarner et al. 2014).

Together they proposed a set of benchmarks useful to guide clinicians and consumers in differentiating the diverse probiotic products on the market. Recommendations for definition of probiotic included the microbial species that have a reasonable expectation of delivering benefits for the wellbeing of the host (as reported in properly controlled trials), used only on products that deliver live micro-organisms with a suitable viable count of well-defined strains (dead micro-organisms, microbial products, microbial components do not come under the probiotic classification). All probiotics must be safe for their

intended use. Any specific claim beyond “contains probiotics” must be further substantiated. Undefined faecal microbiota transplants need to be kept outside the probiotic framework

Being live bacteria, probiotics are definitely more delicate than prebiotics. Prebiotic is a specialized plant fiber that beneficially nourishes the “good” bacteria already in the gut. As for the concept of probiotic, a group of both academic and industry experts (in the ILSI Europe Prebiotic Expert Group and Prebiotic Task Force, respectively) met to define the prebiotics (Roberfroid, Gibson et al. 2010). In 2010, a new definition was published for the prebiotic effect: “the selective stimulation of growth and/or activity(ies) of one, or a limited number of microbial genus(era)/species in the gut microbiota that confer(s) health benefits to the host”. No food products were clearly classified as prebiotic during that symposium, but the prebiotic concept was established as a scientific fact. Nutritional elements, such as plant fibers, which the body is not able to digest, can act as a fertilizer to promote the growth of many of the good bacteria in large bowel or colon. Usually the dietary fibers contained in prebiotic supplements are inulin and/or oligofructose. The use of prebiotics has been shown to improve insulin sensitivity either directly because their fermentation at the colonic level modulate systemic short chain fatty acids levels or indirectly through bacteria which, in turn, provide health benefits. Research on prebiotics has demonstrated that the important health benefits provided can extend from lower gut health to overall well-being (Roberfroid, Gibson et al. 2010). Several studies have been conducted using prebiotics in the context of a variety of diseases, including obesity-associated disorders. Beneficial effects on the metabolic phenotype, usually body weight evaluation, energy homeostasis, energy intake, adipogenic effects, inflammatory markers, are evaluated in response to the microbiota modulation, using probiotics, but also prebiotics and food (da Silva, dos Santos et al. 2013) (De Vadder, Kovatcheva-Datchary et al. 2014) (Everard and Cani 2014). However, further studies are required to prove and optimize the potential effectiveness of this type of nutritional intervention.

Since probiotics are taken orally, this confers increased value to the benefits provided by bacterial supplementation. Products that cause a selective modification of gut microbiota, favoring normobiosis or reducing the risk of dysbiosis and associated pathologies, have been demonstrated promising in some preliminary studies. For example the beneficial effect of certain probiotics could be explained through the regulation of GPR120 at the intestinal epithelial level (Fredborg, Theil et al. 2012). For these reasons, at present, health and food industries are engaging in huge financial tasks towards scientific research in order to find and guarantee a bacterial strain property and use.

RESEARCH PROJÉT: PURPOSE AND RESULTS

A. Hypothesis and models used

Obesity, insulin resistance and the associated metabolic complications are among the greatest causes of morbidity and premature mortality today. To understand the appropriate functioning of adipose tissue is essential for human health. In chronic pathologies, such as fat excess (obesity) or fat loss (lipodystrophy), the physiology of adipose tissue is deeply altered. Studying the capacity of adipose tissue in maintaining or restoring physiological homeostasis, in the context of such functional disturbances, can reveal new common molecular and genetic pathways implicated in the MetS and help to conceive new therapeutic strategies for the associated metabolic disturbances.

For a decade now, obesity has been seen as an inflammatory disease characterized by a chronic low-grade inflammatory state. Despite the proved association between inflammation and metabolism, the source and type of triggering factors activating the immune system at the level of the different metabolic organs remain unknown. In addition, the beneficial potential of dietary interventions to prevent and/or attenuate the severity of liver and adipose tissue inflammation and the associated metabolic alterations remains to be determined.

The overall purpose of our investigations was to explore the capacity of an organism to respond to a nutritional challenge and to evaluate the consequences at local and systemic level, consequences that could be positive or negative for the organism health. The complexity of this approach requires a global vision of the body physiology, linking nutrition (functional foods) to the complex of metabolic organs and activities. Because the immune system, with the classical first response represented by inflammation, is central to “self defense” in case of external attacks, from viruses to nutrients (excess), and because it is now recognized that it monitors the metabolic state of tissues, we focused on its response in metabolic altered conditions. In particular we ought to answer to those broad questions directing our attention specifically on white adipose tissue and the intestinal tract, two large reservoirs of immune cells, to explore tissue plasticity in response to nutritional intervention.

In the present project, two animal models were employed.

- **Study I:** a lipotrophic mouse model induced by t10,c12-Conjugated Linoleic Acid administration (CLA).
- **Study II:** a mouse model of high fat diet (HFD)-induced obesity, with or without bacterial administration.

The study of these animal models is integrated in the context of adipose tissue alterations, in which inflammation, immune cells, angiogenesis and tissue remodeling have a role. The interactions of the different stromal components, such as preadipocytes, fibroblast-like, endothelial cells, vascular smooth muscle cells, neurons, and immune cells, with adipocytes have not been fully elucidated, not even during the most studied obesity. As described in the Introduction part, adipocyte dysfunction is due to hypertrophy in obesity, whereas after drastic fat mass loss, e.g. following HIV-infection, or in lipodystrophy, adipocyte dysfunction is due to lipotrophy. Despite the contrasting alterations, these

two pathologies present similar complications, in particular insulin resistance. The direct or indirect effects of adipocyte dysfunction on the metabolic profile of the animals represented the common question we aimed to answer in both studies.

In the specific context of **Study I**, through the acute administration of t10,c12-CLA, previously reported to induced a lipotrophic state in mice (Poirier, Shapiro et al. 2006), we evaluated the ability of WAT to cope with this nutritional external stressor, dealing with local (molecular, cellular, structural) and systemic (plasma parameters) changes associated with the drastic fat mass loss induced by this isomer. Adipose tissue plasticity was also evaluated after treatment disruption in relation to insulin alterations. In particular the immune response of WAT was characterized by the analysis of the adipose tissue stromal fraction in two different depots, subcutaneous (inguinal - sWAT) and visceral (peri-uterine - pWAT) WAT. The actions of innate and adaptive immune responses were characterized by flow cytometry analysis in CLA-treated mice in comparison with the control group (LA-treated). Since infiltrating immune cells are known to have a profound effect on adipocytes, we decided to look for correlations between immune cell quantity (and phenotype) and WAT structural alterations. We focused in particular on macrophages (ATMs), the most represented population in WAT of CLA-treated mice, to identify their potential involvement in the ECM accumulation and/or tissue repair/resolution (Olefsky and Glass 2010). Finally the hypothesis that the gastrointestinal tract and its microflora could be affected by CLA administration (as dietary stress) was tested, in order to consider potential endotoxemia in influencing local (WAT) and systemic perturbations.

In the specific context of **Study II**, using the HFD-induced model of obesity, we analyzed the metabolic consequences of WAT hypertrophy in a positive energy balance setting, that mimics the chronic low-grade inflammation associated to human obesity. In addition, through the concomitant administration of specific probiotics, we investigated the potential beneficial effects of gut microbiota changes (direct) or metabolites (indirect) on adiposity and metabolic perturbations associated to obesity.

In both studies, open questions were addressed:

- How adipose tissue copes with a chronic or acute nutritional stress?
- What are the systemic consequences of adipose tissue response to a nutritional stress?
- Are local and/or systemic effects of a nutritional stress mediated, at least in part, through gut microbiota derived signals?

In obesity, the altered interactions between adipocytes and the stromal components, in particular the immune cells, lead to changes in the release of adipokines, cytokines as well as FFA setting up an inflammatory environment (Rosen and Spiegelman 2006), (Ouchi, Parker et al. 2011). In both visceral and subcutaneous adipose tissues, macrophages are viewed as important actors of the inflammatory process taking place in obesity (Lumeng, Deyoung et al. 2007), (Zeyda, Farmer et al. 2007), (Weisberg, Hunter et al. 2006). Directly and/or indirectly affected by the deregulated adipokine milieu, macrophages released locally cytokines known to have a profound effect on adipocytes and the other stromal components (Lumeng, Bodzin et al. 2007), (Zeyda, Farmer et al. 2007). In both studies the levels of these circulating factors were evaluated to better understand the effects of the two different nutritional interventions at the systemic level. Glucose and insulin levels were also considered.

It has been reported that the structure of consumed fat (emulsion vs. free oil) changes the extent of endotoxemia (Mani, Hollis et al. 2013). Factors like diets inappropriately high in saturated fat and also bacterial infections have been associated with insulin resistance. Indeed the direct administration of lipopolysaccharide (LPS), a wall component of Gram-negative bacteria and well-known toll-like receptor 4 (TLR4) agonist, was shown to cause insulin resistance, recapitulating metabolic changes associated with high fat feeding (Cani, Amar et al. 2007). Accumulating evidence indicates that the gut microbiota might be involved in the inflammatory and metabolic perturbations linked to obesity (Ley, Turnbaugh et al. 2006), (Turnbaugh, Ley et al. 2006) and even diabetes (Larsen, Vogensen et al. 2010) (Cani, Possemiers et al. 2009) (Cani, Osto et al. 2012). A diet rich in fat causes changes in the composition of the gut microbiota, possibly weakening the gut barrier function and consequently this might affect the host immune response (Pendyala, Walker et al. 2012). Chronic endotoxemia (the presence of endotoxins such as LPS in blood) occurs in obesity and the complex of CD14 and TLR4 on the surface of innate immune cells is engaged in the recognition of circulating gut-originated antigens, mainly LPS.

The hypothesis that acute administration of the specific lipids we used (LA and CLA) could affect gut microbiota and directly or indirectly affect gut permeability was explored through preliminary screening of microbiota and RT-PCR analysis of gut tissues. On the other hand, the hypothesis that the administration of specific probiotics could positively influence the microbiota composition and consequently improve the obesity-associated metabolic alterations was investigated. In two experimental settings, the potential anti-obesity effects of CLA (Study I) and probiotics (Study II) were studied in relation to inflammation and metabolism in the context of adipose tissue and intestine cross-talk.

B. Study I: Adipose tissue plasticity in response to short-term trans 10, cis12 conjugated linoleic acid (CLA) administration in mice

The objectives of the present study were:

- To determine the effects of a short-term administration of t10,c12-CLA on body weight, fat mass and circulating metabolic variables in C57Bl/6 female mice;
- To evaluate WAT cellular and molecular responses to t10,c12-CLA, with a particular focus on the dynamic of the WAT immune infiltrate;
- To evaluate the reversibility of the integrated response of the different WAT components after t10,c12-CLA withdrawal;
- To investigate potential intestinal alterations induced by t10,c12-CLA administration.

In this study, the previously characterized mouse model of lipotrophic state induced by trans-10,cis-12 isomer conjugated linoleic acid (CLA) was employed to evaluate the ability of WAT to control the alterations induced by an acute nutritional stressor. Female mice are known to present higher susceptibility to CLA than male mice, although the mechanisms accounting for this disparity remain unknown. Moreover, in humans, because healthy women have more adipose tissue than men, they are more profoundly affected by lipodystrophy, with greater metabolic dysregulation (Huang-Doran, Sleigh et al. 2010). For these reasons, female C57Bl6 mice were employed for the study. The model has been

developed by our team in collaboration with INSERM U866 NUTOX (Dijon, France). The experimental design is presented in the publication and synthesized in Figure 27.

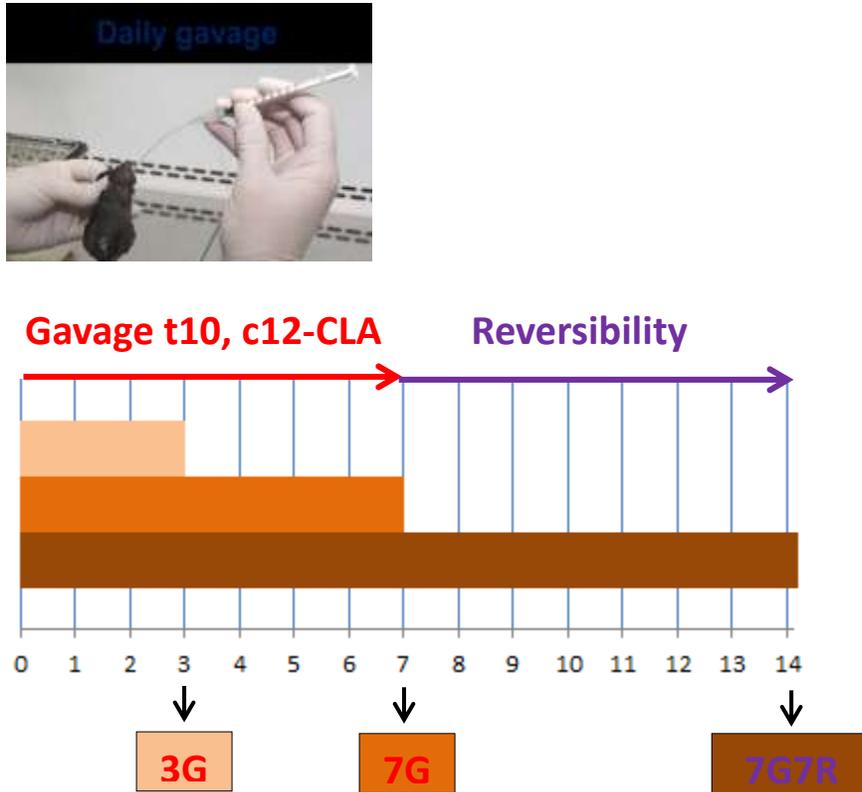


Figure 27. Study I: Experimental design.

G = LA or CLA daily gavage - R = no treatment

C. PUBLICATION 1

Adipose Tissue Adaptive Response to t10,c12 Conjugated Linoleic Acid Engages alternatively activated M2 macrophages

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Running title: M2 macrophages in CLA injured adipose tissue

List of nonstandard abbreviations

LA: linoleic acid

t10,c12-CLA: *trans10,cis12* isomer of linoleic acid

ECM: extracellular matrix

WAT: white adipose tissue

sWAT: subcutaneous inguinal white adipose tissue

pWAT: peri-uterine white adipose tissue

SVF: stroma vascular fraction

Abstract

In mice, nutritional supplementation with the *trans10, cis12* isomer of linoleic acid (t10,c12-CLA) promotes lipoatrophy, hyperinsulinemia and macrophage infiltration in white adipose tissue (WAT). We explored the dynamics of these interrelated responses over two consecutive 7-day periods of t10,c12-CLA administration and withdrawal. t10,c12-CLA down-regulated lipogenic and lipolytic gene expression and increased collagen deposition, although with no evidence of cross-linking. An abundant CD45⁺ cell infiltrate, comprising prominently CD206⁺CD11c⁻ macrophages, was found in WAT in association with an anti-inflammatory gene signature. Infiltration of NK and dendritic cells contributed to WAT innate immune response to t10,c12-CLA. Less abundant adaptive immune cells colonized WAT, including B, NK T, $\gamma\delta$ T and $\alpha\beta$ T cells. By contrast, Treg abundance was not affected. Interruption of treatment allowed recovery of WAT mass and normalization of insulinemia, coincident with regain of WAT homeostasis owing to a coordinated reversion of genic, structural and immune deregulations. These data revealed a striking resilience of WAT after a short-term metabolic injury induced by t10,c12-CLA, which relies on alternatively activated M2 macrophage engagement. Additionally, the temporal links between variations in WAT alterations and insulinemia upon t10,c12-CLA manipulation strengthen the view that WAT dysfunctional status is critically involved in altered glucose homeostasis.

Key words: lipoatrophy; hyperinsulinemia; fibrosis; lipophagy.

INTRODUCTION

It is broadly admitted that accumulation of pro-inflammatory macrophages is a key component of adipose tissue (WAT) response to nutrient overload in obesity. Through the release of an array of pro-inflammatory chemokines and cytokines, macrophages are thought to perpetuate and propagate inflammation in WAT, leading eventually to a chronic pathological state. Although opposite in terms of excess *versus* deficiency of fat mass, obesity and lipodystrophy share common WAT alterations, including inflammation and macrophage infiltration as shown in several lipodystrophic mouse models (1-3) and in patients with HIV-associated lipodystrophy (4-6). Enhanced extracellular matrix deposition and fibrosis are additional signatures of WAT pathology in both conditions (4, 7, 8). These local alterations are accompanied by systemic inflammation and insulin resistance, the most prevalent outcomes of WAT dysfunction common to obesity and lipodystrophy (9).

The time frame required to mount WAT macrophage accumulation and insulin resistance is usually long, ranging from months in mice fed a high fat diet to years in human obesity. Conversely, caloric restriction attenuates WAT inflammation and improves glycemic status in human and mouse obesity after prolonged periods of weight reduction or stabilization (10-14). These observations enlighten WAT plasticity in response to long-term modifications of energy balance and its close link with coordinated changes in metabolic status.

Conjugated linoleic acids (CLA) collectively refer to a group of linoleic acid derivatives with several positional and geometric isomers. Among those, the *trans10,cis12* CLA (t10,c12-CLA) exerts a potent and rapid body weight and fat mass reducing effect in mice (15, 16). Contrary to caloric restriction, t10,c12-CLA administration does not improve metabolic status, but provokes hyperinsulinemia and systemic insulin resistance (15-18). We previously showed that this phenotype is temporally linked with marked macrophage infiltration in peri-uterine WAT in lean female mice (18). In this lipodystrophic model, macrophage recruitment was unexpectedly rapid, since the presence of cells positive for the macrophage marker F4/80 was detected in WAT after 3 days of t10,c12-CLA administration. A similar rapid macrophage infiltration was reported after 3 days of caloric restriction in obese mice, preceding a progressive reduction in macrophage number over a 2 month-period (14). These concordant observations suggest that WAT has the capacity to engage an innate immune response over a short period of nutritional stress.

Since t10,c12-CLA leads to WAT macrophage infiltration and insulin resistance as in obesity and lipodystrophy, we ought to use this compound as a tool to explore the dynamics of these interrelated local and systemic responses and to test their potential reversibility in a short-term time frame.

MATERIALS AND METHODS

Animals and treatment. Female 10 week-old C57BL/6J mice (Charles River Laboratory, L'Arbresle, France) were housed under 12-h light/dark cycles (lights on at 7:00 am) with ambient temperature at 23°C. Gavage was performed daily with 0.1 g sunflower oil, whose main component is linoleic acid (LA), or 0.1 g of a mixture with 0.08 g sunflower oil and 0.02 g triglycerides containing 79% of t10,c12-CLA (total CLA isomers 90.4% (79% t10,c12; 7% c9,t11; and 3.1% t/t), oleic acid 2.5%, linoleic acid 0.2%, saturated fatty acids 6.6%, Lodders Croklaan, Wormerveer, Holland). For evaluation of body weight changes and daily food intake, mice were single-housed and monitored longitudinally. Total body fat mass was assessed with Eco-MRI body composition analyzer (Echo Medical System, Houston, TX, USA). The morning after the last gavage, blood was collected from orbital sinus in anesthetized mice, which were killed by cervical dislocation to collect subcutaneous inguinal (sWAT) and peri-uterine (pWAT) adipose tissue, liver, jejunum and colon. Pieces of WAT were snap frozen in liquid nitrogen before being stored at -80°C, fixed in paraformaldehyde or submitted to collagenase digestion. Care of animals was in accordance with institutional animal care committee guidelines (University of Bourgogne, Dijon, France n°7812).

Immunohistochemistry. Fixed WAT sections of 5 µm were mounted on glass slides and processed according to standard procedures. Immunohistochemical staining was performed with an antibody targeted to CD68 (MCA1957; AbD Serotec, Oxford, UK). Section images were taken by a Zeiss 20 Axiostar Plus microscope, acquired by a TRI CCD camera and processed using Perfect Image V7.7, as previously described (19).

Picrosirius red staining. Slides of WAT biopsies were stained with picrosirius red. Collagen deposition was measured by histomorphometry using an Alphelys platform (Histolab Software, Plaisir, France). Quantification of collagen deposition was expressed as the mean ratio of fibrous tissue area stained with picrosirius red/total tissue surface in 10 random fields at ×10 magnification for each biopsy.

Hydroxyproline assay. Hydroxyproline was evaluated with a colorimetric assay Kit (BioVision, Inc., Milpitas, CA, USA), with some modifications. Frozen WAT biopsies were homogenized in 37°C 6N HCl at a concentration of 0.1 mg/μl and heated at 95°C for 20 hrs. The samples were left for 1 hour at room temperature and centrifuged to collect 10 μl of supernatant. After evaporation, samples were resuspended in 10 μl distilled water before addition of chloramine T reagent. An equal volume of DMAB reagent was added and incubated at 60°C for 90 min. Absorbance was read at 560nm.

RNA isolation and quantitative RT-PCR analysis. Total RNA was isolated with RNeasy kits (Qiagen, Valencia, CA, USA). Gene expression was determined by SYBR Green real-time PCR on 25 ng cDNA. Target gene expression was normalized to the geometric mean of three housekeeping genes (S18, cyclophyllin and RPLP19) and then expressed as fold induction by the comparative CT method using LA-treated control group as reference. Primers for mouse genes are shown in Table S1.

Flow cytometry analysis. Cells of the stroma vascular fraction (SVF) of sWAT and pWAT were obtained by collagenase digestion (20). Cells were incubated in erythrocyte lysis buffer and resuspended in endotoxin-free PBS supplemented with 3 % FCS and 2mM EDTA (FACS buffer). SVF cells were incubated with anti-CD16/CD32 (eBioscience, San Diego, CA, USA) for 15 minutes to block Fc receptors and stained with appropriate monoclonal antibodies for 30 minutes at 4°C in the dark. APC-eFluor780-labeled anti-CD45 (30-F11), PE-Cy7-labeled anti-F4/80 (BM8), eFluor450-labeled anti-CD11c (N418), FITC-labeled anti-CD206 (C068C2), FITC-labeled anti-CD3 (17-A2), PerCP-Cy5.5-labeled anti-CD4 (RM4-5), eFluor450-labeled anti-CD8 (53-6.7), PE-labeled anti-TCRγδ (GL-3), Alexa-Fluor700-labeled anti-NK1.1 (PK136) and APC-labeled anti-B220 (RA3-GB2) (all from eBioscience) were used to characterize SVF cell surface markers. To characterize intracellular expression of the transcription factor Foxp3, SVF cells were first stained for CD45, CD3, CD4 and CD8 surface markers, fixed and permeabilized with Foxp3 fixation/permeabilization kit for 20 minutes and incubated for 30 minutes with PE-labeled anti-Foxp3 (FJK-16s, eBioscience). After staining, cells were washed with FACS buffer and acquired on a FACS LSRII flow cytometer using FACS Diva software (BD Biosciences, San Jose, CA, USA). Data analysis was performed with FlowJo 10.7 software (Tree Star, Ashland, OR, USA).

Blood variables. Glucose was evaluated on peripheral EDTA-treated blood with a commercially available kit (Cayman Biochemical, Ann Arbor, MI, USA). Plasma insulin was measured with the ALPCO ultrasensitive kit (Alpco, Salem, NH, USA). Adiponectin and leptin were quantified by ELISA (R&D Systems, Minneapolis, MN, USA).

Fatty acid profile analysis. 20 mg of sWAT and pWAT, 50 mg of intestinal tissue (jejunum and colon) and 100 mg of liver were homogenized in methanol–chloroform (1/2 v/v). Homogenates were subjected to alkaline hydrolysis followed by methylation, as previously described (21). Fatty acid methyl esters were quantified by gas-liquid chromatography (Focus GC, Thermo-Finnigan, Interscience, Belgium). Identification and quantification of each peak were made by comparison of retention times with standards (Alltech Associates; except CLA isomers from Nu-Chek Prep).

Statistical analysis. Results are expressed as mean \pm standard error of the mean (SEM). Normal distribution of data was tested with Shapiro-Wilk test. Differences between groups were determined using unpaired Student's t test or Mann-Whitney test for variables normally or not normally distributed, respectively. Relationships between variables were assessed using Spearman correlation. All statistical analyses were performed with GraphPad Prism 5.0 Software (GraphPad Software, Inc, La Jolla, CA, USA). A p value < 0.05 was considered statistically significant.

RESULTS

Local and systemic reversible metabolic alterations.

Body weight decreased steadily during t10,c12-CLA administration (Fig 1A) with no major change in food intake (Fig 1B), and returned rapidly to pre-treatment values upon t10,c12-CLA withdrawal. Reflecting lipoatrophy, the percentage of MRI-evaluated fat mass was reduced after 3 days of t10,c12-CLA administration and remained low thereafter (Table 1). Accordingly, the weight of WAT pads decreased, with a more pronounced reduction of subcutaneous inguinal (sWAT) than deep peri-uterine (pWAT) WAT depot (Table 1). After 7 days without t10,c12-CLA, pWAT mass was normalized to the values found in LA-treated control mice, while sWAT remained reduced. Adipocyte diameters were modestly affected by t10,c12-CLA administration or removal (Table 1).

At systemic level, insulin concentrations increased progressively (Table 1). At day 7, insulin circulating levels were 8-fold higher than in control mice and sharply decreased back to normal values upon t10,c12-CLA withdrawal. These data establish a direct link between hyperinsulinemia and t10,c12-CLA administration. No concomitant change in glycemia was consistent with the development of systemic insulin resistance.

Fatty acid profiling revealed that t10,c12-CLA prominently accumulated in WAT, where its concentrations per gram of tissue were 8 to 20 fold higher than in intestine or liver (Fig 1C). This tissue-specific difference pointed out WAT as the main metabolic tissue targeted by the CLA isomer. After 7 days of t10,c12-CLA, several adipokines were markedly down-regulated in pWAT and returned to normal levels of expression upon t10,c12-CLA removal (Fig 1D). A similar pattern of regulation was found in sWAT (data not shown). Adiponectin followed the same variations at gene level and in the circulation (Fig 1D, Table 1). Unlike other adipokines, however, leptin mRNA and plasma levels were not significantly changed upon t10,c12-CLA manipulation. This suggests that t10,c12-CLA dictates adipocyte secretome mostly by targeting transcript levels, leaving secretion pathways virtually unaltered.

Besides adipokines, genes functionally involved in *de novo* lipogenesis and lipolysis were profoundly down-regulated by t10,c12-CLA (Fig 1E). Upon t10,c12-CLA withdrawal, mRNA levels rebounded, although not always back to the values of LA-treated mice. The mRNAs of two lipogenic enzymes, Fatty acid synthase (FAS) and Acetyl-CoA-carboxylase (ACC), were normalized (FAS) and even up-regulated (ACC) in pWAT, but remained decreased in sWAT in line with less efficient mass recovery in this subcutaneous fat depot. In sharp contrast with the marked down-regulation of lipogenic and lipolytic genes, genes implicated in lipophagy, including the transcription factor Tfeb, its target, the lysosomal acid lipase (Lipa) and the mitochondrial protein UCP2, were up-regulated in both sWAT and pWAT in response to t10,c12-CLA (Fig 1E). The reversibility of this regulation identified t10,c12-CLA as the trigger of lipophagic genes overexpression in WAT.

Reversible WAT macrophage accumulation.

Immunohistochemistry analysis of sWAT and pWAT showed that cells labeled by the pan-macrophage marker CD68 accumulated after 3 days and continued to increase after 7 days of t10,c12-CLA (Fig 2A, B). In pWAT, the number of CD68⁺ cells dropped abruptly upon t10,c12-CLA disruption, back to the low level found in LA treated mice. In sWAT, however,

residual CD68 immunolabeled cells persisted. Coordinated changes in CD68 gene expression and CD68⁺ cell number were found in both WAT depots (Fig 2C).

To substantiate these observations, we analyzed cells contained in the stroma vascular fraction (SVF) of WAT by flow cytometry. The number of CD45⁺ cells in this non-adipose cellular fraction increased markedly in response to 7 days of t10,c12-CLA administration (Fig 2D). Roughly 50% of these cells were positive for the F4/80 macrophage marker and this proportion was not significantly affected by t10,c12-CLA, supporting a large infiltration of macrophages in both adipose depots (Fig 2E).

In this lipotrophic model, as opposed to the inverse relationship found in obesity, macrophage abundance was negatively correlated with WAT mass (Table 2). Nevertheless, as in obesity, adiponectinemia and insulinemia were respectively negatively and positively associated with WAT macrophage accumulation (Table 2). Of note, these relationships were independent of subcutaneous or deep WAT localization.

Reversible collagen deposition.

Picrosirius red labeling revealed that t10,c12-CLA induced collagen deposition in WAT. This response was more pronounced in sWAT than in pWAT (Fig 3A,B). Increased collagens mostly in sWAT was confirmed by hydroxyproline accumulation (Fig 3C). Upon t10,c12-CLA interruption, collagen labeling decreased in both depots, although less efficiently in sWAT than in pWAT. Gene expression analysis identified fibronectin and collagen 1 as the ECM components selectively increased in both adipose depots, with the additional contribution of collagen 3 in sWAT (Fig 3D). In line with histological data, ECM gene expression decreased sharply upon t10,c12-CLA withdrawal. Several ECM remodeling related genes followed a similar pattern of regulation, including the proteases Mmp12 and cathepsin S, the protease inhibitor Timp-1 and galectin 3 (Fig 3E). Despite increased collagen deposition, collagen fibril bridging enzymes were not found overexpressed but rather down-regulated (Lox, lysyl oxidase) or unchanged (Loxl2, lysyl oxidase-like 2) in response to t10, c12-CLA (Fig 3F). The expression of profibrotic factors, such as TGF- β 1, inhibin beta-A and Ctgf, was modestly changed (Fig 3G). Although TGF- β 1 mRNA level slightly increased in sWAT, this transcriptional profile makes unlikely the induction of a fibrotic phenotype in fibroblasts and potentially other cell types in WAT. Supporting this possibility, Acta2 gene expression, a marker of fibrotic activation, did not

correlate with fibronectin gene expression (Fig 3H). By contrast, strong correlations between fibronectin and Cd68 mRNA were found in both adipose depots, suggesting implication of macrophages in the ECM remodeling response to t10,c12-CLA (Fig 3H).

Alternatively-activated M2 profile of WAT macrophages.

We determined the phenotype of t10,c12-CLA-induced WAT macrophages through flow cytometry analysis of the cell surface markers CD11c and CD206 in the F4/80⁺ cell population. In both WAT depots, over 80 % of the F4/80⁺ cells were CD206⁺CD11c⁻ in LA treated mice (Fig 4A-C). In response to t10,c12-CLA, this proportion only slightly decreased, coincident with the appearance of a minor subpopulation of CD206⁺CD11c⁺ cells. Both cell types markedly increased in number per gram of WAT, in line with F4/80⁺ cell accumulation with t10,c12-CLA (Fig 4D). Whatever the fat depot, CD206⁺CD11c⁻ cells represented the prominent macrophage subset in t10,c12-CLA treated mice. Up-regulation of a series of M2-associated gene markers confirmed the prevalence of alternatively activated macrophages in WAT (Fig 4E). All M2 markers decreased sharply upon t10,c12-CLA withdrawal. In sharp contrast, M1 markers were virtually not affected by t10,c12-CLA manipulation. The M2/M1 ratio estimated through Arg1/iNOS mRNA increased 3-fold in sWAT and nearly 20-fold in pWAT and was normalized with t10,c12-CLA disruption (Fig 4F). Anti-inflammatory cytokine IL-1ra and Il-10 mRNA levels dramatically increased in response to t10,c12-CLA, overshadowing slight elevation of pro-inflammatory Tnfa and Il-1 β gene expression in both WAT depots (Fig 4G). Similar to M2 macrophage markers, anti-inflammatory cytokine gene expression dropped abruptly upon treatment interruption, demonstrating striking dependency on t10,c12-CLA administration. These data show that WAT reacts to t10,c12-CLA by engaging a marked and reversible M2-like anti-inflammatory response.

Innate and adaptive immune cell profile in WAT.

Beside macrophages, other innate immune cells, including NK cells (CD3⁻ NK1.1⁺) and dendritic cells (F4/80⁻ CD11c⁺) contributed to the t10,c12-CLA-induced CD45⁺ cell infiltrate in WAT (Fig 5A), along with less abundant NKT cells (CD3⁺ NK1.1⁺), T cells and B cells (Fig 5B). In pWAT, the proportions of CD8⁺ and CD4⁻ CD8⁻ cells inside the $\alpha\beta$ T cell population slightly increased, while the frequency of CD4⁺ cells decreased in response to t10,c12-CLA administration (Fig 5C). A similar profile was found in sWAT (data not shown). When

expressed per gram of WAT, the number of each cell subset tended to increase, reaching statistical significance only for CD8⁺ and CD8⁻CD4⁺ cells in pWAT (Fig 5D). The frequency of Foxp3⁺ Tregs inside the CD4⁺ T cell subset was unchanged by t10,c12-CLA, amounting 8.2 ± 1.8 and 4.9 ± 0.64 % (n = 3) in the LA group and 8.8 ± 1.2 and 6.1 ± 1.4 % (n = 5, ns) in the t10, c12-CLA group in sWAT and pWAT, respectively. Finally, a small population of TCRγδ⁺CD3⁺ T cells was enhanced in both WAT depots (Fig 5E, F).

Specific chemokine profile and cell proliferation in WAT.

The large t10,c12-CLA-induced infiltrate of macrophages raised the question of their origin, either from increased recruitment of circulating monocyte precursors, *in situ* proliferation or both. Gene expression analysis revealed that t10,c12-CLA induced a specific chemokine regulation, with marked increase of Ccl7, Ccl2, osteopontin (Opn) and Cxcl10 mRNA and lower stimulation of Ccl5, Cxcl2 and Cxcl3 genes in both WAT depots (Fig 6A,C). Changes in chemokine gene expression were driven by t10,c12-CLA, as shown by their normalization with treatment interruption. These data suggest that t10,c12-CLA promotes an environment favoring blood cell recruitment in WAT. However, *in situ* proliferation cannot be excluded. Indeed, when we investigated Ki-67 gene expression as a marker of cell proliferation, its mRNA level increased in proportion with Cd68 mRNA in both sWAT and pWAT (Fig 6 B,D). Interestingly, Ki-67 gene expression was still observed enhanced after t10,c12-CLA removal, when Cd68 gene expression was normalized. This suggests that SVF non-immune cells might actually proliferate in WAT during t10,c12-CLA treatment and recovery period.

DISCUSSION

Here, whereas t10,c12-CLA administration in lean mice provokes a lipotrophic syndrome along with macrophage accumulation and collagen deposition in WAT, we demonstrate that these alterations are reversible, at least in the short term. The results highlight an appropriate extracellular matrix remodeling with alternatively activated M2 macrophage accumulation in WAT. This reversible phenomenon reflects the ability of WAT to adequately adapt to a metabolically altered environment. We suggest that WAT M2 macrophage accumulation represents an early and adaptive response to environmental insult. Under persistent metabolic stress, M2-macrophages could become outnumbered by M1-macrophages, leading to chronic inflammation and insulin resistance.

As previously reported and confirmed here, mice receiving t10,c12-CLA developed marked hyperinsulinemia with no change in glycemia, reflecting a state of systemic insulin resistance (15-18). In this model, as opposed to obesity, altered glucose homeostasis occurred in face of M2 macrophage accumulation and high M2/M1 ratio. Our data do not support a protective role for WAT M2 macrophages against metabolic deregulation occurring at this early stage. This situation is apparently at odds with studies showing that several procedures increasing the M2/M1 ratio in WAT, through thiazolidinediones (22, 23), dietary ω -3 fatty acids administration (24) or in response to nematode parasitic infection (25) (26) for example, improved glycemic status in obese mice. In these latter conditions, reduction of M1 pro-inflammatory macrophages rather than M2 macrophage accumulation might be the prominent trigger of metabolic improvement.

M2 macrophages are thought to be implicated in ECM remodeling and collagen turnover required for tissue repair in response to injury (27-29). In the t10,c12-CLA model, ECM deposition was associated with alternatively activated macrophage accumulation and dramatic up-regulation of the macrophage elastase MMP12. This appeared to be a rapid and adaptive response as opposed to pathological fibrosis. First, and most importantly, collagen accumulation and enhanced ECM-related gene expression were reversible, as soon as 7 days after we stop t10,c12-CLA administration. Second, enzymes involved in collagen cross-linking were not up-regulated, suggesting that their relative deficiency limited the formation of stable fibrils. Third, there was no major induction of several well established pro-fibrotic factors and no evidence of pro-fibrotic activation of fibroblasts in WAT. Surprisingly, this occurred despite marked up-regulation of Lgals3, a β -galactoside-binding lectin, which has been implicated in myofibroblast activation and fibrosis in kidney and liver (30, 31), but could play a distinct role in WAT. It was previously reported that feeding mice a t10,c12-CLA enriched diet induced ECM deposition in mammary fat pad (32). Our data additionally revealed depot selectivity in collagen deposition, which was more pronounced and long-lasting in subcutaneous sWAT than in visceral pWAT. Importantly, the persistence of increased ECM deposition in sWAT was associated with delayed tissue mass regain after t10,c12-CLA removal. This gives further support to the notion that abnormal collagen deposition in WAT contributes to adipocyte dysfunction (8, 33).

Recent studies have established that a specific immune profile prevails in WAT of lean mice and that obesity induces marked deregulation of cell frequency and/or phenotype (34, 35). How WAT immune cells interact with each other and influence the biology of non immune cells,

including adipocytes, is not firmly established. Through gain and loss of function mouse studies, it has been proposed that some cells, including NK cells (36), B cells (37, 38) and $\gamma\delta$ T cells (39) promote WAT dysfunction and metabolic disturbances, while others, such as NKT cells (40) and Tregs (41, 42) contribute to maintain WAT homeostasis and improved metabolic status. In our model, the immunological profile of WAT was markedly and specifically modified in response to t10,c12-CLA-induced injury. The prominent response was the primary recruitment of innate immune cells. As a whole, the immune cell infiltrate driven by t10,c12-CLA contained a mix of cells shown to be protective or deleterious in mice models. Although not tested, the contribution of neutrophils, which transiently colonize WAT in the early phases of diet-induced obesity (43) and/or eosinophils, which sustain WAT macrophage M2 activation (44) might add further complexity to WAT immune response to t10,c12-CLA. Future exploration will specify the implication of each cell types in WAT dysfunctional state and ensuing glycemic deregulation induced by t10,c12-CLA administration.

Although it is well established and confirmed in the present study that t10,c12-CLA accumulates selectively in WAT, it is currently not known how this CLA isomer impinges upon transcriptional programs in adipocytes. In our experimental setting, genes functionally implicated in both lipogenic and lipolytic metabolic pathways were severely down-regulated after 7 days of t10,c12-CLA administration. The reversibility of these alterations indicates that metabolic functions were restored in a majority of adipocytes when t10,c12-CLA was removed. Our data suggest that induction of lipophagy through overexpression of *Lipa* (45), is part of a physiological response to t10,c12-CLA-induced catabolic phenotype in adipose cells. Fatty acids released through *Lipa* then could serve as alternative energy substrates, owing to t10,c12-CLA-induced overexpression of *UCP2* (46, 47). In line with our current observations, a coordinated and marked increase in *Lipa* and *UCP2* gene expression was previously found through WAT transcriptomic analysis (48). Strikingly, this up-regulation was detected after only 2 days of t10,c12-CLA oral supplementation in mice, indicating that induction of lipophagy might represent one of the earliest metabolic switch induced by t10, c12-CLA.

Whether adipogenic precursors contribute to tissue resilience after t10,c112-CLA injury remains to be assessed. We observed that high levels of *Ki-67* gene expression persisted in pWAT after t10,c12-CLA withdrawal, when immune cells were not detected any more in WAT. Thus, it is possible that adipocyte progenitors proliferate in this situation, as recently demonstrated in a mouse model of $\beta 3$ adrenergic receptor agonist-mediated adipose cell death

(49). In this study, PDGFR α ⁺ progenitor cells expressing the osteopontin receptor CD44 were recruited and proliferated in response to M2 macrophage-derived osteopontin. This suggests that the M2 macrophage accumulation and marked overexpression of osteopontin that we observed in response to t10,c11-CLA might contribute to progenitor proliferation, particularly in pWAT. Increased cell apoptosis (15, 18) could support this process and be part of the triggering events for M2 macrophage recruitment (50).

To conclude, this study revealed WAT ability to mount a rapid and reversible response to a short-term nutritional insult, relying primarily on induction of an anti-inflammatory program and alternatively-activated M2 macrophage recruitment. The components of this response were strikingly distinct from WAT alterations reported in obesity, which comprise inflammation, M1 macrophage accumulation and fibrosis. Further studies will help to assess whether the capacity of WAT to adapt to a disturbed metabolic environment, enlightened in t10,c12-CLA treated mice, is lost under chronic and persistent metabolic constrain leading to adipocyte dysfunction in obesity or lipodystrophy. Nevertheless, despite WAT resilience, t10,c12-CLA induced metabolic, structural and immune alterations were associated with rapid increased of insulinemia, adding support to the view that WAT dysfunctional state is a crucial to trigger glucose homeostasis alteration.

ACKNOWLEDGEMENTS

The authors thank Remi Selleslagh and Sonia Mutel for expert technical assistance.

Ws also thank Isabelle Dugail, Danièle Lacasa and Charles Caër for helpful discussion. This study was funded by the National Agency of Research (Adipofib to KCs' team), French Foundation for Medical Research (Equipe FRM DEQ20120323701 to KC's team) and by a grant from Groupe Lipides Nutrition (GLN) to MGM. This work has benefited from a French government grant managed by National Agency of Research (Institute of Cardiometabolism and Nutrition, ICAN, reference ANR-10-IHU). PhD fellowships were allocated to ST by the EU-Metacardis FP7 program. We thank Association Française d'Etude et de Recherche sur l'Obésité (AFERO) for financial support to MP. The work performed by ND and CD was supported upon granting by the Fonds National de la Recherche Scientifique in Belgium (FNRS Crédit aux Chercheurs 1.5121.12F). CD benefited from a grant from Institut Danone Belgique as a salary.

No potential conflicts of interest relevant to this article were reported.

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Table 1: Morphometric and blood metabolic variables in mice receiving linoleic acid (LA) or t10,c12-CLA by daily gavage for 3 or 7 days, followed by 7 days of recovery without treatment.

		Linoleic acid (LA)			t10,c12-CLA		
		3 gavages	7 gavages	7 gavages + recovery	3 gavages	7 gavages	7 gavages + recovery
Body composition							
	Body weight (g)	20.1 ± 0.43	19.6 ± 0.29	20.0 ± 0.18	20.1 ± 0.39	18.4 ± 0.32*†	19.1 ± 0.45
	Fat mass (%)	13.0 ± 1.25	13.7 ± 1.26	12.2 ± 1.63	9.74 ± 0.48*	8.67 ± 0.77*	10.6 ± 2.39
Adiposity							
	sWAT (g)	0.28 ± 0.02	0.26 ± 0.02	0.26 ± 0.03	0.21 ± 0.02*	0.14 ± 0.01*†	0.10 ± 0.01*†\$
	pWAT (g)	0.38 ± 0.03	0.30 ± 0.04	0.34 ± 0.07	0.25 ± 0.02*	0.21 ± 0.02*	0.33 ± 0.06\$
	Adiposity index (%)	3.28 ± 0.23	2.83 ± 0.17	2.98 ± 0.48	2.29 ± 0.17*	1.81 ± 0.12*†	2.15 ± 0.31
Adipocyte diameter							
	sWAT (µm)	35.1 ± 3.85	34.4 ± 3.51	34.2 ± 1.37	24.2 ± 1.92*	29.4 ± 0.98†	29.4 ± 1.79*
	pWAT (µm)	59.2 ± 5.53	46.7 ± 6.97	48.2 ± 5.54	47.6 ± 1.27	39.6 ± 1.20†	47.7 ± 4.21
Plasma variables							
	Glucose(mmol/l)	9.2 ± 0.52	9.2 ± 0.60	7.7 ± 0.60	8.6 ± 0.41	9.6 ± 1.1	10.4 ± 2.2
	Insulin (ng/ml)	0.43 ± 0.13	0.46 ± 0.12	0.47 ± 0.12	1.0 ± 0.46	3.9 ± 1.0*†	0.65 ± 0.21\$
	Adiponectin (µg/ml)	15.2 ± 2.1	18.5 ± 2.7	20.6 ± 1.5	9.7 ± 0.84*	6.6 ± 0.53*†	13.9 ± 1.3*†\$
	Leptin (ng/ml)	3.5 ± 0.59	4.5 ± 0.65	5.8 ± 0.75	3.0 ± 0.50	4.3 ± 0.77	7.0 ± 1.3†

Data are expressed as means ± SEM (n = 6-12 mice); Adiposity index was calculated as the ratio of sWAT + pWAT weights over body weight in percentage; *p < 0.05 vs. LA; † p < 0.05 vs. 3 gavages; \$ p < 0.05 vs. 7gavages.

Table 2: Spearman's correlation of macrophages accumulation with adipose tissue weight and

		F4/80 ⁺ cells (n / g WAT)		CD68 ⁺ cells (n / 100 adipocytes)	
		sWAT	pWAT	sWAT	pWAT
Adipose tissue weight	r	-0.77	-0.55	-0.52	-0.41
	P	< 0.001	< 0.001	< 0.001	0.006
Plasma adiponectin	r	-0.51	-0.43	-0.84	-0.74
	P	0.002	0.007	< 0.001	< 0.001
Plasma insulin	r	0.72	0.63	0.61	0.53
	P	< 0.001	< 0.001	<0.001	0.002

biomarker of glycemic status

Figure legend

FIG 1. Administration of t10,c12-CLA induces a reversible lipotrophic syndrome and targets WAT secretory and metabolic function. Female 10 week-old C57BL/6J mice received t10,c12-CLA by daily gavage for 7 days (7G) followed by 7 days without treatment (7G7R). Body weight (**A**) and food intake (**B**) were followed daily during experimental periods. Data are means \pm SEM; LA, white circle, n = 12; CLA, black circle, n = 12. * $P < 0.05$ vs. LA. **C:** Amounts of t10,c12-CLA in sWAT, pWAT, jejunum, colon and liver after one gavage with LA or t10,c12-CLA. **D:** Adipokine gene expression analysis in pWAT by RT-qPCR. Results are expressed as fold mRNA change relative to LA values set to 1. **E:** Gene expression analysis for a panel of metabolic enzymes and transcription factors. Results are shown as fold decrease (- n) or increase (+ n) over LA values. Data are means \pm SEM (n = 5-10 per group). * $P < 0.05$ vs. LA.

FIG 2. t10,c12-CLA provokes a rapid accumulation of macrophages in WAT after 3 (3G) and 7 (7G) gavages, which is reversed after 7 days without treatment (7G7R). Macrophage abundance was assessed by immunohistochemistry using anti-CD68 antibody. **A:** Representative images of sWAT and pWAT; original magnification $\times 20$. **B:** quantification of CD68⁺ cells normalized to 100 adipocytes. Data are means \pm SEM; n = 5-15 per group; * $P < 0.05$ vs. LA at same time point. **C:** RT-qPCR analysis of CD68. Data are means \pm SEM; n= 3-10 per group; * $P < 0.05$ vs. LA at same time point. **D-E:** Quantification of CD45⁺ (D) and F4/80⁺ cells (E) of the stroma vascular cell fraction (SVF) of sWAT and pWAT after 7 gavages with LA or t10,c12-CLA by flow cytometry. Data are means \pm SEM (n=3-10 per group). * $P < 0.05$ vs. LA.

FIG 3. Extracellular matrix deposition is induced in WAT after 3 (3G) and 7 (7G) gavages with t10,c12-CLA and is reversed after 7 days without treatment (7G7R). **A:** Morphological aspect of sWAT and pWAT observed after picrosirius red labeling. Original magnification 20 \times . **B:** quantification of labeled surface in per cent of total biopsy area for sWAT and pWAT. Data are means \pm SEM for n = 6-16 per group. **C:** Hydroxyproline content in WAT (LA, n = 3; CLA, n = 5). **D-G:** Gene expression analysis for ECM components (D), tissue remodeling (E) and bridging (F) enzymes, and profibrotic factors (G) was performed in sWAT and pWAT by RT qPCR. Results are expressed as fold

mRNA change relative to LA values set to 1 and shown as mean \pm SEM for n = 5-10. * P < 0.05 vs. LA. **H**: Spearman correlations between fibronectin mRNA and Cd68 or aortic smooth muscle actin (*Acta2*) mRNA levels expressed as fold change over LA set to 1 in sWAT and pWAT.

FIG 4. t10,c12-CLA administration favors alternative activated M2 macrophage accumulation in WAT, in association with an anti-inflammatory transcriptional profile. Cells expressing CD11c or CD206 in F4/80⁺ gated cells were quantified by flow cytometry of WAT stroma vascular cell fraction. **A-B**: Representative flow cytometry results. **C-D**: Quantification of cells in percentage (C) and in number per gram of WAT (D) (n = 3-10) * P < 0.05 vs. LA. **E**: Gene expression analysis for M2 and M1 macrophage markers in sWAT and pWAT by RT qPCR. **F-G**: Confirmation of M2-like macrophage accumulation by the ratio of representative phenotype markers Arg1/iNOS (F) and associated cytokines in WAT (G). Results are expressed as fold change relative to LA values set to 1 and are shown as mean \pm SEM for n = 5-10. * P < 0.05 vs. LA.

FIG 5. Cells of innate and adaptive immunity contribute to the immune infiltrate induced by t10,c12-CLA in WAT. Flow cytometry analysis of the stroma vascular fraction of sWAT and pWAT was performed to quantify immune subpopulations. **A**: NK and dendritic cells increased in pWAT and sWAT. **B**: Increased abundance of B cells, NKT and $\alpha\beta$ T cells per gram of WAT (n = 5-10). **C**: Percentage of CD4⁺, CD8⁺, CD4⁺CD8⁻ in $\alpha\beta$ T cells provided in pWAT from a representative experiment. **D**: Amounts of CD4⁺, CD8⁺, CD4⁺CD8⁻ in $\alpha\beta$ T cells per gram of WAT (n = 5-15). **E**: Percentage of $\gamma\delta$ T cells in CD3⁺ cells provided in pWAT from a representative experiment. **F**: Amounts of $\gamma\delta$ T cells per gram of WAT (n = 3-10). * P < 0.05 vs. LA.

FIG 6. t10,c12-CLA-induced macrophage infiltrate is potentially regulated by chemotactic recruitment and *in situ* proliferation evaluated by Ki-67 gene expression. **A-D**: Gene expression analysis was performed for a panel of factors related to immune cell recruitment and for Ki-67 and Cd68 in sWAT (A, B) and pWAT (C, D). Results are expressed as fold mRNA change relative to LA values set to 1. All data are means \pm SEM (n=5-10 per group). * P < 0.05 vs. LA.

Fig 1

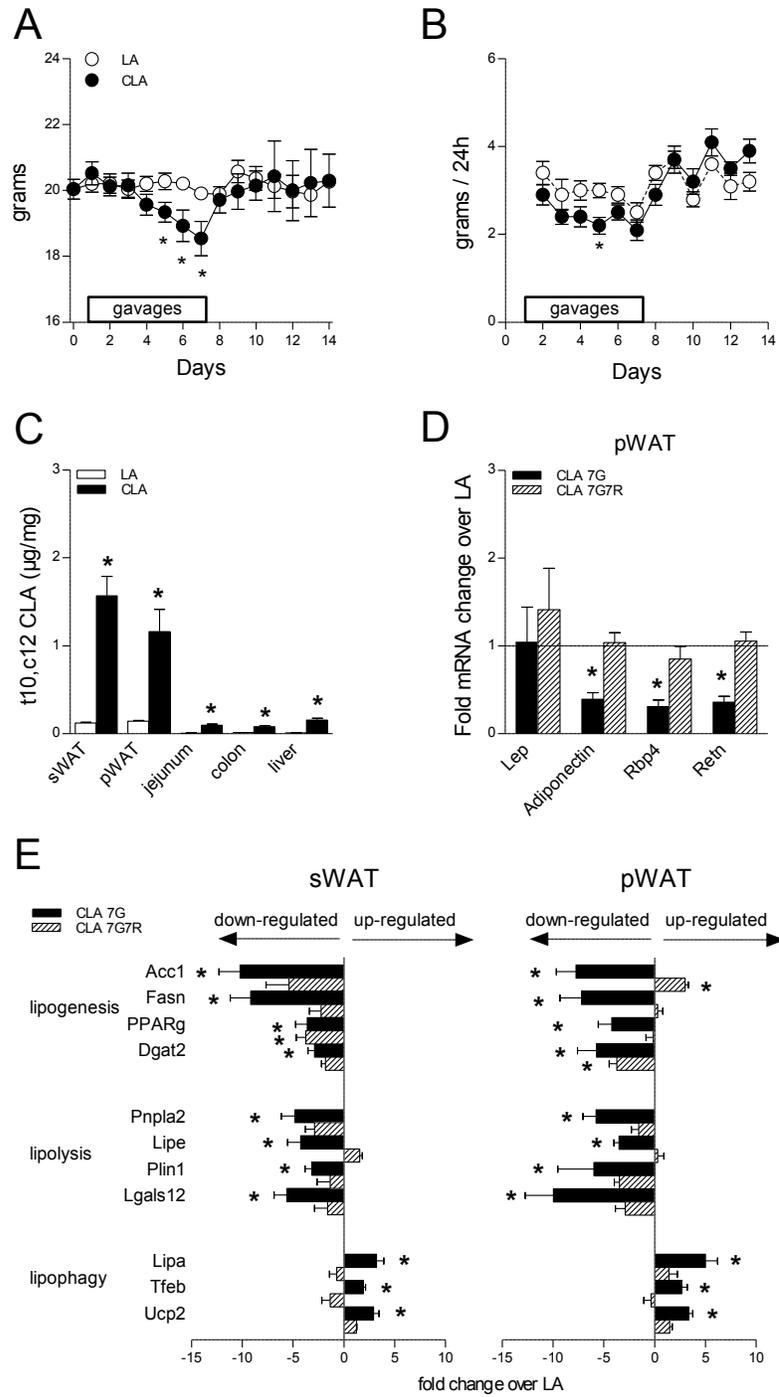


Fig 2

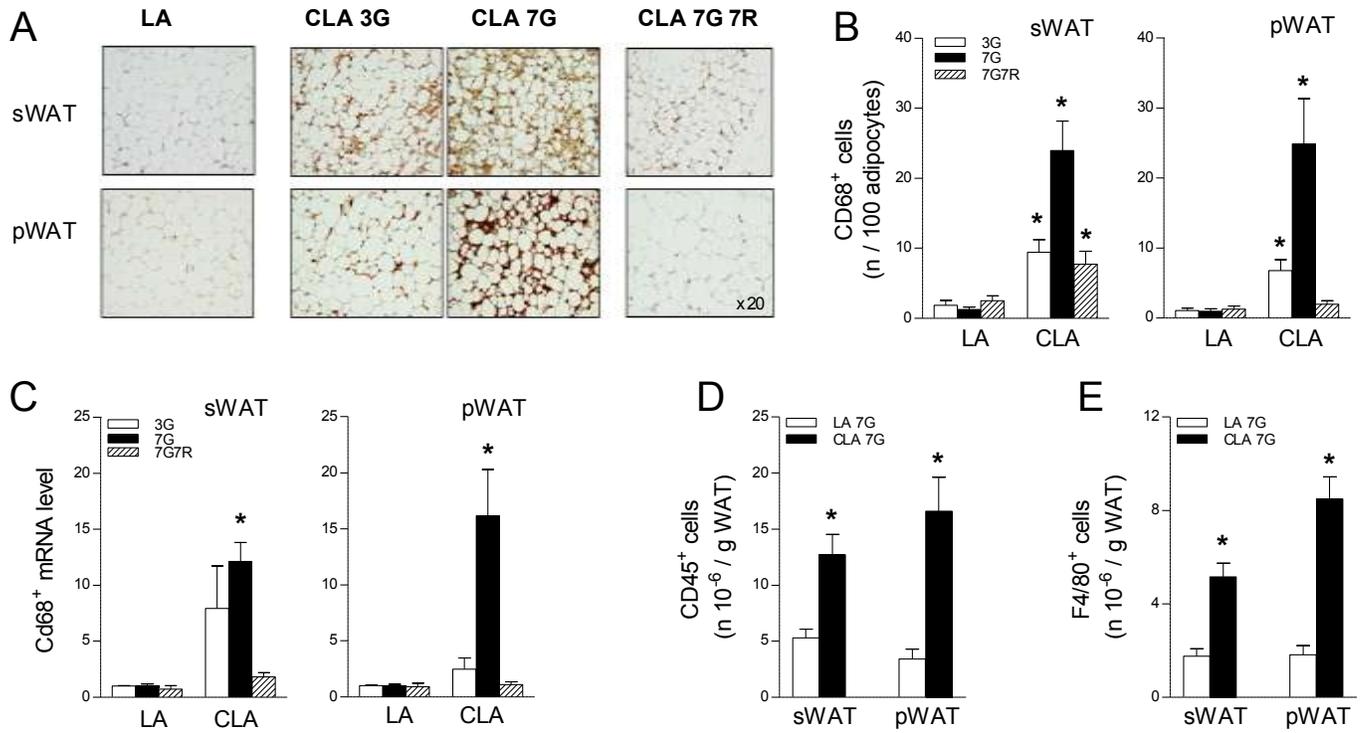


Fig 3

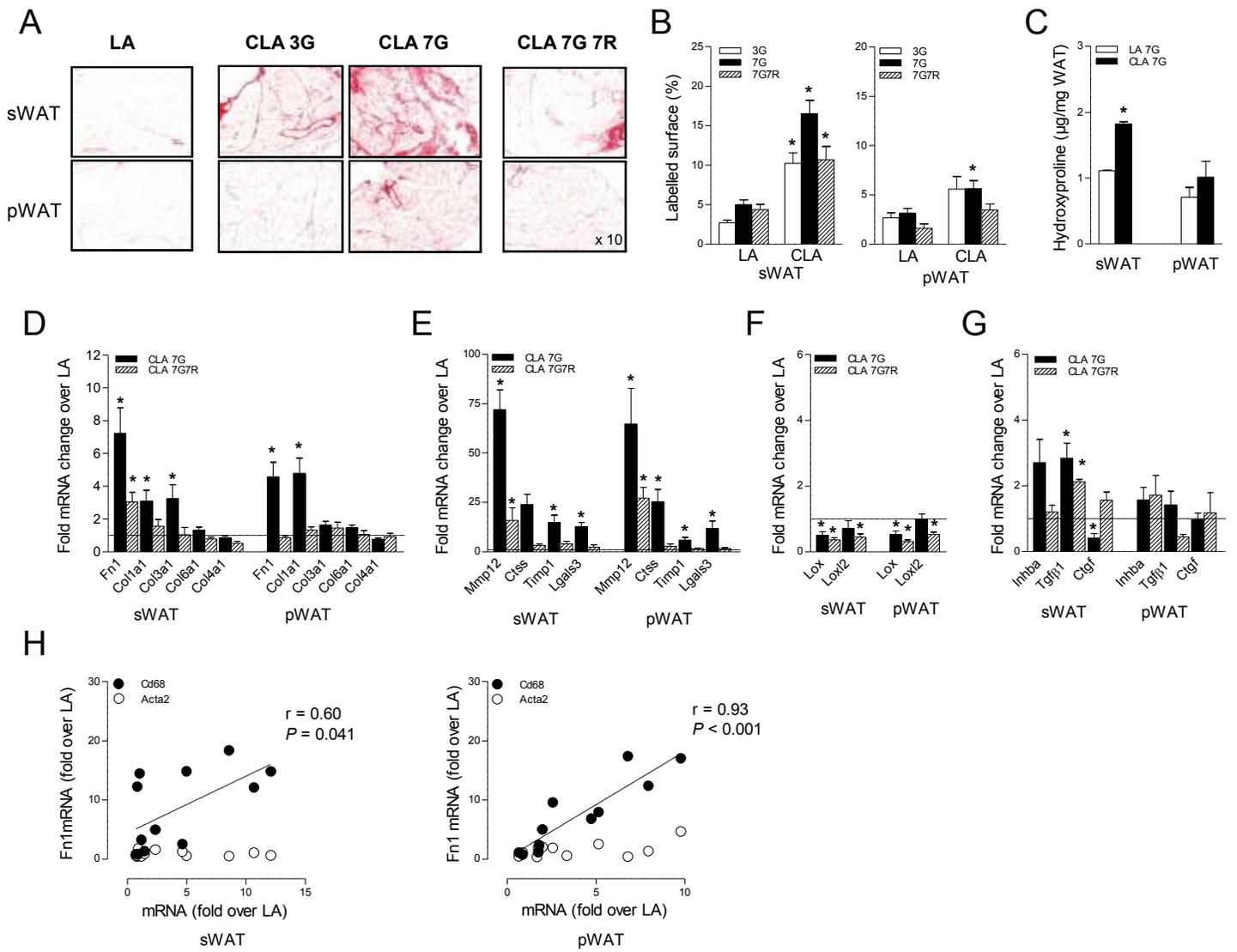


Fig 4

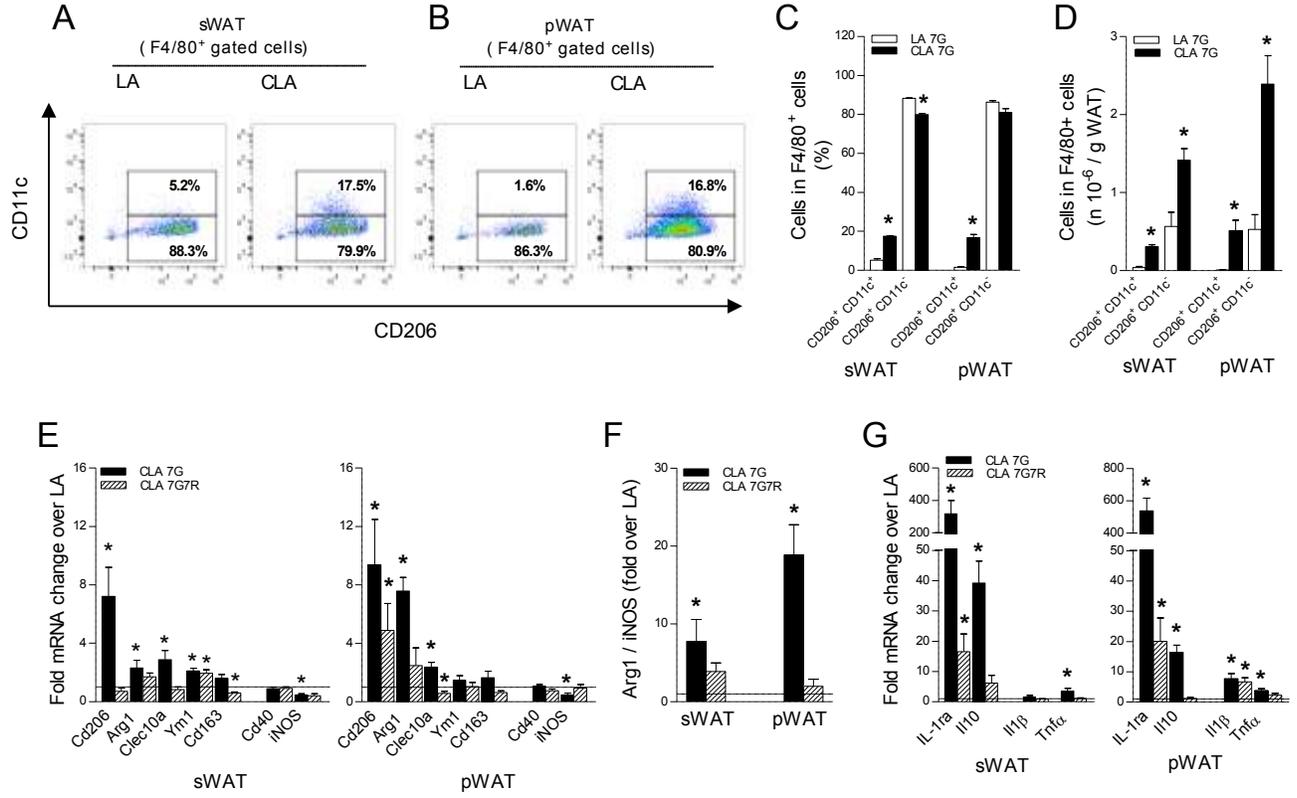


Fig 5

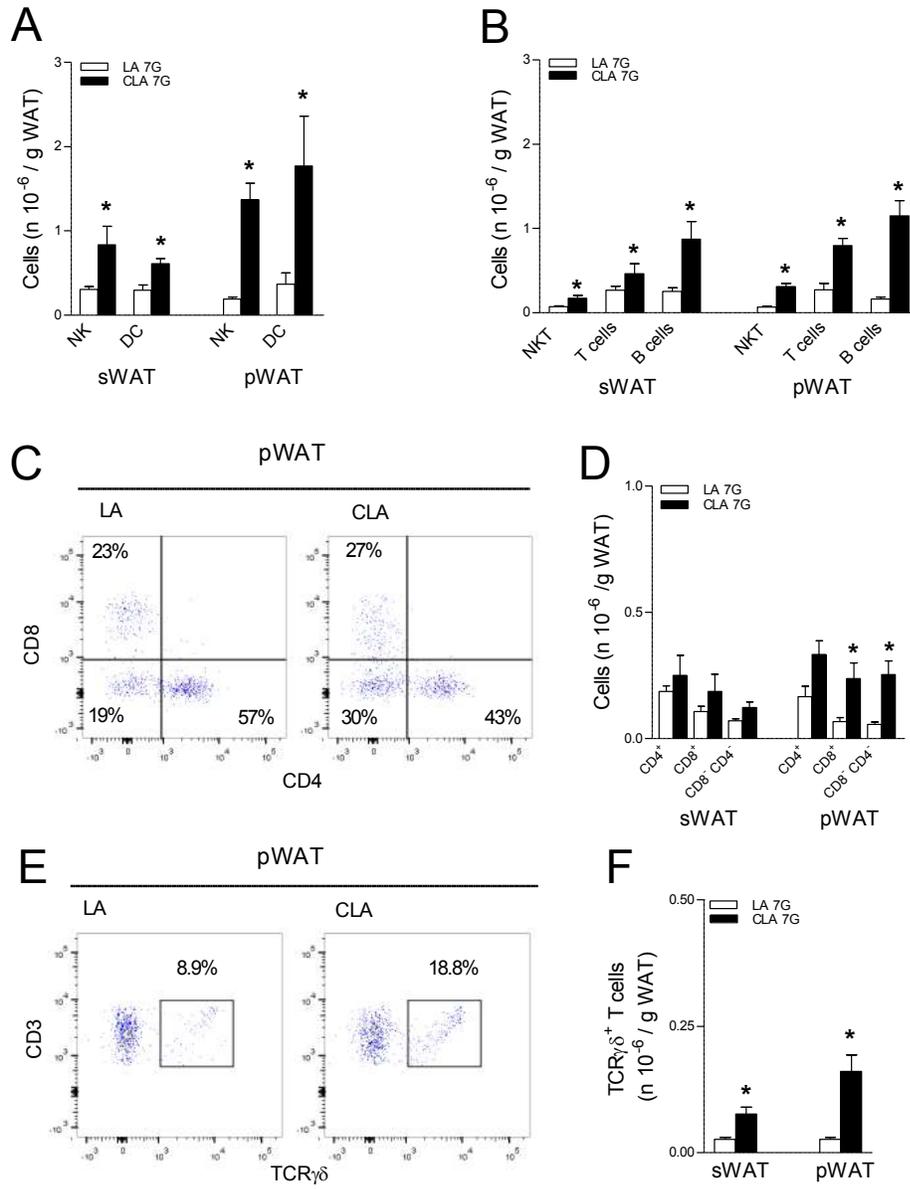
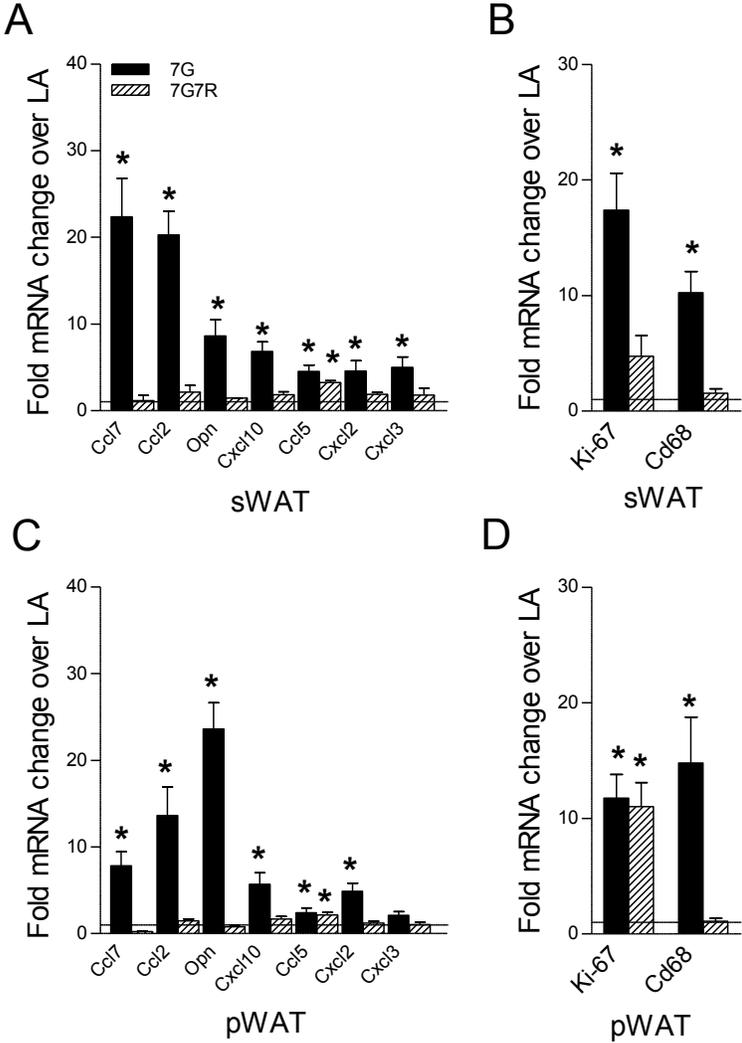


Fig 6



D. Complementary results of study I

1. WAT nutrient-sensing in response to CLA

The systematic effort to identify the potential link between metabolism and immunity in the effects caused by CLA is reported in Publication 1. Additional findings will be reported in this paragraph, aimed at exploring the capacity of WAT to biologically respond to t10,c12-CLA nutritional challenge through regulation of genes known to mediate the interplay between nutrient-sensing pathways and immune system. Results for Toll like receptor (TLR) and G-protein coupled receptor (GPR) expression will be presented in the different tissues of mice treated for 7 days with t10,c12-CLA (daily gavage = G7). As shown in Figure 28, CLA, as other saturated fatty acids, upregulated the expression of specific TLR-family members (TLR1, TLR2, TLR9) in both WAT depots. These results are not surprising, considering that TLR9 is expressed in most of the tissues at high levels and together with TLR1 and TLR2 is mostly expressed on immune cells or immune cell rich tissues, such as bone marrow, lymph nodes and spleen. However, it was surprising that the classically FFA-activated TLR4 was totally not affected by CLA. Certainly these observations at the mRNA level would need to be further supported by protein measurements.

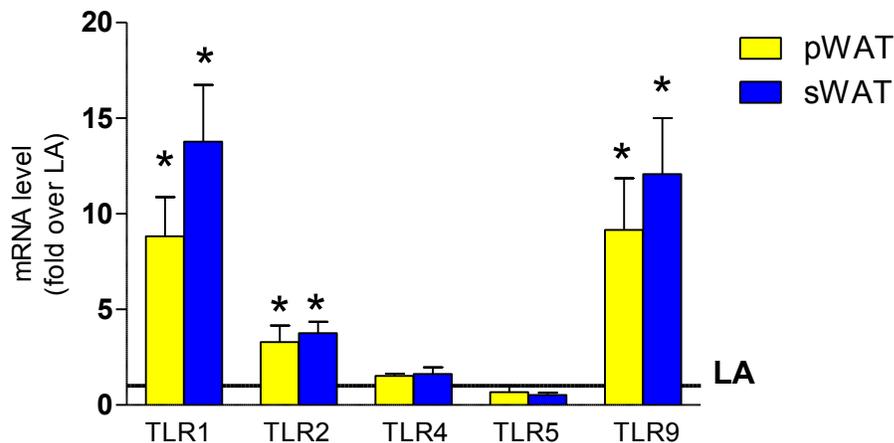


Figure 28. Adipose tissue expression of genes encoding TLRs after 7 CLA gavages.

Data are shown as fold change over LA values set to 1. pWAT: peri-uterine adipose tissue; sWAT = inguinal adipose tissue. All data are means (n=4-5/group) and t-bars indicate SEM. *p < 0.05 is significant compared to LA control group.

Because FFA have been demonstrated to act as ligands of several GPCRs, we evaluated the effect of CLA on their tissue expression, although aware that their functions are still largely unknown. In particular, we evaluated GPR43 that is activated by short-chain FFA. Obesity increases GPR43 expression in the subcutaneous adipose tissue of HFD-fed mice (4 weeks) and inulin-type fructans (nondigestible/fermentable carbohydrates) counteract the HFD effects through microbiota changes (Dewulf, Cani et al. 2011). GPR43-deficiency was shown protective toward HFD-induced obesity (Bjursell, Admyre et al. 2011). We evaluated GPR109A and GPR81, adipocyte cell surface receptors that enable the cells to "sense" metabolic changes in the environment and respond through lipolytic

regulation and release of free fatty acids and pro- or anti-inflammatory adipokines. Chronic obesity (60% high fat diet for 11 weeks (Wanders, Graff et al. 2012) and ob/ob mice (Feingold, Moser et al. 2011) significantly reduced GPR109A and GPR81 gene expression in WAT. Moreover, acute inflammation (in vivo injections of LPS, zymosan, turpentine) decreased GPR81 expression in WAT of C57BL/6 mice (Feingold, Moser et al. 2011). We evaluated GPR116, which was shown to have a role in controlling adipocyte biology, in a WAT specific conditional GPR116 knockout mouse (Nie, Hui et al. 2012). In this study, GPR116 WAT deletion caused a pronounced glucose intolerance and insulin resistance in mice, especially when challenged with a high fat diet (Nie, Hui et al. 2012). Finally, we evaluated GPR120 that encodes an n-3 PUFA receptor/sensor and is activated by medium- and long-chain FFA. As presented in the introduction, recent studies indicated that GPR120 has a key role in sensing dietary fat in WAT and dysfunction of GPR120 is associated with obesity and insulin resistance in both humans and rodents (Ichimura, Hara et al. 2014). Interestingly, we discovered that the expression of all the different GPRs was coordinately down-regulated in response to CLA in the three adipose depots: inguinal (sWAT), peri-uterine (pWAT) and retroperitoneal (rWAT) (Figure 29).

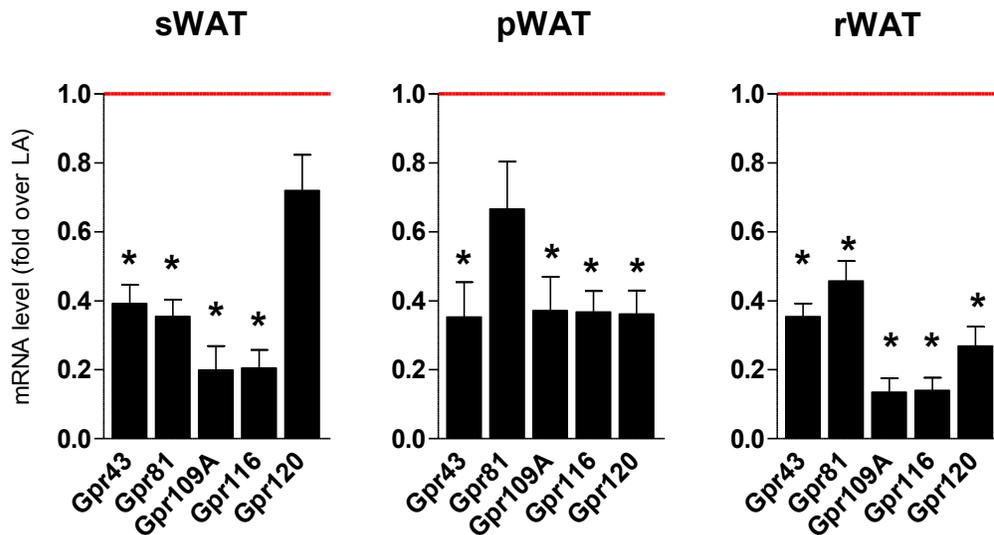


Figure 29. Tissue expression of genes encoding GPRs after 7 CLA gavages.

Data are shown as fold change over LA values set to 1. pWAT: peri-uterine adipose tissue; sWAT = inguinal adipose tissue. All data are means (n=6-10 / group) and t-bars indicate SEM. *p < 0.05 is significant compared to LA control group.

Currently, the local consequences of this striking and consistent GPRs down-regulation is difficult to interpret, given the lack of knowledge of their specific implication in adipose tissue normal or altered biology. The reduction we observed for GPR120 at the mRNA level in WAT was unexpected. In particular, in relation to the anti-inflammatory environment observed in WAT in response to CLA (upregulation of IL-1ra and Il10 mRNAs), we had anticipated that CLA could have an anti-inflammatory effect through GPR120 activation, possibly like ω 3 fatty acids (DHA). In a study from Olefsky's team, GPR120, expressed in macrophages and mature adipocytes, was shown to inhibit TLR2, TLR4 and TNFR-mediated inflammation through DHA (Talukdar, Olefsky et al. 2011). The precise mechanism of GPR120 action

pointed to the exclusively dependency on β -arrestin 2 (β -arr2) (Talukdar, Olefsky et al. 2011). Consequently, the expression of β -arr2 was evaluated in our experimental model. Interestingly mRNA level of β -arr2 was found upregulated in response to CLA (from 2 to 6 fold in pWAT and sWAT, respectively). Figure 30 shows that classical pro-inflammatory cascades are largely mediated by TNF- α and TLR4 receptors and the downstream inflammatory effects require activation of NF- κ B and JNK, via the interaction between TGF-beta activated kinase 1 (TAK1) and TGF-beta activated kinase 1 binding protein 1 (TAB1) (Talukdar, Olefsky et al. 2011). It would be interesting to explore if some of the effects of t10, c12-CLA could be mediated by similar mechanisms. The activation of GPR120 by CLA (instead of DHA) could recruit β -arr 2 to the cytosolic putative binding sites on GPR120, and the GPR120- β -arr 2 complex would internalize and through TAB1 sequestration could participate to the inhibition of inflammation (represented by the strong up-regulation of Il10 and IL-1ra, in association with the M2 macrophage accumulation, in WAT of CLA-treated mice).

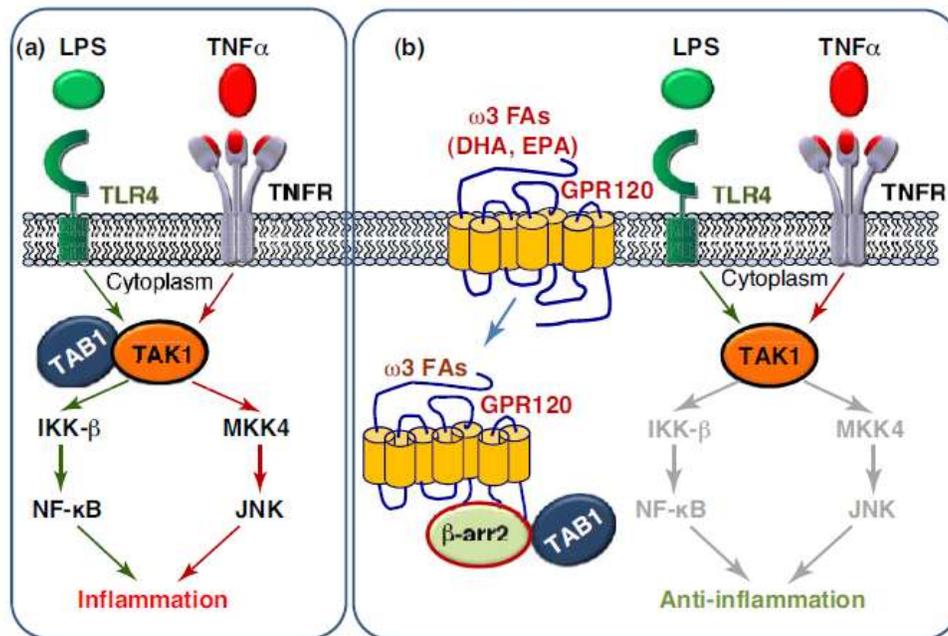


Figure 30. Mechanism by which activated GPR120 inhibits inflammation in the macrophage (Talukdar, Olefsky et al. 2011).

Of note, when CLA treatment was dismissed, the expression of the GPCRs cell membrane receptors fully moved or trended back to levels found in LA-treated mice in most of the cases, with some depot specific peculiarity. Gene expression analysis of WAT in earlier time points then after 7 gavages would allow understanding if the changes at the mRNA level reported in the above article and as complementary data precede and influence the depot specific response in term of fat mass reduction and fat mass recovery when the CLA treatment is dismissed, if WAT is analyzed before than after 7 days of treatment interruption.

These findings remain simple observations at this stage and they will require further exploration through full protein analysis, since it is known that there is not a strict correlation between gene expression and

protein level for these factors. Moreover only few GPCRs are currently well characterized, so the potential role of these receptors remains to be explored in different organs in physiological and pathological conditions.

2. WAT plasticity in response to CLA

About the capacity of WAT to properly respond to environmental metabolic changes, it is important to underline the differences between depots. In sWAT the magnitude of collagen accumulation indicated a more intense ECM remodeling compared to deep adipose depots. This indicated that tissue homeostasis was not fully restored in sWAT after CLA disruption (7 days without treatment after 7 gavages), consistently with the unrecovered fat mass. Instead both pWAT and rWAT fully resolved the CLA-induced alterations. One of the differences between inguinal and deep WAT is that the inguinal depots enclose the inguinal group of lymph nodes (LN) (Figure 31).

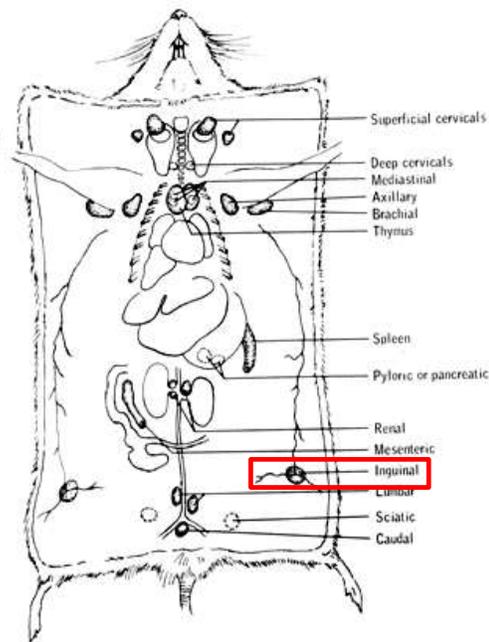


Figure 31. Scheme reporting localization of the lymphatic system in a mouse.

One possible hypothesis is that inguinal lymphoid cells, nestled in the “fibrofatty” tissue, and their (possible) cytokine secretion, could influence the lipolysis (Pond and Mattacks 1995) and consequently the fibrotic process in response. One interesting observation we made during the inguinal WAT collection was that the size of LN was drastically reduced by CLA treatment. In order to avoid interference from leukocytes present in LN during FACS analysis, LN were carefully excised and removed from sWAT before preparation of stromal-vascular fraction. After 7 gavages it was almost not possible to identify sWAT LN, that looked “empty”, compared to LN from LA-treated animals. This observation suggests that LN could possibly supply the massive WAT immune cell accumulation observed at 7G. Unfortunately, we did not quantify LN cellularity counting the cells, so this hypothesis remains to be checked. Gene expression analysis of RNA isolated from the collected LN could possibly reveal more about LN content and potential signals at various time points (3G, 7G, 7G7R).

Another factor extremely important to recover “healthy” fat is oxygenation. The role of hypoxia in WAT fibrosis associated to obesity is well documented (Sun, Tordjman et al. 2013). The senescence of endothelial cells, along with decreased vessel density (Pasarica, Gowronska-Kozak et al. 2009), has been suggested as a possible cause of the modified oxygen tension and inflammation observed in hypertrophied WAT (Rouault, Pellegrinelli et al. 2013). Certainly adipose tissue vasculature has a critical role in ensuring that the tissue has appropriate access to oxygen. In fact, during adipose tissue expansion, proangiogenic activity proves to be beneficial for adipose tissue functions (Sun, Wernstedt Asterholm et al. 2012). Overexpressing vascular endothelial growth factor (VEGF) in adipose tissue of HFD-fed mice was shown to be protective against obesity and insulin resistance (Elias, Franckhauser et al. 2012). In particular, transgenic mice presented increased M2 anti-inflammatory accumulation, with fewer M1 proinflammatory macrophages compared to control group, suggesting that the anti-inflammatory milieu could contribute to maintain insulin sensitivity (Elias, Franckhauser et al. 2012). In our study, the administration of CLA caused WAT ECM accumulation, in association with early and drastic M2-like macrophages infiltration (see figure 3 in Publication 1). After CLA treatment disruption, the profibrotic transcriptional program engaged during CLA administration was dismissed, with all the genes involved in ECM remodeling and macrophage accumulation declining back to the control level (LA-treated group) (see figure 3 in Publication 1). Together with those genes, VEGF-A, a potent angiogenic factors, and CD31, also known as platelet endothelial cell adhesion molecule (PECAM-1), involved in blood vessel formation, were evaluated (Figure 32).

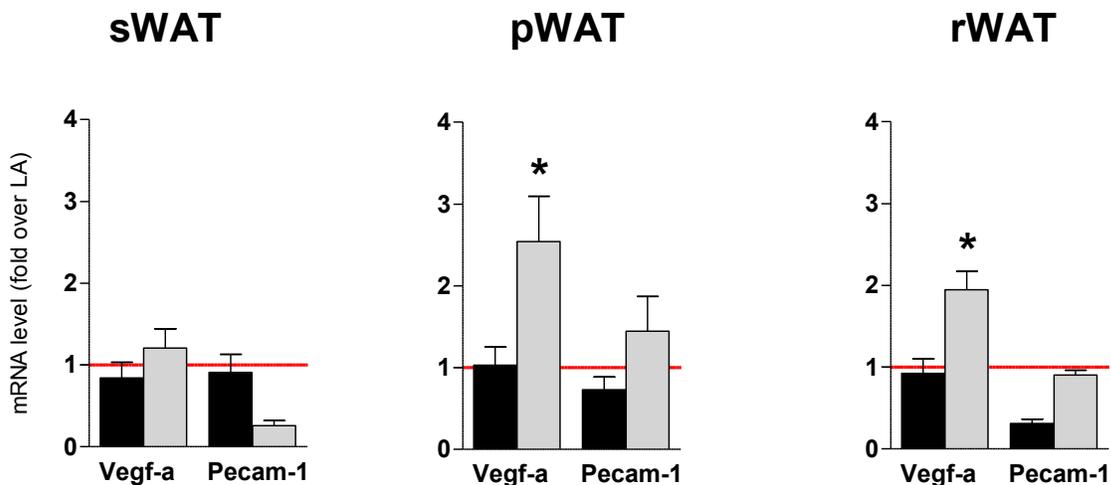


Figure 32. Tissue expression of genes encoding Vegf-a and Pecam-1/CD31.

Data are shown after 7 gavages with CLA (black bars) and after 7 gavages followed by 7 days of CLA dismissal (grey bars) as fold change over LA set to 1. sWAT = inguinal adipose tissue- pWAT = peri-uterual- rWAT = retroperitoneal adipose tissue. All data are means (n=7-10 / group) and t-bars indicate SEM. *p < 0.05 is significant compared to LA control group.

The expression of these angiogenesis markers was unchanged or reduced during CLA treatment (Figure 32), indicating that the M2-like macrophage accumulation was independent from VEGF-A (Elias,

Franckhauser et al. 2013). However, interestingly, after CLA removal both VEGF-A and PECAM-1 showed upregulation exclusively in deep adipose depots (Figure 32). Although slight, this depot-specific increase suggests that new vessel formation possibly accompanies the recovery in fat mass observed at the visceral level. It would be interesting to complete these observations by visualization of vascular network in different adipose depots.

The precise mechanisms inducing the lipotrophic state caused by CLA have not been elucidated. The rate of cellular stress and cell death induced by CLA (apoptosis, necrosis and/or pyroptosis) has not been fully explored in vivo (Kennedy, Martinez et al. 2010) and it is possibly elevated as in obesity (Cinti, Mitchell et al. 2005). Temporary local hypoxia could be induced by CLA in association with adipocyte dysfunction. Consequently we investigated hypoxia-inducible ATF3 in comparison with other members of the ATF family of transcription factors, known to be activated by the stress. An integrated stress response specifically induced by t10,c12-CLA was highlighted through microarray analysis of 3T3-L1 preadipocytes treated for 4 and 24 h (LaRosa, Riethoven et al. 2007) and supported this hypothesis. Interestingly we found that ATF3 was the only ATF member markedly upregulated, compared to ATF4 and ATF6 (Figure 33). A role in adipocyte hypoxia-mediated mitochondria dysfunction in obesity has been shown recently for ATF3 (Jang, Son et al. 2013). In this latter study HIF-1 α and ATF3 were increased in WAT of HFD-induced obese mice compared with control lean mice, whereas mitochondria-related genes were significantly reduced (i.g. NRF-1, PGC-1 α , COX1).

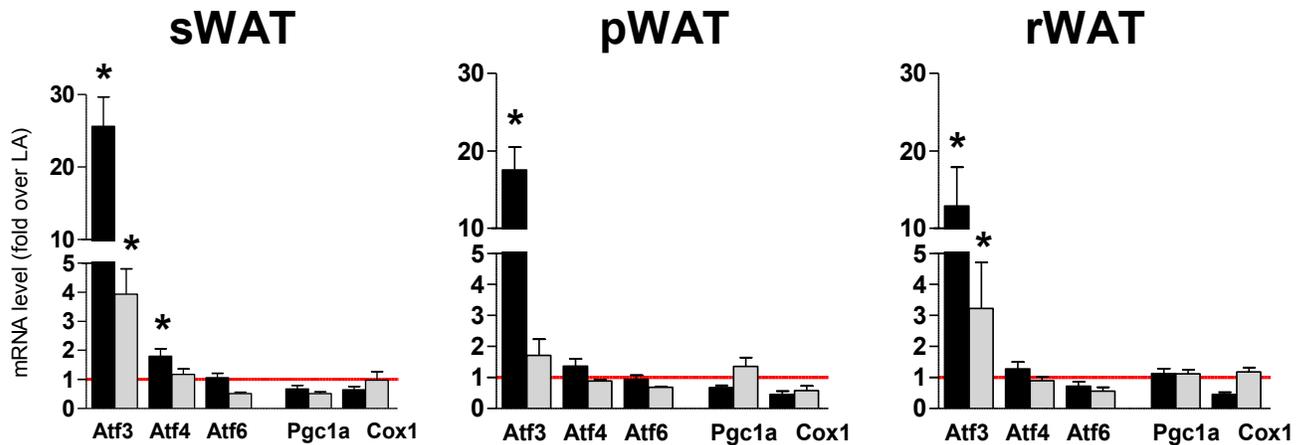


Figure 33. Tissue expression of stress-related (ATFs) and mitochondrial-related (Pgc1 α , Cox1) genes.

Data are shown after 7 gavages with CLA (black bars) and after 7 gavages followed by 7 days of CLA dismissal (grey bars) as fold change over LA set to 1. sWAT = inguinal adipose tissue- pWAT = peri-uterual- rWAT = retroperitoneal adipose tissue. All data are means (n=7-10/group) and t-bars indicate SEM. *p < 0.05 is significant compared to LA control group.

Similarly, in WAT of CLA-treated mice, PGC-1 α and COX1 were unchanged or reduced by the treatment (Figure 33), although HIF1 α was unchanged (not shown). The increased mRNA level of ATF3 suggested

that ATF3 may contribute to the inhibition of adipocyte differentiation as reported in hypoxic conditions in 3T3-L1 cells, in which lipid staining with oil red staining was decreased and adipogenic genes reduced (Jang, Kim et al. 2012), similarly to ACC and FAS in response to CLA. After 7 days of CLA treatment disruption, ATF3 remained upregulated only in sWAT. Subcutaneous WAT is the adipose depot that does not recover fat mass after CLA suspension, possibly due to unfavorable regulation of adipogenesis. Recently it has been demonstrated using 3T3-L1 cells that ATF3 interacts with PPAR γ and represses PPAR γ -mediated transactivation, possibly playing a role in inhibition of adipocyte differentiation (Jang and Jung 2015). Our results in vivo could be in line with those in vitro findings, since the expression of PPAR γ remained highly down-regulated in sWAT (7G -54% and 7G7R -65% reduction compared to LA-treated mice), but not in pWAT (7G -58% and 7G7R -8% reduction compared to LA group). Therefore, in our model, the inhibition of PPAR γ -regulated adipocyte differentiation by ATF3 (Jang and Jung 2015) could be one of the possible mechanisms explaining the inability of sWAT to recover fat mass after CLA disruption, in contrast to the two other WAT depots. Moreover, since ATF3 has been recently linked to macrophage lipid body formation (Gold, Ramsey et al. 2012), the upregulation of this gene could represent a potential intersection point between lipid metabolism and inflammatory pathways in these cells highly infiltrating WAT after CLA administration. It would be interesting to further explore the role of this factor in WAT, in particular in light of a very recent study reporting a key role for ATF3 in experimental models of fibrosis (Gold, Ramsey et al. 2012).

3. Effects of CLA administration on transcription factors of the IRF family

The family of interferon regulatory factors (IRFs) has been implicated as critical transcription factors that sense a variety of environmental stresses (Sato, Taniguchi et al. 2001). In particular, IRF5 has a key role in the activation of genes encoding pro-inflammatory cytokines in antiviral and immune response, being generally involved downstream of the TLR-MyD88 signalling pathway (Takaoka, Yanai et al. 2005), (Krausgruber, Saliba et al. 2010). IRF5 has been shown highly expressed in human M1 macrophages, with a crucial role for conversion from M2 to M1 macrophages in response to LPS (Krausgruber, Blazek et al. 2011). In human obesity and diet-induced obese mice, WAT, mostly infiltrated by M1 macrophages displays elevated expression of IRF5 (Publication 3). In mice, this factor was shown to alter adipose tissue immune profile, promoting M1 macrophage accumulation, and to contribute to extracellular matrix deregulation specifically in deep adipose depots. For the reported implication of this specific IRF member in WAT immune and structural alterations, the expression of IRF5 and other IRFs family members was evaluated in WAT of CLA treated mice (Figure 34). Of note IRF5 and IRF7 were specifically up-regulated factor, in a coordinate manner in the three explored WAT depots. Recently, using two murine models of obesity, IRF7 has been shown to be implicated in the deterioration of glucose and lipid metabolism (Wang, Zhang et al. 2013). Being both IRF5 and IRF7 implicated primarily in innate immunity, their increased expression in WAT could represent the increased innate infiltrate observed in response to CLA. However, the metabolic role of these factors in conditions of WAT dysfunctions, such as the lipotrophic state provoked by CLA administration, deserves further exploration.

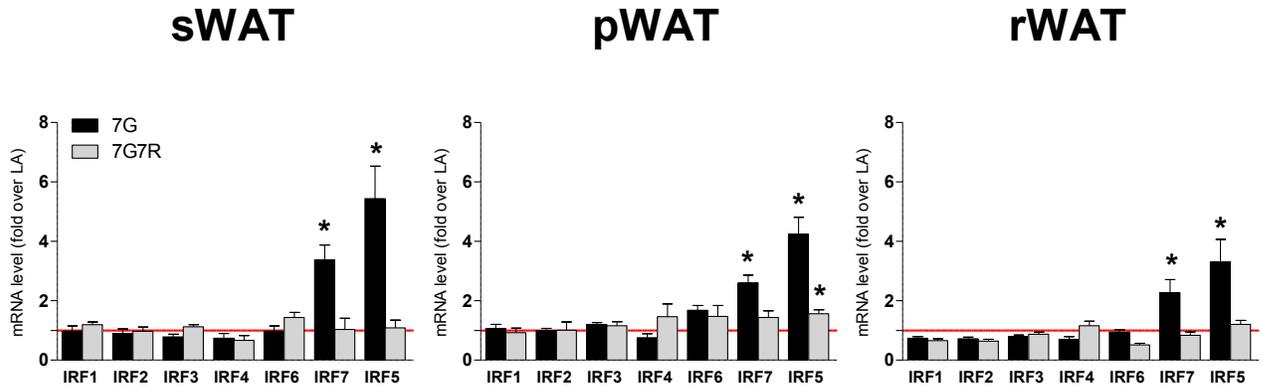


Figure 34. Tissue expression of IRF family members.

Data are shown after 7 gavages with CLA (black bars) and after 7 gavages followed by 7 days of CLA dismissal (grey bars) as fold change over LA set to 1. sWAT = inguinal adipose tissue- pWAT = peri-uterar- rWAT = retroperitoneal adipose tissue. All data are means (n=7-10/group) and t-bars indicate SEM. * $p < 0.05$ is significant compared to LA control group.

4. Effects of CLA administration on the intestine: barrier function

Complementary studies were performed to evaluate the effects of CLA on gut immunity and permeability, not to exclude a potential action of dietary fat on gut leakiness and consequently on WAT alterations. Gastrointestinal tract is the most common route of food and drug administration and that means it is generally the first organ to be exposed to external attacks. The diet is known to modulate the production of pro- and anti-oxidant species, reactive nitrogen species and radicals, potentially contributing to toxicity level (Lobo, Patil et al. 2010). All these diet-derived components are known to favor local inflammation and potentially alter the local immune functions (Chamulitrat 1999). High fat diet in particular is reported to activate immune cells, mast cells in particular, and Toll-like receptors (Ji, Sakata et al. 2011), (Kim, Gu et al. 2012). Along with this, sufficient evidence has proved that lipids induce inflammation in the course of obesity development.

To explore the relationship between nutrient absorption and intestinal inflammation, we made a preliminary evaluation of the health status of enterocytes and the intestinal immune cell infiltration in response to CLA administration by RT-PCR. The hypothesis was that, being a lipid, CLA would possibly induce acutely gut inflammatory responses similar to the one reported in response to chronic high-fat feeding (Cani, Possemiers et al. 2009). Gene expression analysis of immune cell markers used to evaluate WAT revealed that CLA did not induced a more sustained lymphocyte (CD3) and macrophage (CD68) accumulation compared to LA, in intestinal tissues (Figure 35).

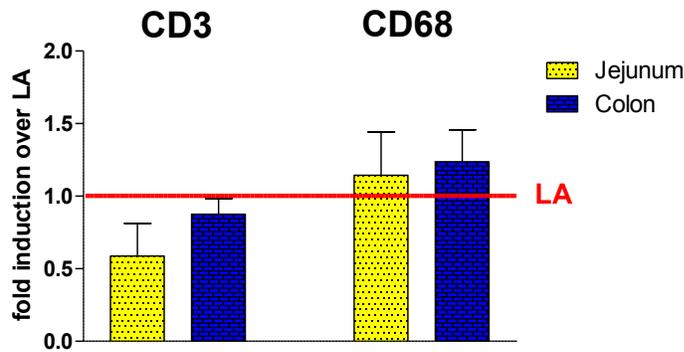


Figure 35: Tissue expression of lymphocyte (CD3) and macrophage (CD68) markers.

Data are shown after 7 gavages with CLA as fold change over LA set to 1. All data are means (n=5/group) and t-bars indicate SEM.

However, CLA clearly modulated members of the toll-like receptor family in intestine and colon with a quite distinct profile (Figure 36) compared to adipose tissue (Figure 28). In jejunum, after 7G, CLA tended to increase the expression of TLR4 and TLR5, not surprisingly for this last receptor, since it is mainly expressed in intestinal epithelial cells. Whereas, the response in the colon was driven by TLR9, suggesting that CLA could possibly favor an intestinal epithelial inflammatory response, possibly due to increased pathogenic bacteria DNA recognition (Ewaschuk, Backer et al. 2007) (Kruis, Batra et al. 2014).

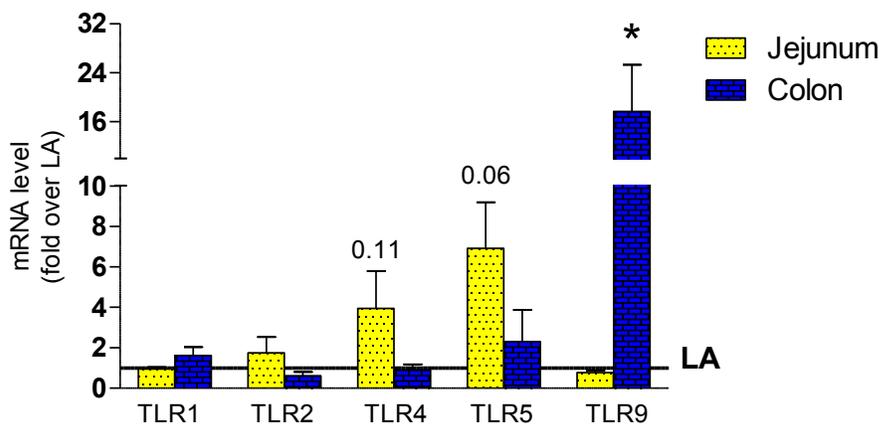


Figure 36: Intestinal expression of genes encoding TLRs after 7 CLA gavages.

Data are shown after 7 gavages with CLA as fold change over LA set to 1. All data are means (n=5/group) and t-bars indicate SEM. *p < 0.05 is significant compared to LA control group.

The activation of both TLR4 and TLR5 mobilize the nuclear factor NF-κB and stimulates tumor necrosis factor-alpha production, a pathway known to be critical in activating the inflammatory response (de La Serre, Ellis et al. 2010), (Shi, Kokoeva et al. 2006). In the intestine of the CLA-treated mice both molecular actors TLR4 and TNF-α were engaged at the mRNA level. In fact, coupled with a 4-fold

increase of TLR4, a significant upregulation of TNF α (5x) was observed in jejunum, in association with reduced expression of genes coding for tight junction proteins, claudin2 (CLDN2 gene), occluding (OCLN gene) and zonula occludens 1/ZO-1 (TJP1 gene) (Figure 37). These data suggest that, despite the modality of administration (short-time frame and transient exposure), CLA could possibly initiate an inflammatory response by binding to TLR4 and increasing TNF- α expression in the jejunum.

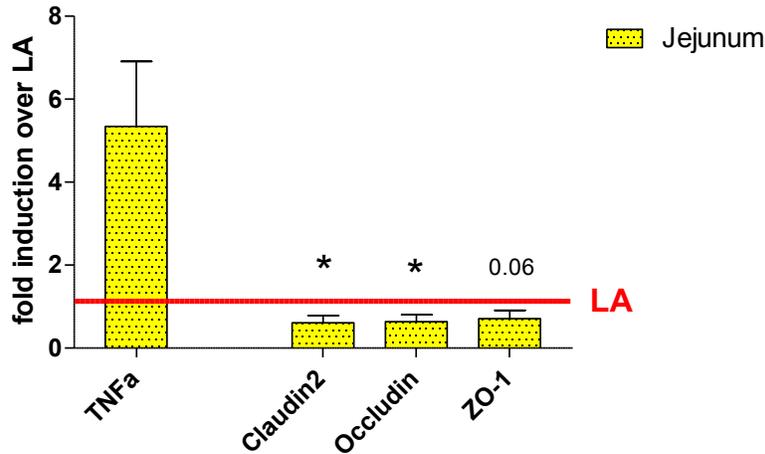


Figure 37: Tissue expression of TNF α and TJ protein markers (claudin2, occludin, ZO-1) in jejunum of mice treated with CLA for 7 days.

Data are shown after 7 gavages with CLA as fold change over LA set to 1. All data are means (n=5/group) and t-bars indicate SEM. * $p < 0.05$ is significant compared to LA control group.

This very powerful and potentially destructive inflammatory cytokine (TNF α) could participate in altering intestinal barrier on a longer time course, similarly to what occurs in Chrown-like pathology (Roulis, Armaka et al. 2011) or in response to a high fat diet (Lam, Ha et al. 2012) (Suzuki and Hara 2010). Interestingly, the dramatic reduction of circulating adiponectin induced by CLA could be also associated to a “leaky gut” phenotype, since the anti-inflammatory role of adiponectin has been demonstrated in intestinal inflammation in other settings, together with different metabolic dysfunctions (Yamamoto, Kiyohara et al. 2005) (Saxena, Baliga et al. 2013). Moreover, it is important to consider mesenteric WAT, the visceral fat depot located in the mesenterium of the intestine. Investigate the effects of CLA on this intestinal fat tissue compartment for activation, secretion and the infiltrating immune cells would be important, since it is known to play an important role for proper intestinal barrier function. Unfortunately, this has not been possible due to the drastic reduction of WAT and near absence of mesenteric WAT caused by CLA after 7 gavages, when mRNA tight junction proteins were observed reduced. In contrast with the chronic administration of HFD in the murine model of obesity, the stressful effect of this fatty acid on the gut is limited due to the short time frame and possibly due to the administration modality as well. A longer time course would possibly increase the risk of an inflammatory immune engagement in the gut wall, which over time could result in epithelial damage (leaky gut). Long-chain PUFA are known for their propensity to easily oxidize and generate lipid peroxidation byproducts, so longer administration of CLA could determine the same risk of inducing leaky gut and endotoxemia observed in other contexts (de La Serre, Ellis et al. 2010) (Erridge, Attina et

al. 2007) (Cani, Amar et al. 2007). The ligands recognized by TLRs are varied. Although the bacterial component LPS is considered the main ligand of TLR4, followed by production of proinflammatory cytokines, the activation by the t10, c12 -CLA is not excluded.

Some genes known to regulate lipid metabolism at the intestinal level were evaluated. In particular, fatty acid binding protein 2 (Fabp2), known to be expressed exclusively in enterocytes in the small intestine, histone deacetylase 1 (Hdac1), important for intestinal epithelial cell homeostasis, scavenger receptor class B, member 1 (SR-BI), suggested to be involved in the absorption of dietary cholesterol in rodent, plus other genes specific for lipoprotein metabolism, such as apolipoprotein B (Apob), apolipoprotein A-IV (Apoa4), mannoside acetylglucosaminyltransferase 2 (Mgat2), microsomal triglyceride transfer protein (Mttp), were studied. No major modifications were observed at the mRNA level at the time point examined (7G) for these genes in jejunum and colon of CLA-treated mice.

5. Effects of CLA administration on the intestine: microbiota

Through 16S rRNA pyrosequencing, the microbiota of mice receiving LA and CLA was assessed at different time points, following the hypothesis that fatty acid administration, even when short, could alter it. The association of gut microbiota with a disease does not mean causation. However understanding the potential role of microbiota, depending on the composition, could open up new avenues to treat or mitigate the disease itself (Nguyen, Vieira-Silva et al. 2015). Diet components, in particular fatty acids, have been shown to impact the microflora richness and functions. The effect of lipids in the lumen or through direct action on the enterocytes and through activation of mast cells has been reported to provoke pro-inflammatory cytokine release. However our understanding on the onset of inflammation at the intestinal level is not complete without taking into account the gut microbiota. This point has been highlighted by the use of germ-free mice, that, despite a diet rich in fat, do not show upregulation of inflammatory markers, such as TNF- α and nuclear factor-kappa B.

During the course of the CLA project, fecal samples were collected from individual mice treated by LA and CLA at different time points in multiple experiments. Samples were stored at -80°C until metagenomics analysis was performed in INRA, thanks to the collaboration with MICALIS (Unit for food and gut microbiology for human health). The metagenomics analysis was based on microbial 16S rRNA gene sequencing to determine bacterial composition in fecal samples of 88 mice. Because the number of mice per group was low (n=4), we decided to pool the data from close days, 1-2G (=baseline), 3-4G, 6-7G (=7G) and 7G5-9R (=7G7R) for the two treatments LA versus CLA, to gain statistical power. The statistical analysis was performed by our collaborator Julien Tap (INRA) based on the pre-defined objectives of the study.

Microbial species were organized using the concept of enterotypes, a way of analyzing the ecological organization of gut microflora in mice (distinction between resistant and non-resistant microbiota). Three main clusters were found, with more or less 30% of the mice within each enterotype (Figure 38):

- M1 = prédominance de *Barnesiella* et *Alistipes*
- M2 = prédominance de *Barnesiella*, *Alistipes*, *Clostridium* et *Lachnospiracées*
- M3 = prédominance de *Bacteroides* et *Barnesiella*

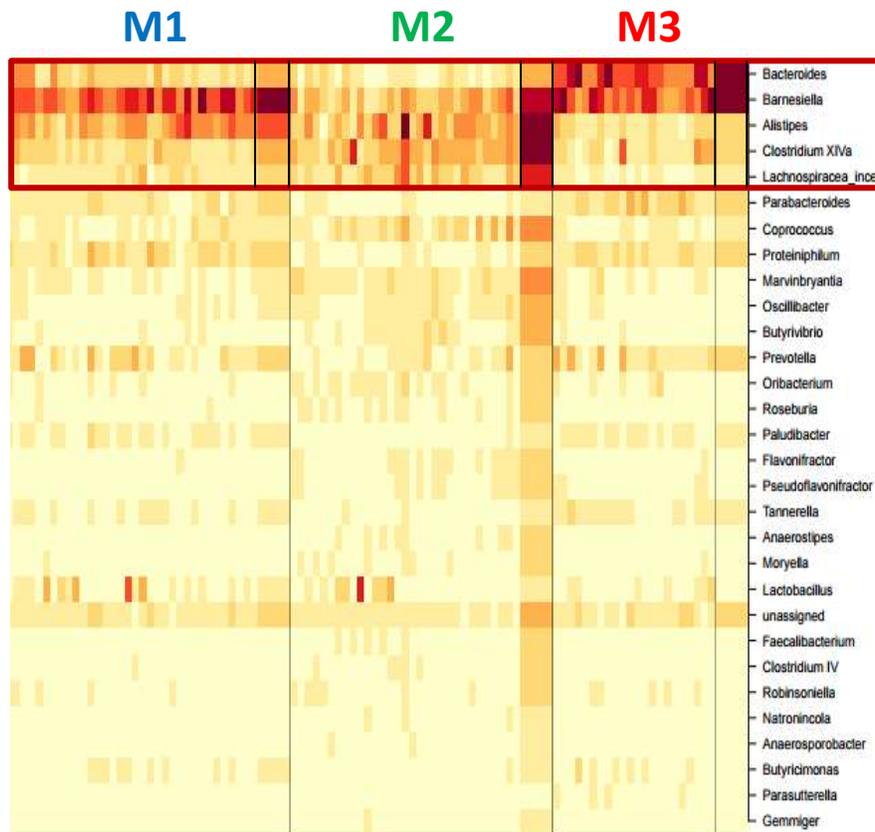


Figure 38. Microbial species organized in three distinct enterotypes designed M1, M2 and M3.

Each column identifies the microbial species of one mouse, with the average for each enterotype group indicated on the right (black rectangles).

Interestingly, the enterotype M3-Bacteroides, which was found the less rich in bacterial species, was particularly low in the CLA-treated mice, as indicated by the absence of red (M3) dots (CLA) (Figure 39). Bacteroidetes is a genus of Gram-negative, obligate anaerobic bacteria, found in lower amount in obese subjects, even if with some contradictory findings (Aron-Wisniewsky and Clement 2014). Our preliminary results could suggest a similarity between the effects of short-term t10,c12-CLA administration and obesity (i.e. chronic high fat consumption) on gut microbiota composition. However the sharp differences between human and animal environment and behavior, above all, clearly downsize this observation.

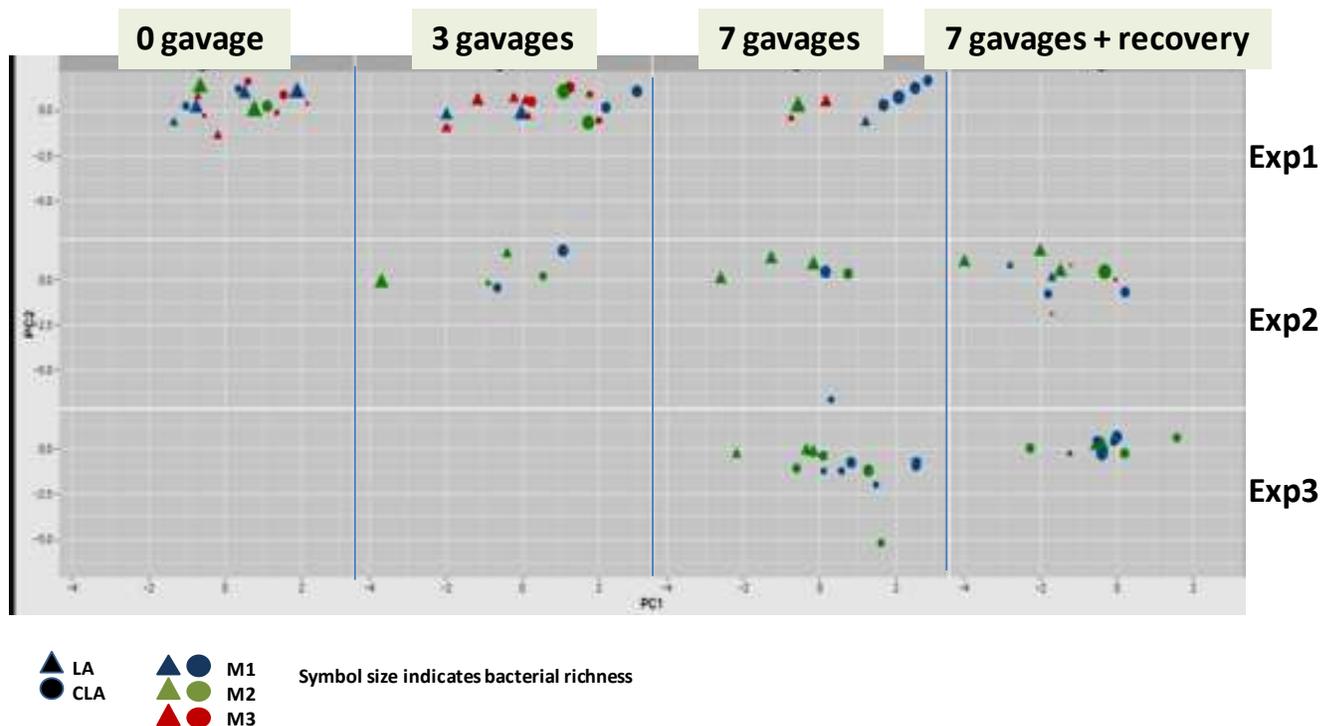


Figure 39. Number of mice with a specific enterotype in three experiments.

A limitation of the analysis is that no feces were collected before starting the treatment; also, microbiota changes are not correspondent to the same animals followed over time. However, we tried to distinguish a potential effect of CLA on microflora, making a distinction between mice that received only 1 gavage (1G), that we considered “baseline” (= 0 gavage), and mice in the groups 3G and 7G (Figure 40). The p values reported on top of the Figure 40 represent the independence test khi2, showing that there is dependence between enterotypes and gavages in CLA-treated mice, but not for LA. This figure shows that CLA treatment modified gut microbiota composition in mice, with a clear absence of the M3 enterotype (*Bacteroides-Barnesiella*) after 7 gavages. Other differences between time points within the same treatment or between treatments were not significant. These results could indicate that the microbiota progressively change in response to CLA, becoming more enriched in *Barnesiella* and *Alistipes* (M1 enterotype) and losing *Bacteroides* (M3 enterotype).

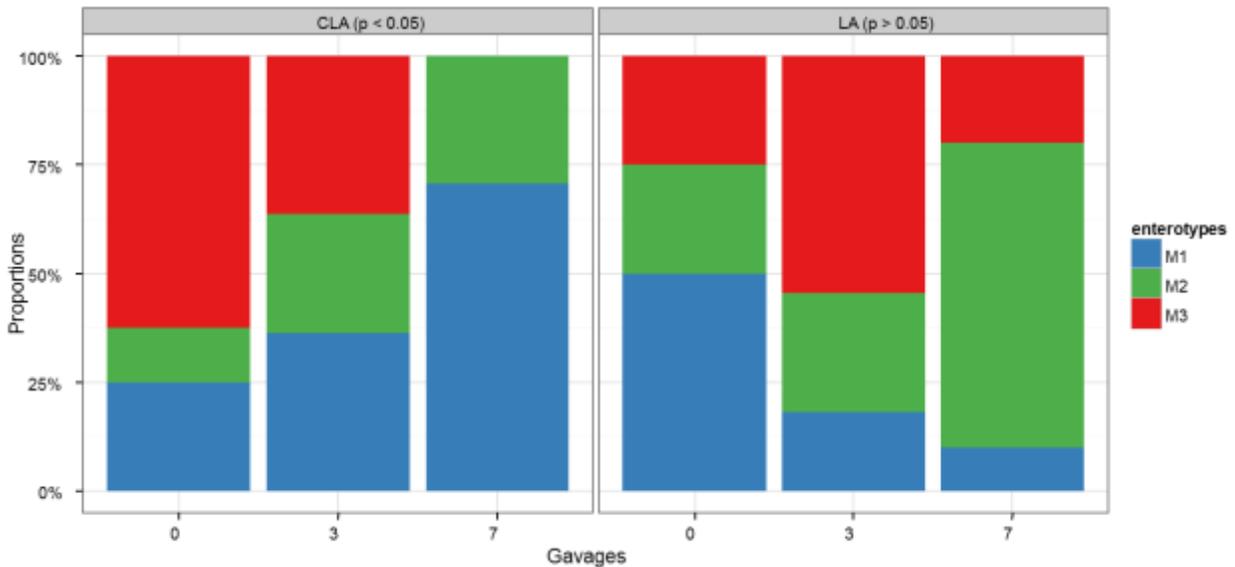


Figure 40. Proportions of the three enterotypes in mice gavaged with either CLA or LA.

0 gavage: n=16; 3 gavages: n=11; 7 gavages: n=16. Enterotype proportions are significantly affected in the CLA group (Chi2 analysis).

These are preliminary results and further studies will be needed to assess the possible consequences of gut microbiota changes on the animal metabolic phenotype. Gut microbiota changes would need to be confirmed through further exploration and characterization. For example, to obtain a more accurate picture about what happens at the enterotype level in response to CLA, the same metagenomics analysis would be necessary over time following the same mouse to avoid any confounding effect. This would allow associating the microbiota composition with the mouse immuno-metabolic phenotype with or without CLA (from fat mass reduction or ECM remodeling to insulin level or WAT altered immunity).

6. Reversibility: permeability and intestinal ecology

The potential effects of CLA administration on intestinal barrier integrity suggested by mRNA changes observed at 7G were completely reversed 7 days after CLA removal (7G7R). Tight junction proteins recovered levels observed in the LA-treated group, whereas TLR4 and TNF- α upregulations were abolished (data not shown).

The importance of the intestinal epithelial barrier is emphasized by the alterations in paracellular permeability and tight junction functions observed in inflammatory bowel disease (IBD) and colon cancer. In future experiments, it would be important to support these observations during and after CLA treatment, evaluating paracellular permeability through more specific methods, i.e. FITC-dextran administration and quantification at the circulating level 4 hours after gavage (Gupta, del Barco Barrantes et al. 2014) or measuring paracellular flux mounting intestinal biopsy specimens in Ussing chambers (Clarke 2009). Moreover, it could be relevant to study how the host immune system could influence gut microbiota composition, known that the opposite process has long been recognized (Zhang, Sparks et al. 2015). This approach could be possible evaluating microbiota richness in

immunodeficient mice, such as Rag1 mice (no mature B and T-cells) or in highly immunocompromised SCID mice, for example, treated with LA and CLA mice. Finally, to conclude, the specific variations of intestinal ecology depending on treatment or time frame could be further enlighten through stratification of the mice based on the CLA-induced alterations (i.e. low versus high insulin level or low versus high percentage of fat mass loss), similarly to patient stratification in human metabolic diseases (i.e. clinical parameters, such as BMI or HOMA-IR, homeostatic model assessment for insulin resistance).

7. Effects of CLA administration on the liver

Alterations of liver homeostasis were also considered in response to CLA, but only marginally. No changes were observed by gross examination of the organ, in particular no steatotic appearance drawn our attention. Gene expression analysis was performed in liver of CLA-treated mice and compared to LA group. No alterations were observed at the level of immune infiltrate. However, *Mcp1* and *IL1 β* were upregulated (5.4- and 6-fold, respectively) in association with the lipogenic genes *Acc* and *Fas* (5.4- and 4.5-fold, respectively). These preliminary results indicate that even such a short-term administration of CLA is able to initiate an inflammatory response in liver, possibly due to the concomitant hepatic lipid accumulation, as demonstrated by tissue fatty acid analysis (see Figure 1C in the publication).

8. Are the CLA effects on WAT indirectly mediated by macrophages?

The direct action of CLA on human and murine adipocytes has been demonstrated by several studies, the majority of which conducted by the research group headed by Prof M McIntosh, in Emory University (Atlanta, GA, USA) (Brown, Halvorsen et al. 2001) (den Hartigh, Han et al. 2013). Although aware that WAT is the primary target of CLA *in vivo*, we decided to perform complementary *in vitro* experiments using macrophages, the most rapidly engaged and dominant immune cell population in WAT of CLA-treated mice. The hypothesis behind these experiments was that CLA could be directly “sensed” by macrophages and possibly affects the activation state of these cells, potentially elucidating one of the mechanisms of CLA-induced alterations in WAT. We hypothesized that macrophages could autonomously respond to LA or CLA stimulation by changing their polarity from M0 to M1 or M2 status. We also explored the possibility that LA or CLA has an anti-inflammatory effect on LPS-primed macrophages.

Methods: Linoleic acid and conjugated linoleic acid isomers were obtained from Matreya, LLC, PA, USA. Fatty acids were solubilized in ethanol and stored in aliquots at -20°C . Prior to each experiment, LA and CLA were conjugated to fatty acid- and endotoxin-free albumin (BSA) at a molar ratio of 10:1 (fatty acid : albumin) in serum-poor medium (1mM of BSA fatty acid free). Fatty acid-albumin complexes were freshly prepared at the physiological concentration of 50 μM (Kennedy, Martinez et al. 2010).

The effects of LA or t10, c12-CLA were investigated *in vitro* on wild-type bone marrow-derived macrophages (BMDM) from wild-type mice. The bone marrow-derived macrophages were selected by cell adherence. BM-derived cells were plated at the concentration of $1 \times 10^6/\text{ml}$ in 12 wells plates. After 2 hours all the floating cells (in particular lymphocytes) were eliminated, by replacing the medium by a differentiation medium (30% L929 medium, 10% horse serum, 1% antifungal and 1% antibiotics). The differentiation medium was changed every 2-3 days, until when macrophages were confirmed fully

differentiated by microscopic examination (10-12 days). Macrophages were then stimulated with LA or CLA (50 μ M solution in 1mM BSA) alone or together with lipopolysaccharides (LPS 10 ng/ml) in a 1% serum-enriched medium. After 24h of stimulation, the supernatants were collected in order to evaluate protein secretion and macrophages on the bottom were resuspended in RLT buffer with 1% β -mercaptoethanol and conserved at -80°C for RNA extraction. Gene expression analysis was performed in order to evaluate the potential effects of CLA on macrophage polarization.

The experiment was performed in 5 WT mice, irrespectively of sex, in separate trials and both hypotheses were evaluated by RT qPCR. The expression patterns for genes associated with classical (M1) and alternative (M2) activation was analyzed using the same markers selected to characterized mouse WAT sorted macrophages (Herrero, Shapiro et al. 2010). For the M1 phenotype: iNOS, IL-1 β and CCL2/MCP1; for the M2 phenotype: CD206, arginase 1 (Arg1), IL-1Ra and IL-10, were the used markers.

Results: An average of 1.8-fold increase was observed in CLA treated BMDM over the untreated cells set to 1, whatever the gene of interest, whereas for LA there was no change. Both LA and CLA failed to polarize macrophages toward the M1 or M2 activation state. These results were somehow expected. First of all because it is known that the primary target of CLA is the adipocyte, then because the macrophage expression of the above listed M1-M2 genes is tightly regulated by exogenous stimuli, such as Th1 cytokine IFN γ (for M1) and Th2 cytokines IL-4 and IL-13 (for M2). None of those cytokines was either found upregulated in WAT after CLA or secreted in the ex-vivo WAT explant medium (24h-48h culture of WAT from CLA-treated mice – data obtained by Luminex, not shown). Moreover, IL6 and IL1Ra, chosen as representative of pro- and anti-inflammatory cytokines, respectively, were measured by ELISA in the medium of stimulated BMDM and levels were in the lowest range of the standard curve. In parallel to polarization, the effects of LA and CLA stimulation were evaluated on receptors potentially able to “sense” lipids, such as TLRs (TLR2, TLR4) and GPCRs (GPR120, GPR116, GPR109A) and on chemokines (CCL2, CCL5) able to recruit other immune cells. Once again, the expression of these genes was minimally altered by CLA, being CCL5 the most affected factor (2-fold increase compared to untreated cells). On the other hand, macrophages strongly responded to LPS stimulation, showing for example for CCL5 a 220-fold increase, independently of the presence of LA/CLA. No specific effects of LA or CLA were observed when LPS was used as activating factor, possibly due to the overwhelming effects of LPS on macrophages (data not shown).

Since no major effects were observed using “naïve” macrophages, we decided to evaluate the effects of CLA on pre-polarized macrophages, trying to mimic a more physiological situation, with a possible predominance of M1 or M2 macrophages depending on the pathophysiological state of the tissue. This experimental setting (in vitro polarization) was performed one time only using 1 WT male mouse, so these results are preliminary observations. Bone marrow-derived macrophages were differentiated as described above. After macrophages were fully differentiated, BMDM were stimulated for 24h to activate the specific phenotype with no stimulus for control group (M0), IFN γ (10 ng/ml) for M1 phenotype and IL4 (5 ng/ml) for M2 phenotype

After 24h, 200 μ l of medium was collected for further analysis and replaced with the same amount of LA or CLA at the usual concentration (50 μ M). Following 24h stimulation with LA or CLA, medium was collected and macrophages were processed as described above. The gene expression analysis reported

for this experiment is the result of one single experiment (from one wild-type mouse bone marrow). In Figure 39, the key markers for the M1 phenotype (iNOS, IL1 β) and for the M2 phenotype (Cd206, arginase 1) indicated that macrophages were efficiently polarized by the recombinant cytokines used. No significant effects of CLA were observed in unpolarized macrophages, M0. Looking at inducible nitric oxide synthase (iNOS) expression, cytokine-polarized M1 cells showed a 2-fold increase of iNOS when stimulated with CLA, whereas in cytokine-polarized M2 cells iNOS was undetermined. Similarly IL1 β expression trended higher in M1 cells stimulated with CLA (1.4-fold increase). On the contrary, in the M2 cells IL1 β expression was decreased (4-fold reduction) by CLA. When the classical M2 markers, mannose receptor CD206 and arginase 1 (Arg1) were analyzed, we found them expressed only in M2 cells. However, CLA stimulation could possibly differentially affect their regulation (Figure 41). Based on these preliminary observations at the mRNA level, it is tempting to assume that t10,c12-CLA is sensed by macrophages, possibly affecting their transcriptional profile depending on their activation state within the tissue. However these results will need to be confirmed by other experiments *in vitro*, in order to exclude or not a direct action of t10, c12-CLA *in vitro* and *in vivo*. Moreover the cytokine secretion of the different macrophage phenotype in the presence or absence of LA and CLA stimulus will need to be analyzed for this and other *in vitro* experiments.

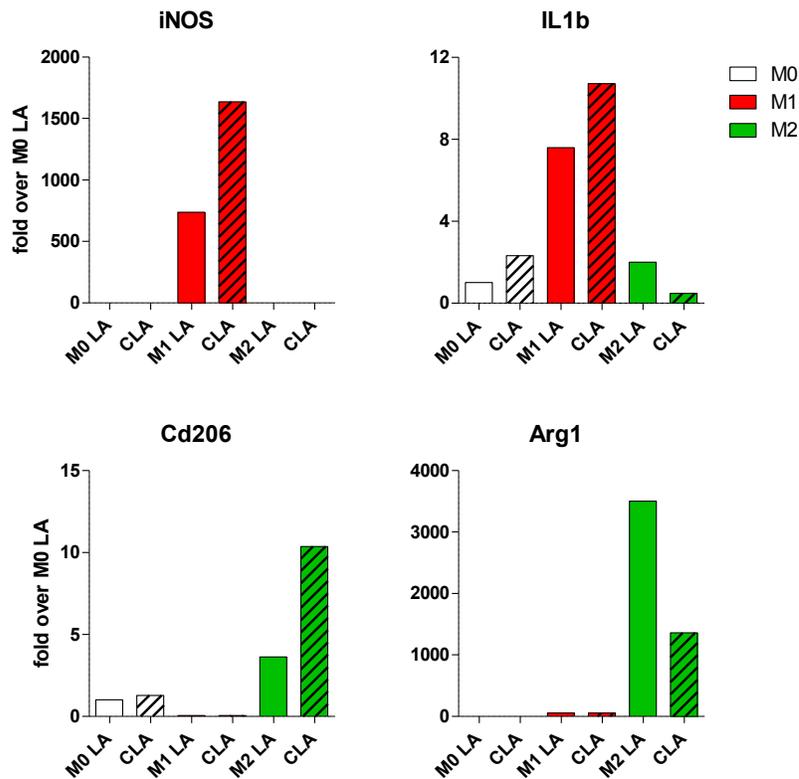


Figure 41. Gene expression analysis in vitro polarized BMDM stimulated with LA or t10, c12-CLA.

Data are shown as fold change over unpolarized macrophages M0 stimulated with LA set to 1. IFN γ (10 ng/ml) was used to polarize M0 toward M1 phenotype (red bars) and IL-4 (5 ng/ml) toward M2 phenotype (green bars).

The main limitation of these *in vitro* studies is that typically differentiated bone marrow macrophages respond differently than WAT-infiltrating macrophages to high concentrations of lipids and FFA. Tissue macrophages are in fact a group of cells that share common progenitors and that develop in that specific lipid-rich context (Xu, Grijalva et al. 2013). However, since in the CLA *in vivo* experiments most of the macrophage accumulation in WAT was due to infiltration (proved by marked WAT upregulation of recruiting factors – see above publication), working with BMDM seemed a reasonable model to answer to the question about the potential role of macrophages to mediate the effects of CLA in WAT. Finally, despite these results were not obtained from macrophages isolated directly from different WAT depots, t10, c12-CLA does not seem to differentially affect the transcriptional profile of macrophages compared to linoleic acid, suggesting that macrophage polarization is most probably not the primary mechanism affecting the local response of WAT.

E. Conclusions

The magnitude of changes produced by dietary factors is known to be low and cumulative. CLA administration can be considered a diet-induced metabolic insult, since 7 gavages are sufficient to provoke a fat mass decrease, associated with a drastic reduction in circulating adiponectin and massive insulin secretion. Our results demonstrated that CLA caused alterations with great differences among different cell types, mainly targeting adipose tissue. Despite a series of pathways critical for WAT homeostasis (adiponectin, resistin, RBP4, Plin1, CD36, ATGL, AHL, PPAR γ) were completely or almost completely downregulated in response to CLA, adipocytes survived potentially owing on upregulation of lysosomal acid lipase (Lipa) and transcription factor EB (TFEB), known to control a process called lipophagy. The regulation of one single transcription factor of multiple steps of lipid catabolism may be used by adipocytes to tightly control the lipid flow (Settembre, De Cegli et al. 2013). It is tempting to speculate that the process of lipophagy allowed maintaining energetic homeostasis during CLA treatment through mitochondrial oxidation of fatty acids released through Lipa action, given upregulation of the uncoupling protein-2 (UCP2).

The CLA-induced rapid and massive immune cell accumulation in WAT was possibly driven by endogenous signals linked to stress (ATF3) or hypoxia/apoptosis (Tsuboyama-Kasaoka, Takahashi et al. 2000). Complementary cell culture studies with bone marrow-derived macrophages allowed us to exclude a direct action of CLA on the most represented cell population infiltrating WAT after CLA administration. The interesting observation is that WAT immune cell recruitment, in particular M2-like activated macrophages, positively correlates with ECM accumulation. This correlation suggests an active role for these innate immune cells in what is a WAT adaptive response, possibly participating to homeostasis restoration. The almost total reversibility of the deleterious metabolic alterations, in particular insulin resistance, provoked by this CLA isomer revealed an unexpected resilience of WAT to an acute nutritional stressor. The results concerning the intestinal tract revealed that the acute administration of CLA provoked a local inflammatory response, as indicated by TLR4 and TNF- α upregulation, although with unchanged immune gene (Cd3 and Cd68) expression level. Intestinal

inflammation was associated with a reduced expression of tight junction proteins in the jejunal tract. These preliminary observations suggest that part of the effect of CLA in WAT could be mediated by the potentially increased intestinal permeability. Further investigations could evaluate the degree of “leaky gut” measuring potential increased bacterial translocation (blood and tissue endotoxin level) and its effects on local and peripheral immune cell activation in association to WAT alterations (depending on depot localization). Finally, through metagenomics analysis, microbiota modifications in response to CLA could be analyzed over time following single animals in order to better define a microbial phenotypic signature induced by CLA treatment. The link between microbiota composition and the local and systemic immunity could be explored in relation to the animal metabolic state.

To conclude, dietary fatty acids can modulate adipose tissue immunity and remodeling, inducing transient modifications in an acute experimental setting. Once again the cellular plasticity of WAT in response to a varying nutrient environment allows this tissue to quickly adapt, effectively maintaining the control over glucose homeostasis.

STUDY II: PROBIOTIC ADMINISTRATION IN DIET-INDUCED OBESE MICE: EFFECT OF BODY WEIGHT GAIN AND GLYCEMIC STATUS.

A. Purpose

This study was designed in the context of prevention and treatment of obesity and the associated metabolic alterations. Beneficially altering gut microflora, through daily administration of specific probiotics, might provide long-term beneficial effects against obesity (Stenman, Waget et al. 2014), (Chen, Guo et al. 2014). This hypothesis gave us the opportunity to investigate the role of the indirect crosstalk between adipose tissue and intestine, and in particular the impact of local and systemic inflammation on the metabolic alterations associated with obesity.

The objectives of the presented study were to evaluate the effects of probiotics in HFD-fed mice on:

- Body weight gain and adiposity over a 12 weeks time course;
- Glycemic status: glycemia, insulinemia, insulin sensitivity and glucose tolerance;
- Systemic metabolic variables and triglyceride concentration in plasma and liver.

B. The experimental model

In the present study, the experimental model used is the murine high fat diet-induced obesity model. The study design implicated the administration of specific probiotics concomitant to HFD (60% calories derived from fat). The strategy of daily administration of specific strain of probiotics at 10⁹ CFU in daily drinking water was tested in male wild-type mice (C57BL/6J) under HFD for 12 weeks. The effects of the probiotic supplementation were evaluated over time for the different variables examined in comparison to HFD-fed age and sex-matched mice.

1. Methods

Probiotics: Frozen dry pellets of probiotics were reconstituted immediately before use. Every day, bacterial pellets (probiotic X and Y) were thawed 10 minutes in ice and 10 minutes at room temperature prior the addition of a specific drinking water volume in order to have a resulting concentration of 10⁹ cfu in 100 ml inside the biberon. Control group (CTL) received drinking water alone.

Animals: Thirty male 8 weeks old C57BL/6J mice were housed 5/cage (8 cages) with free access to food and water (12-h daylight cycle with light on at 7:00 am) in ICAN animal facility. Mice were given a high fat diet containing 60% Kcal from fat (Research Diets, Inc. New Brunswick, NJ, USA). After 2 week of acclimation, mice received Water (control) or bacterial strains resuspended in Water for 12 weeks. Water suspensions were prepared fresh daily. Daily water intake was recorded daily for the first two weeks and thereafter once a week, to make sure bacterial suspension did not change drinking habits. Mice were assigned to 3 treatment groups, CTL, probiotic X (10⁹ cfu/per day/mouse), probiotic Y (10⁹ cfu/per day/mouse).

Study design: Body weight and food intake were measured weekly. Food intake was determined per cage by weighing the food left in the food container after 1 week. In addition, the weight of food consumed per cage was followed daily during one week. On weeks 0, 2, 4, 6 and 8, non-fasted blood samples were drawn from the tail between 9:00 and 10:00 am and analyzed for glucose concentration using a glucometer (Roche, Basel, Switzerland). Body composition was analyzed by NMR using EchoMRI-100TM equipment (Echo Medical Systems, Houston, TX, USA) on week 11.

Insulin and glucose tolerance tests: Insulin (ITT) and oral glucose (OGTT) tolerance tests were performed at week 10 and 11, respectively in unanesthetized mice. For insulin tolerance tests, insulin (Actrapid, 100UI/ml; Novo Nordisk, Copenhagen, Denmark) was administered i.p. (0.5 U/kg) after 5h of food removal. For oral glucose tolerance test, glucose (10 mmol/kg) was administered by gavage after 16h of fasting. Glycemia was determined with a glucometer (Roche, Basel, Switzerland) from blood obtained at the tail 15, 30, 60 and 90 min after insulin or glucose administration.

Tissue collection: For tissue and blood collection, mice were anesthetized with isoflurane and killed by cervical elongation after 3h of food deprivation the day after the last probiotic administration. Mice from each treatment were equally separated in 3 groups and sacrificed on consecutive days. Inguinal, epididymal and retroperitoneal adipose pads were weighted to calculate an adiposity index (ratio of adipose tissue weight to body weight in %). Liver was also weighted and reported to body weight. Intestinal (caecum) content was collected, together with a part of intestine (jejunum) and colon, and stored at -80°C. Plasma samples prepared from orbital sinus blood samples, collected using EDTA capillary tubes, were stored at -20°C before use. Insulin, leptin and adiponectin concentrations were measured using immunoassay kits (R&D Systems, Minneapolis, MN, USA). Triglycerides levels were determined in plasma and liver lysate using a commercial kit (Sigma-Aldrich, St. Louis, MO, USA).

Statistical analysis: Comparison between groups was performed by one-way ANOVA using JMP software (SAS, Cary, NC, USA). When this analysis revealed statistically significant difference ($p < 0.05$), groups were compared by Student's t test.

2. Effects of probiotics on body weight and adiposity

Before starting the experiment, mice were randomized based on body weight (BW) in the 3 different groups, not to present any difference at baseline. In Figure 42, BW increase over the 12 weeks of HFD is reported in grams and percentage. As expected, the mice in the control (CTL) group developed obesity and presented values of body weight and weight gain expected for similar duration of the specific HFD. The mice receiving probiotic X (from here identified as X) had significantly higher BW compared to the CTL group in weeks 5, 7, 8 and 10. Whereas mice receiving probiotic Y (from here identified as Y) had significantly higher BW compared to the CTL group from week 4 to week 12 (+1.80g to 6.40g). Percentage of fat mass was significantly higher in the Y group compared to the CTL group (7.21% of difference in means).

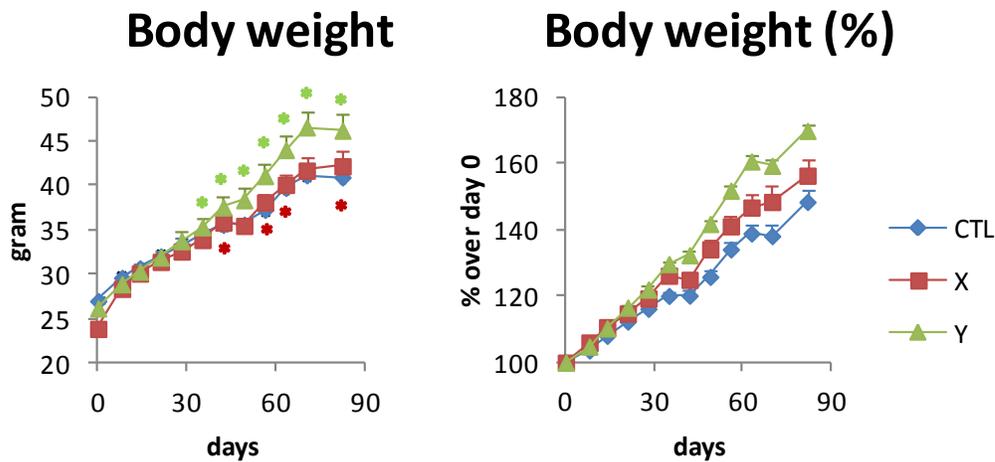


Figure 42. Body weight gain during probiotic treatment in diet-induced obese mice.

*Obese mice were fed a high-fat diet (HFD) with 60 energy % fat. All data are means (n=10/group) and t-bars indicate SEM. * $p < 0.05$ when X (red *) and Y (green *) treatment groups are significant compared to control group.*

The body weight difference of group Y was confirmed when body composition was analyzed by quantitative magnetic resonance system (Echo Medical System, Houston, TX, USA – EcoMRI) at week 11 of HFD. In fact, as shown in Figure 43, percentage of lean mass was significantly lower in the Y. group (-8.41% of difference in means) and percentage of fat mass significantly higher (7.21% of difference in means) in the Y group compared to the CTL group. Values of lean and fat mass for the mice in the CTL group were consistent with previous reports after 12 weeks of HFD feeding.

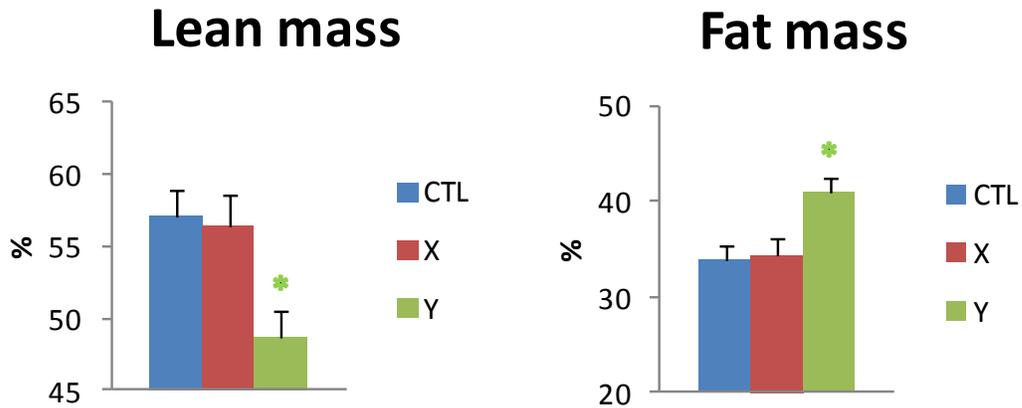


Figure 43. Body composition analysis (%) was measured with EchoMRI.

All data are means (n=10/group) and t-bars indicate SEM. *p < 0.05 is significant compared to control group.

At the sacrifice, WAT from different depots was carefully excised and weighted, as shown in Figure 44. Inguinal pad weight was significantly higher in the Y group compared to the CTL group, whereas no difference was observed in the other adipose depots. This observation suggested that the higher inguinal WAT weight might explain alone the increased percentage in fat mass in the Y group quantified by EcoMRI, also indicated by total WAT, as a result of the sum of the three adipose depots (Figure 44). Adiposity index, calculated as total WAT (gram) divided by body weight (gram) of each animal did not show any differences between groups (data not shown).

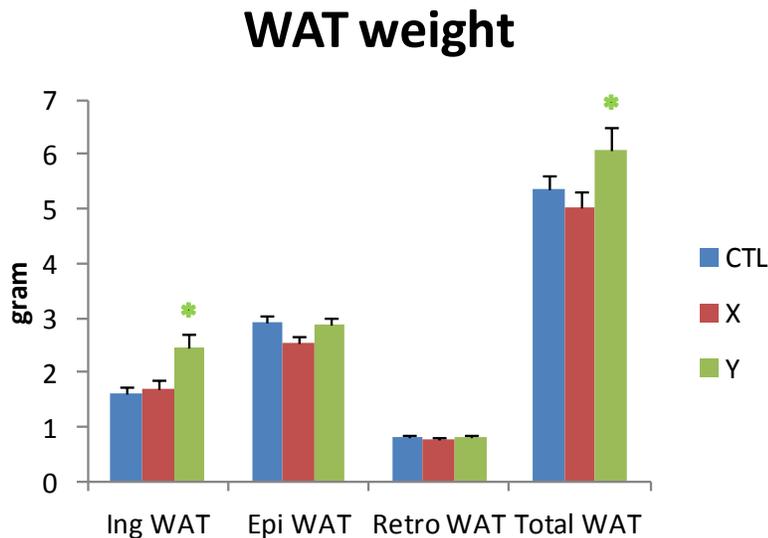


Figure 44. White adipose tissue depot weight

Weight of different WAT depots (Ing = inguinal, Epi = epididymal and retroperitoneal = Retro) from HFD-fed mice without (CTL) or with probiotic treatment (X or Y). All data are means (n=10/group) and t-bars indicate SEM. *p < 0.05 is significant compared to control group.

3. Effects of probiotics on glycemic status

Prolonged high fat diet feeding is known to induce insulin resistance and to promote hyperglycemia. Control group mice presented both these metabolic alterations induced by HFD in expected range. Blood glucose was measured every two weeks in fed-state mice and no differences were observed between treatment groups (data not shown). To evaluate more directly whether IR developed, metabolic tests (ITT and OGTT) were performed in the 3 groups at the end of the HFD time frame. The mice in the control group presented expected responses to ITT and OGTT tests for mice under HFD. Insulin (ITT) tolerance test was performed also at week 10. Overall there was no significant difference between groups. However a trend for lower insulin sensitivity in the Y group was observed and confirmed by the area under the curve over time (Figure 45).

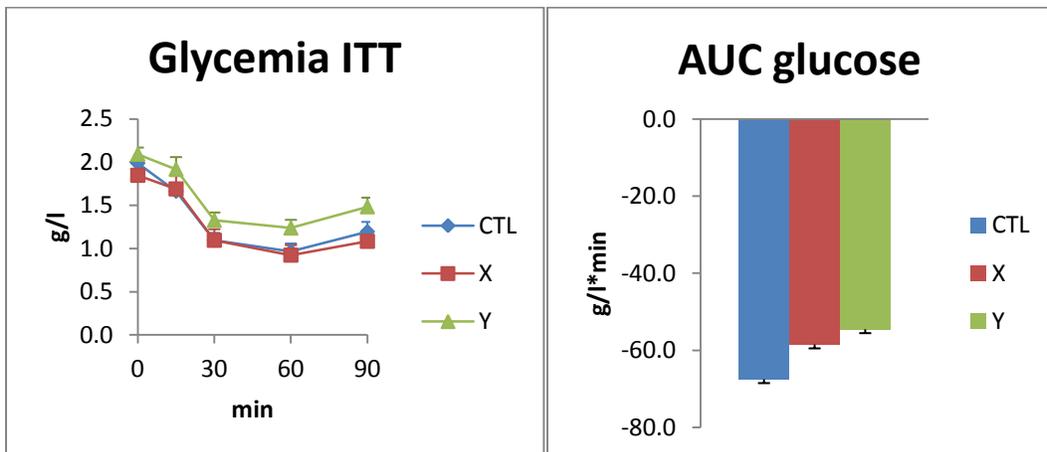


Figure 45. Insulin tolerance test.

Curve of insulin tolerance test and areas under the curve (AUC) of ITT are presented. All data are means ($n=10/\text{group}$) and t-bars indicate SEM.

Oral glucose (OGTT) tolerance test was performed at week 11. Glucose response curves were not statistically different between groups (Figure 46).

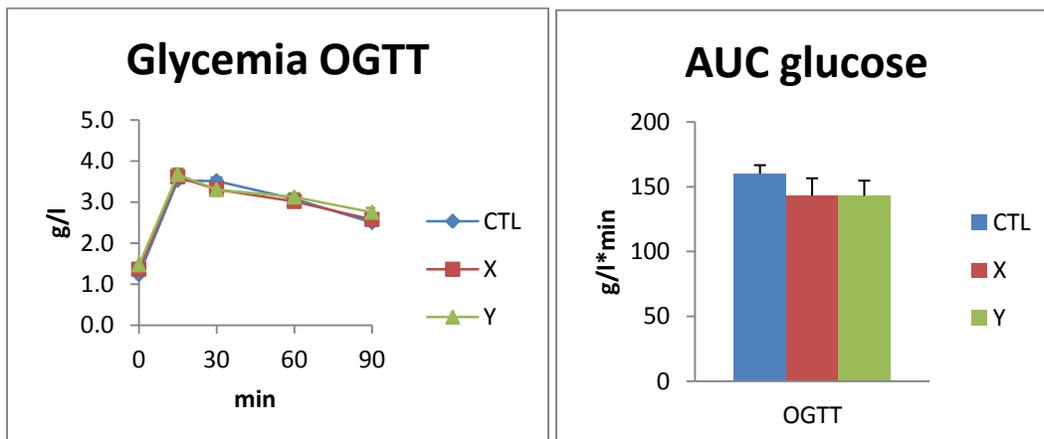


Figure 46. Oral glucose tolerance test.

Curve of oral glucose tolerance test and areas under the curve (AUC) of OGTT are presented. All data are means (n=10/group) and t-bars indicate SEM.

4. Effects of probiotics on plasma variables

Circulating factors were measured in HFD-fed mice and mice either treated with probiotic X or Y or untreated (control group). Insulin levels were quite variable, despite the use of an ultrasensitive kit (Figure 47). Few mice in the probiotic groups presented very high values (1 in group X and 4 in group Y). The high values found for those mice are acceptable, in particular after 12 weeks of HFD, so the insulin graph in Figure 47 shows the groups X and Y with all the mice. However no statistical significance was found compared to the CTL group due to the high standard deviations. Equal amounts of circulating adiponectin were found in the 3 groups, whereas plasma leptin was significantly higher only in the Y group (Figure 47), in accordance with higher BW and fat mass.

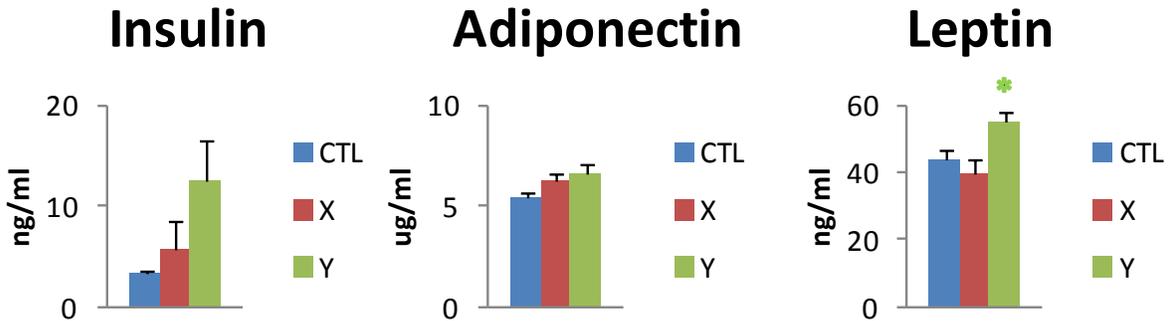


Figure 47. Plasma variables.

Adiponectin, leptin and insulin levels were measured in the HFD-fed mice without (CTL) or with probiotic treatment (X or Y). All data are means (n=10/group) and t-bars indicate SEM. *p < 0.05 is significant compared to control group.

Plasma triglyceride levels did not show any difference between the different groups, whereas liver presented significantly higher TG levels in the Y group compared to the CTL group (5.15 mol/g of difference in means) (Figure 48).

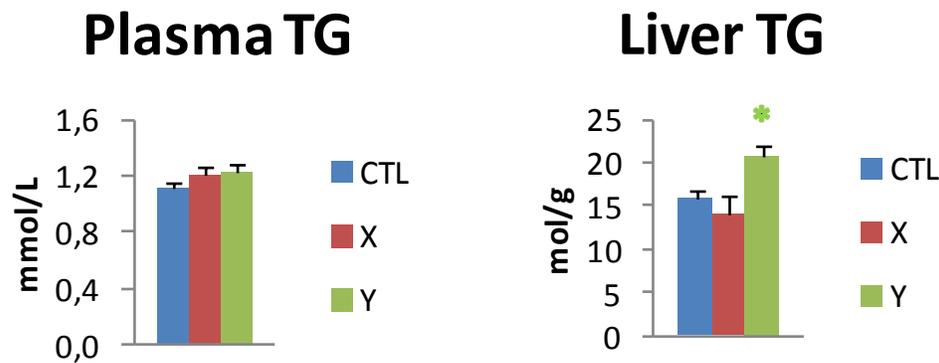


Figure 48. Triglycerides concentration in plasma and liver lysate.

*Triglyceride levels were measured in plasma and liver lysate from HFD-fed mice without (CTL) or with probiotic treatment (X or Y). All data are means (n=10/group) and t-bars indicate SD. *p < 0.05 is significant compared to control group.*

C. Conclusions

The current study shows that probiotics, the Y strain in particular, failed to promote a favorable metabolic phenotype in high fat diet-fed mice. Administration of X and Y probiotic strains in drinking water resulted in increased weight gain as compared to the control group starting after week 4 of HFD. Increased percentage of fat mass was found in the Y group, in association with higher inguinal white adipose tissue weight and circulating leptin levels. Additionally, liver triglyceride content was increased in the Y group, although without significant alteration of plasma triglycerides. No differences were observed for systemic adiponectin levels. Bacterial supplementation did not affect glucose homeostasis in any of the two groups. Glucose tolerance was not different between groups. However, a trend toward systemic insulin resistance was found in the Y group. Although drinking water supplementation is a less aversive method compared to the often reported probiotic administration by daily gavage, measurement of probiotic viability by flow cytometry revealed that probiotics were increasingly stressed (about 1-3%) or dead (6-30%) over time, also in relation to the specificity of the strain used. Finally, further examinations of cellular and molecular mechanisms behind the observed phenotype, such as lipid metabolism within the gastro-intestinal tract and white adipose tissue, will surely help to clarify and avoid those detrimental effects in the presence of certain probiotics. To conclude, research on beneficial effects of specific probiotics on metabolic parameters, such as body weight and glucose homeostasis in HFD-induced obese mice, will surely require further investigation.

GENERAL DISCUSSION AND PERSPECTIVES

A. Contribution of adipose tissue to metabolic deregulation

During my PhD project, I performed and contributed to a series of mice studies conceived and executed in the framework of scientific focus of the Nutriomics team headed by Prof Karine Clément. In this research group, there is an active interest in linking adipose tissue dysfunction, metabolic alterations and gut microbiota, with the overall goal of finding potential therapeutic targets for preventing and/or treating obesity-associated disorders. My prominent contribution is the demonstration of an unexpected capacity of adipose tissue to adapt to an acute nutritional stress, with a major contribution of M2 anti-inflammatory macrophage recruitment (Publication 1, discussed below). This study strengthened the primacy of adipose tissue integrity for glucose homeostasis and enlightened the role of adipose tissue innate immune profile to control inflammation. In a second study, contrary to our expectation, we were unable to demonstrate a beneficial role of probiotic administration to alleviate body weight gain and insulin resistance induced by a high fat diet (Study II, discussed below). Beside, several potential targets were recently identified in adipose tissue by Karine Clément's team. This includes the matrix protease Cathepsin S, the pro-inflammatory transcription factor IRF5 and DAPK2, a kinase regulating autophagy. In this context, I contributed to studies in which high fat diet obese mice were used to substantiate the implication of these targets in fat mass regulation and metabolic homeostasis. A general scheme shown below (Figure 49) recapitulates these contributions, in the context of obesity and lipodystrophy-linked insulin resistance.

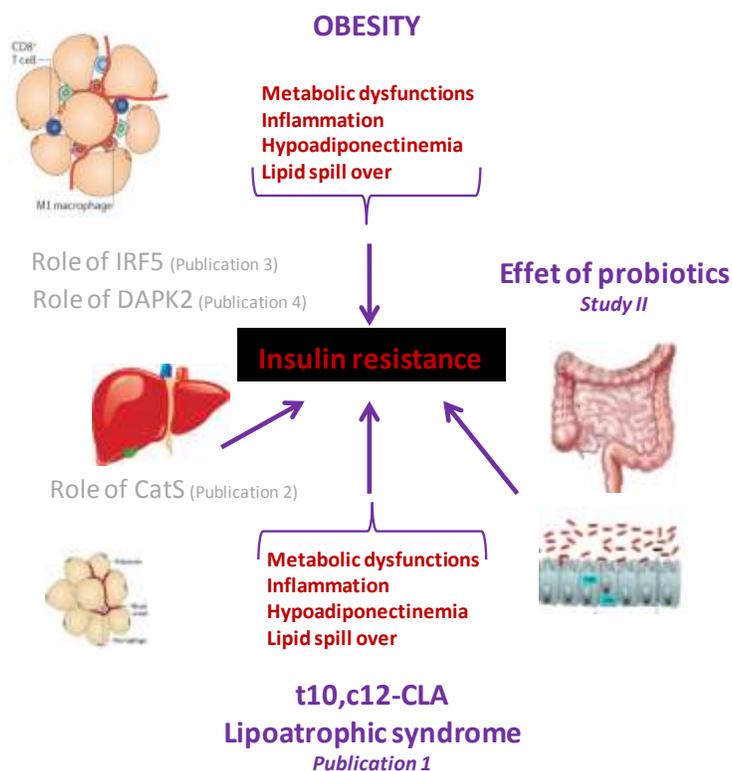


Figure 49. Obesity- and lipodystrophy-induced insulin resistance.

Phenotypic characterization of Cathepsin S deficient mice reported in Publication 2 revealed that mice displayed improved glycemic status and remained glucose tolerant when fed a high fat diet. However, contrary to our initial hypothesis, this was not related to adipose tissue dysfunction or immune alteration. By contrast, a clear liver phenotype was discovered, in which reduced hepatic glucose production was linked to reduced hepatocellular respiration. Although the mechanisms relying this effect were not deciphered, this study identified inhibition of Cathepsin S as a potential mean to improve glycemic status in obese subjects.

The transcription factor IRF5 was found increased in the adipose tissue of obese mice and humans, specifically in visceral depots. In mice, this factor was shown to alter adipose tissue immune profile, promoting M1 macrophage accumulation, and to contribute to extracellular matrix deregulation (Publication 3). IRF5 was up-regulated in adipose tissue of CLA treated mice, although we did not detect any site-specific difference in this model. Further investigations of the effect of CLA on adipose tissue in IRF5 deleted mice are likely to provide new information regarding the role of this transcription factor on adipose tissue dysfunction and ensuing metabolic deregulation.

I contributed to show that DAPK2 gene expression is actually reduced in the adipose tissue of obese mice, as found in human obesity (Publication 4). The role of this kinase in adipocyte was investigated, showing that it could be implicated in attenuation of autophagy in the adipocytes of obese subjects. We did not detect any change in DAPK2 gene expression in our model of CLA treated mice, potentially due to a short-term administration. The role of autophagy in adipose tissue cellular remodeling in obesity and lipodystrophy is not firmly established. The CLA model used in the PhD program could be useful to address this specific question.

B. Adipose tissue plasticity and metabolic consequences

A wealth of studies using distinct mode of CLA administration, i.e. as mixture or isolated isomers, in the food or by gavage and at different doses, have helped to establish a general scheme of CLA-induced systemic and local alterations. All studies converge to the view that adipose tissue represents the main metabolic tissue targeted by CLA and that the t10,c12-CLA isomer is the main culprit in the deleterious effects of these compounds. The table below (Figure 50) summarizes the current knowledge regarding the pleiotropic effects of this specific isomer.

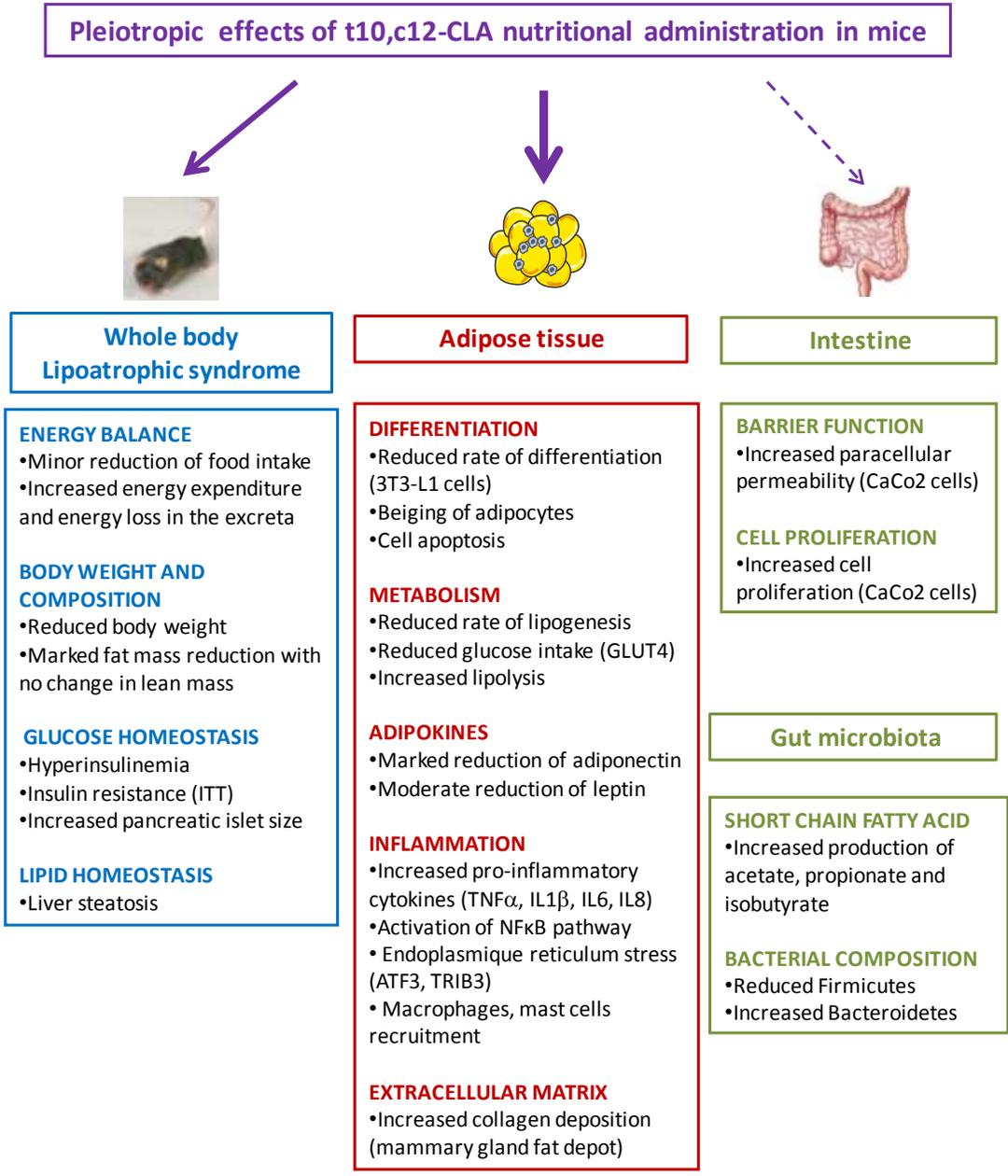


Figure 50. Pleiotropic effect of the t10,c12-CLA isomer in mice.

In study I, we showed that most of these alterations, with the exception of liver steatosis, were acquired quickly in response to high doses of the t10,c12-CLA isomer and were strikingly reversible after short-term interruption of the treatment.

This work as a whole allowed us to better recognize the central role of white adipose tissue in the regulation of glucose homeostasis. In particular, Study I pointed out the integrated involvement of several processes highly tuned to protect WAT homeostasis. Processes intrinsic to the cell itself (adipocyte), including adipokine secretion, mitochondrial fatty acid oxidation and lipolysis, or extrinsic,

such as immune cell dynamics, allow WAT to effectively adapt to metabolic changes. Through this study, we discovered that, in response to an acute nutritional stressor, WAT and glucose homeostasis could be rapidly regained. When the stress is more chronic, as in obesity, certain intrinsic and extrinsic processes, so tightly correlated with metabolism, might become irretrievably out of tune and the dysfunctional state of WAT difficult, if not impossible, to reverse.

During this thesis project, we used the t10, c12-CLA isomer to cause a lipotrophic syndrome in mice (Clement, Poirier et al. 2002) and evaluate the ability of WAT to control the local and systemic changes provoked by this external stressor. To answer to the question, we analyzed the reversibility of the previously reported WAT alterations caused by the specific isomer, macrophage accumulation and tissue remodeling (Clement, Poirier et al. 2002) (Russell, McGee et al. 2007). We focused our attention in particular on WAT immunological response, using flow cytometry to analyze the dynamics and the profile of different immune sub-populations. We also evaluated the potential association between WAT cellular and molecular changes with the insulin resistance induced by CLA (Poirier, Shapiro et al. 2006).

The changes in body composition and adipose depot weight were the result of effects of CLA on adipocytes. Through fatty acid analysis we demonstrated that t10,c12-CLA targets mainly adipose tissue. Indeed a marked accumulation of this isomer was found in WAT compared to other peripheral organs, including liver and intestine. Several in vitro studies have described the effects of CLA on adipocyte functions, such as reduction of lipid uptake and storage capacity inhibiting lipoprotein lipase (Park, Albright et al. 1999), reduction of lipogenesis by decreasing expression of SREBP-1c and PPAR- γ and their downstream genes, ACC, FAS, and SCD1 (House, Cassady et al. 2005), increase in apoptosis (Kennedy, Martinez et al. 2010). Microarray profiling of epididymal WAT of mice fed t10c12-CLA for 14 days indicated a significant reduction of mRNAs for major adipocyte regulatory factors, PPAR γ , SREBP-1, lipin 1 in line with the reduced transcript levels for key metabolic pathways in the WAT (LaRosa, Miner et al. 2006). Our results mostly confirmed those metabolic alterations (ACC, FAS, Plin1 severely down-regulated). The broad chemokine upregulation reported by LaRosa and colleagues for CXC family members in particular (LaRosa, Miner et al. 2006) was also confirmed by our RT-PCR results in the three adipose depots. This increased local chemotaxis was associated with a rapid and massive WAT macrophage accumulation, combined with fat mass reduction, changes in body composition and reduced circulating adipokines (Park, Albright et al. 1997) (West, Delany et al. 1998), (Poirier, Shapiro et al. 2006). However the infiltration of macrophages we observed as early as after 3 days of CLA administration was not associated with WAT inflammation, in sharp contrast with the previously reported increase of proinflammatory cytokines at the protein level (Poirier, Shapiro et al. 2006) and at the transcriptional level (2- to 100-fold induction of many cytokine transcripts, i.e. IL-6, IL-1 β , TNF ligands (LaRosa, Miner et al. 2006), and the reported activation of NF- κ B pathway in response to CLA (Liu, Purushotham et al. 2007). In fact, in our experimental setting, gene expression analysis of WAT after CLA administration indicated a marked upregulation of the classical anti-inflammatory cytokines, IL-1ra and Il10, in the three distinct adipose depots analyzed, subcutaneous, peri-uterine and retroperitoneal WAT.

Macrophages have been reported accumulating in WAT in response to lipodystrophy (Jan, Cervera et al. 2004) and obesity (Cancello, Henegar et al. 2005). In the response induced by t10,c12-CLA, to further characterized the WAT massive accumulation of macrophages shown by immunolabeling, we used flow

cytometry analysis. The stromal vascular fraction infiltrating WAT was analyzed, with a special focus on macrophage phenotype. As presented in the introduction, once these monocyte-derived cells arrive in the tissue, they can be found in a rich spectrum of polarization state (phenotype). The so called M2 macrophages represent the alternatively-activated phenotype and they are usually “resident” in the tissue. At the completely opposite side of the spectrum we have the M1 macrophages, also called “effector” because they are recruited to resolve inflammation. A precise definition of the functional phenotype of macrophages *in vivo* is complicated to achieve. However, through flow cytometry analysis, the phenotype of t10,c12-CLA-induced WAT macrophages was evaluated, using the cell surface markers CD11c and CD206 in F4/80+ cells. This exploration revealed that the majority of the F4/80+ cells infiltrating WAT during CLA administration were CD206+ CD11c- (M2-like macrophages) compared to LA control mice. In response to t10,c12-CLA, this population increased markedly, along with a less abundant subset of CD206+ CD11c+ (M1-like macrophages), which was virtually undetectable in the LA group. These data indicate that CLA did not induce any “phenotype switch” from M2 to M1 state over time, despite the development of insulin resistance in response to the treatment, in opposition to the model proposed by Lumeng for WAT macrophages in obesity (Lumeng, Bodzin et al. 2007). Moreover, gene expression analysis confirmed these findings with a clear predominance of M2 marker upregulation, well represented by the ratio Arg1/iNOS (3-fold increase in subcutaneous WAT and 20-fold increase in peri-uterine WAT). Lower elevation of pro-inflammatory Tnf α and Il-1 β gene expression was observed in WAT in response to CLA, probable outcome of the slight increase of M1 macrophages in WAT during treatment. However their mRNA levels were boldly weighted down by the marked enhancement of IL-1ra and Il-10, the anti-inflammatory cytokines classically expressed in M2 macrophages, indicating that WAT favors an anti-inflammatory response to the short-term metabolic stress-induced by CLA. The increased M2 macrophage accumulation was combined with increase of other innate immune cells, including NK cells and dendritic cells, although to a lower extent. In parallel cells of the adaptive immunity, such as NKT cells, CD3+ T cells and B cells were also increased in both WAT depots, but remained less abundant than innate immune cells, whereas Tregs remained unaffected by t10,c12-CLA administration. We could have expected a reduction in Tregs abundance, given the link established between Tregs reduction and insulin resistance in mice and humans (Deiuliis, Shah et al. 2011). It is possible that longer exposure to a nutritional stress is required for these cells to be altered in WAT.

The marked prevalence of M2-like macrophage accumulation in response to CLA-induced WAT alterations, including apoptosis (Kennedy, Martinez et al. 2010), is consistent with their scavenger function (Lee, Petkova et al. 2013). That such immune cell accumulation happened in the absence of local inflammation during CLA administration, is supported by the findings of one study in which dietary supplementation of t10,c12-CLA was used for one week in mice (Russell, McGee et al. 2007). They reported leukocyte accumulation in association with tissue remodeling in the mouse mammary fat pad (Russell, McGee et al. 2007). In addition, they suggested the dispensability of inflammation during the tissue remodeling process. Interestingly, using genetically modified mice, the authors showed that CLA induced the same response in wild-type and mice lacking TNF- α or functional mast cells (Russell, McGee et al. 2007). Since one week of dietary supplementation of t10,c12-CLA was sufficient to induce collagen deposition in mammary WAT of mice (Russell, McGee et al. 2007), this prompted us to evaluate WAT extracellular matrix accumulation in the three adipose depots during and after CLA treatment. In our

experimental setting, daily gavage with the same CLA isomer induced a gradually increasing fibrillar collagen deposition in WAT, observed by picosiruis red staining already after 3 gavages and confirmed at 7 gavages also by hydroxyproline content determination.

In obesity, WAT fibrosis is associated to tissue expansion and adipocyte dysregulation (Berry, Jeffery et al. 2014), possibly to limit excessive adipocyte enlargement. Instead when fat mass decreases drastically as in lipodystrophy (due to genetic abnormalities, autoimmunity, or retroviral agents in HIV) or too rapidly as in certain cases after bariatric surgery, WAT remodeling intervenes possibly to safeguard adipocyte structure, to avoid excessive loss of adipocyte lipid content. A beneficial role of extracellular matrix remodeling has been proposed in IRF5 deficient mice, which displayed increased collagen deposition associated with limited visceral WAT expansion and improved insulin sensitivity (Publication 3). In 2010, the team of Karine Clément showed that human WAT fibrosis, in particular peri-cellular fibrosis, is a process with the potential of limiting the lipolytic activity of adipocytes after bariatric surgery (Divoux, Tordjman et al. 2010). This concept was further supported by a more recent study from our group, in which Pellegrinelli et al. have shown that human adipocytes decrease lipolysis and adipokine secretion in a 3D system modeling the fibrotic environment characteristic of obese WAT (Pellegrinelli, Heuvingh et al. 2014). In our model, collagen accumulation was found correlated with mRNA Cd68, suggesting that the prevalent M2-like macrophages could have implicated in this process. The decreased expression of *Lox* and *Loxl2*, two transcriptional targets of HIF1 α , known to be involved in pathological fibrosis (Cox and Erler 2011), and above all the reversibility of WAT cellular and molecular alterations indicate that WAT response to CLA is a physiological adaptation to metabolic changes. Further explorations will certainly allow better understanding the functions of these innate cells in response to WAT alterations, i.e. lipid engulfment or collagen turnover (Madsen, Leonard et al. 2013) or tissue repair (Lee, Petkova et al. 2013).

The role of macrophages in the pathological process of other fibrotic tissues can help to decipher which function these cells could exert in WAT remodeling. For example, in fibrotic liver pathology, in mice in mice infected with *Schistosoma mansoni*, IL-4-activated M2 macrophages have been shown to control collagen synthesis and tissue remodeling, expressing high levels of *Arg1* that catalyzes L-polyamine production necessary for myofibroblast activity (Hesse, Modolell et al. 2001). However, another study using the same human pathology model (schistosomiasis infection) showed that *Arg1*-expressing M2 cells are required for the suppression and resolution of fibrosis (Pesce, Ramalingam et al. 2009). Despite this phenotype over-simplification, the role of these innate cells in exacerbating or resolving WAT fibrogenesis could depend on their respective associated molecular mediators. Indeed, evaluation of the transcriptomic profile and the secretion (cytokines, matrix remodeling enzymes and growth factors) of freshly isolated macrophages from WAT during and after t10,c12-CLA administration would allow to draw a well-defined course of macrophage functionality during tissue remodeling.

Finally, whether macrophages accumulate in WAT through infiltration and/or *in situ* proliferation, depending on the specific experimental context remains an interesting question to further explore. In addition, the localization of the CLA-increased M2-like macrophages (around dying cells or within the cytoplasm of WAT) could also help to clarify their role in CLA-induced WAT alterations.

Tissue homeostasis is controlled by an evolutionarily conserved tissue damage response. Studies in flies, mice, and human models have provided evidence that these highly conserved mechanisms are represented by a secretory program that regulates the tissue repair process when cells are damaged (Neves, Demaria et al. 2015). In the context of chronic progressive diseases, such as obesity and aging, the degeneration of tissue homeostasis (due to diet or age-related decline) becomes chronic and as a consequence the adaptive immune response during tissue injury is amplified and grows uncontrolled. The WAT alterations that we observed in response to t10,c12-CLA present few similarities to what happens after injury, an acute tissue degeneration. During wound healing, damaged cells release growth factors and chemokines that stimulate the proliferation and recruitment of leukocytes that produce pro-fibrotic cytokines, such as interleukin-13 and transforming growth factor- β (TGF- β). In our model, IL-13 had very low mRNA expression levels in WAT and the pro-fibrotic factors, inhibin beta-A and Ctgf were modestly increased in WAT. Only in subcutaneous WAT, TGF- β was significantly increased (3-fold over the LA-treated mice) in association with the extra cellular matrix accumulation observed after 7 gavages with CLA. However in common with the acute wound healing, the magnitude of chemokine upregulation in response to CLA was present and it was disproportionate compared to TGF- β , suggesting that immune cell recruitment overpassed the tissue remodeling process. The WAT alterations we observed in response to CLA possibly preceded ECM accumulation, which was still limited, as was limited in term of time and intensity the injury caused by CLA (7 gavages). We attempted to answer to this hypothesis with a small trial where RNA was isolated from WAT of mice treated for 3 gavages. Gene expression analysis of the three WAT, subcutaneous, peri-uterl and retroperitoneal, was performed. The most affected tissue, therefore possibly the first one, was subcutaneous WAT. Three gavages with the t10,c12-CLA were sufficient to induce transcriptional modifications, as reported by LaRosa et al (LaRosa, Miner et al. 2006). Our analysis showed that in sWAT the most affected genes were the chemokines Ccl7 and Ccl2, highly induced (30 to 10-fold induction, respectively), in association with the pan-macrophage marker Cd68 (8-fold), whereas the two collagens (Col1 and Col3) significantly upregulated in response to 7 gavages with CLA (Publication 1) were unchanged in the earlier time point (3 gavages, data not shown). Interestingly, these data suggest that chemokine and consequently macrophage accumulation preceded ECM remodeling, leaving open the question about the effects of CLA during a longer time course.

It is worth to underline that subcutaneous WAT failed to completely recover fat mass after CLA disruption. This was associated with ECM accumulation and fibronectin gene expression still significant in the reversion CLA-treated group (7G 7R), whereas all the other factors, including chemokines, returned to control level. Another observation made through RT-PCR analysis is that this depot did not present increase of VEGF-A and PECAM-1 after CLA, in contrast with peri-uterl and retroperitoneal WAT, suggesting absence of angiogenesis in line with failure in fat mass recovery. Neovascularization is the process during which stromovascular network expands. During the development as well as expansion of adipose tissue, next to adipocyte growth and proliferation, the concomitance of this process is extremely important (Christiaens and Lijnen 2010). Neovascularization has been deeply explored in the context of breast cancer, in particular, in association with extracellular matrix environment on adipose tissue plasticity (Iyengar, Combs et al. 2003) (Iyengar, Espina et al. 2005). In this context, the ability of WAT to secrete protein and lipid derivatives highly proangiogenic can affect the preexisting vasculature, contributing to a unique growth microenvironment. It would certainly be of

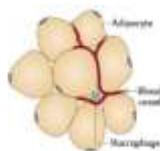
interest to perform similar explorations in the t10,c12-CLA model, i.e. visualization of vascular network in the different adipose depots during and after treatment.

Concomitant or alternative mechanisms possibly implicated in the poor mass recovery in sWAT could rely on reduced fat cell renewal. The inhibition of PPAR γ -regulated adipocyte differentiation by ATF3 (Jang and Jung 2015) could contribute to inability of sWAT to recover fat mass after CLA disruption, in contrast to the two other WAT depots. Whether the recruitment and proliferation of adipose progenitor cells (Lee, Petkova et al. 2013) (Jeffery, Church et al. 2015) is implicated remains to be determined. Interestingly, increased expression of Ki-67, a proliferation-associated marker, is still upregulated in the 7G7R condition, despite the complete regression of infiltrating immune cells in pWAT but not in sWAT. Different approaches could help to further support this preliminary observation. Quantification of preadipocyte replication is possible through adipose tissue *in situ* immunolabeling with Ki-67 antibody or through determination of BrdU incorporation rate in perilipin-positive adipocytes, to assay the rate of formation of new adipocytes (Rigamonti, Brennand et al. 2011). However, unfortunately, the signals triggering an influx of preadipocytes and enhancing their recruitment towards adipogenesis are only beginning to be deciphered, so it is difficult to identify the specific factors that enable WAT to successfully cope with CLA or other external stressors.

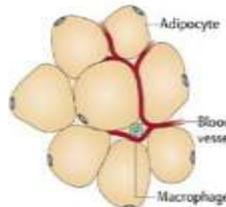
Figure 51 recapitulates the different cellular and molecular mechanisms potentially implicated in WAT depot distinct recovery from CLA-induced injury.

Reversible lipoatrophic syndrome Site-specific recovery after t10,c12-CLA injury

sWAT *Partial recovery*



pWAT *Full recovery*



- Residual metabolic dysfunction (ACC, FAS, PPAR γ)
- Persistent increased ECM (Fibronectin, Col 3, TGF β)
- Persistent macrophage accumulation (CD68)
- *Reduced adipogenesis (PPAR γ , ATF3) ?*
- *Reduced adipose progenitor proliferation (Ki67) ?*
- *Poor neovascularisation (VEGF, PECAM-1/CD31) ?*

Figure 51. Potential biological processes implicated in the poor recovery of subcutaneous WAT after an external injury.

It is only recently that the scientific community increased the attention level on adipose tissue centrality, including extracellular constituents, in relation to the systemic metabolic state. Adipose tissue dysfunction is now recognized as important contributor to the adverse outcomes associated to metabolic diseases. Both lipodystrophy and obesity are characterized by fat mass disturbances and insulin resistance. The results obtained with the t10,c12-CLA-induced lipotrophic syndrome model also suggest primacy of WAT functional integrity for glucose homeostasis. Moreover our data underline a potential common outcome between CLA- and diet induced-WAT and systemic alterations, i.e. adiponectin low levels. In another study insulin resistance was associated with drastic reduction of circulating adiponectin (Purushotham, Wendel et al. 2007). To my knowledge, this is the only study that reported the reversibility of the effects of CLA on WAT mass and hepatic steatosis improvement, when mice were switched to a diet without CLA for the following 30 days. In this experimental design, insulin resistance was abolished concomitantly with adiponectin restoration, suggesting a causal link between those two parameters. This isomer has certain experimental advantages for investigating this link between WAT functionality and systemic metabolism: a rapid time course, the proved reversibility, the control of the stimulus and the specificity, since WAT is its main target.

Finally, the analysis of the microbial composition by 16S rRNA pyrosequencing suggested that even such a short-term administration of t10,c12-CLA possibly alter microbiota in mice, as recently shown (Marques, Wall et al. 2015). The upregulation of the pro-inflammatory TNF α observed in the jejunal compartment could represent the starting point of the intestinal perturbation, caused by CLA directly or indirectly. Unknown endogenous factors could contribute to the microbiota composition changes observed in response to CLA. The initial inflammatory response potentially provoked by a short-administration of this isomer could be amplified if followed by recruitment and/or activation of resident immune cells during longer exposure. This study suggests that mechanisms of t10,c12-CLA action on lipid metabolism might be mediated in part by alterations in gut microbiota composition and functionality. Dietary t10,c12-CLA supplementation for 8 weeks induced a decrease in the proportion of Firmicutes and an increase of Bacteroidetes. In our preliminary results, the enterotype M3, mostly represented by Bacteroides, the less rich in bacterial species, was particularly low in the CLA-treated mice (Figure 40), indicating that longer periods of treatment could change the experimental outcome. Carefull planning of future experiements will be necessary to better evaluate the potential changes at the microbiota level and possibly correlate them with metabolic parameters and WAT cellular and molecular alterations induced by CLA.

C. The future of probiotics to improve metabolic status?

It is now well established that consumption of a high-fat diet and obesity induce changes in the gut microbiota (Erridge, Attina et al. 2007) (Cotillard, Kennedy et al. 2013) (Kong, Tap et al. 2013). Whether modulation of gut microbiota by prebiotics or probiotics could beneficially affect the deleterious metabolic alterations induced by obesity is still controversial. In rodents challenged by a high fat diet,

lactic acid bacteria and bifidobacteria supplementation results generally in reduced body weight gain and adiposity, reduced liver steatosis, improved glycemic status and in anti-inflammatory effects (Amar, Chabo et al. 2011) (Hamad, Sato et al. 2009) (Naito, Yoshida et al. 2011) (Cano, Santacruz et al. 2013) (Miyoshi, Ogawa et al. 2014) (Stenman, Waget et al. 2014) (Poutahidis, Kleinewietfeld et al. 2013) (Wang, Tang et al. 2015). However, some conflicting reports have been published showing increased weight gain and liver enlargement, increased inflammation and/or hyperlipidemia in mice receiving specific *Lactobacillus* strains (Aronsson, Huang et al. 2010) (Angelakis, Bastelica et al. 2012) (Cani, Possemiers et al. 2009).

In Study II, using a high fat diet-induced obesity model (12 weeks), we found that under chronic nutritional stress, the resulting altered metabolic profile is difficult to be reversed. The gut microflora modulation through daily administration of two strains of probiotic was not able to attenuate WAT expansion, hyperglycemia and insulin resistance of HFD-fed mice. The potential beneficial contribution of microbiota to mouse metabolic phenotype could be due to multiple mechanisms, such as increased fatty acid oxidation at the peripheric level, or food intake reduction and/or delayed gastric emptying. In our experimental setting, no difference in food consumption was observed over time, so it is possible that the selected probiotics (X and Y) did not induce this positive effect. In case of mechanisms of action at the tissue level, gene expression analysis of key genes involved in lipid and glucose metabolism will be explored in the main metabolic organs (adipose tissue, liver, intestine). Further exploration, including pyrosequencing of the feces collected over time (before starting probiotic administration and after 6 and 12 weeks of treatment) will allow identifying the impact of the specific probiotic strain used on the intestinal ecology. In particular, one strain over two promoted an unfavorable phenotype, with increased weight gain and fat mass, in association with elevated circulating leptin and liver triglycerides. A limitation of the presented study was that one of the two strains used was supposed to have beneficial effects on the metabolic profile of the HFD-induced obese mice, since it was shown to be “protective” in a previous experimental setting. For this reason, for the studies planned in the next future the use of prebiotics will be introduced as a positive control. Prebiotics have been shown to have consistent beneficial effects on body weight gain, energy homeostasis, energy intake and inflammation, through direct or indirect effects (Everard and Cani 2014) (da Silva, dos Santos et al. 2013) (De Vadder, Kovatcheva-Datchary et al. 2014). The potential effectiveness of this type of nutritional intervention will be tested in parallel to specific probiotic supplementation. Our results indicate that the potential benefits provided by probiotic supplementation need to be further explored in relation to human metabolic diseases, systemically, combining multiple experimental approaches.

D. Conclusions

To conclude, the results obtained with the presented experimental models will need to be reconducted to humans. As far as human consumption is concerned, a definite conclusion for conjugated linoleic acids safety has not been reached yet (Benjamin and Spener 2009). Type of CLA isomer and/or their combination with other polyunsaturated fatty acids, mode of administration, daily dose and duration of consumption are all parameters that remain to be carefully evaluated in relation to gender, age, or ethnic and geographical backgrounds. Similar concerns need to be taken into account for interventions of microflora modulation, including probiotic supplementation. Understanding how different nutritional

choices can impact our health is definitely an important challenge for future research. In particular, looking for the endogenous triggering factors activating the immune system toward pro- or anti-inflammatory responses in the different metabolic organs could open new promising venues for long-term therapeutic interventions in metabolic diseases.

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ANNEXES

During my Doctorate, I participated to three supplemental studies, reported in the Annexes of the present Thesis. One about glucose homeostasis in the context of Cathepsin S deficiency or pharmacological inhibition (Publication 2), one on adipose tissue remodeling in obesity in the context of interferon regulatory factor 5 (IRF5) deficiency (Publication 3) and one on the role of death-associated protein kinase 2 (DAPK2) in the regulation of adipocyte autophagic clearance in human morbid obesity (Annexe 4).

A. Publication 2

Lafarge JC*, Pini M*, Pelloux V, Orasanu G, Hartmann G, Venteclef N, Sulpice T, Shi GP, Clément K, Guerre-Millo M. Cathepsin S inhibition lowers blood glucose levels in mice. *Diabetologia* 2014 Aug;57(8):1674-83.

Abstract:

AIMS/HYPOTHESIS: Cathepsin S (CatS) belongs to a family of proteases that have been implicated in several disease processes. We previously identified CatS as a protein that is markedly overexpressed in adipose tissue of obese individuals and downregulated after weight loss and amelioration of glycaemic status induced by gastric bypass surgery. This prompted us to test whether the protease contributes to the pathogenesis of type 2 diabetes using mouse models with CatS inactivation.

METHODS: CatS knockout mice and wild-type mice treated with orally active small-molecule CatS inhibitors were fed chow or high-fat diets and explored for change in glycaemic status.

RESULTS: CatS deletion induced a robust reduction in blood glucose, which was preserved in diet-induced obesity and with ageing and was recapitulated with CatS inhibition in obese mice. In vivo testing of glucose tolerance, insulin sensitivity and glycaemic response to gluconeogenic substrates revealed that CatS suppression reduced hepatic glucose production despite there being no improvement in insulin sensitivity. This phenotype relied on downregulation of gluconeogenic gene expression in liver and a lower rate of hepatocellular respiration. Mechanistically, we found that the protein 'regulated in development and DNA damage response 1' (REDD1), a factor potentially implicated in reduction of respiratory chain activity, was overexpressed in the liver of mice with CatS deficiency.

CONCLUSIONS/INTERPRETATION: Our results revealed an unexpected metabolic effect of CatS in promoting pro-diabetic alterations in the liver. CatS inhibitors currently proposed for treatment of autoimmune diseases could help to lower hepatic glucose output in obese individuals at risk for type 2 diabetes.

My implication in this study has been important as proved by the co-first authorship. * (JCL et MP, 1st author mentioned in the original article). My contribution is related to the experimental and the reviewing part. I contributed to the preparation of the paper through critical discussion.

B. Publication 3

Dalmas E, Toubal A, Alzaid F, Blazek K, Eames HL, Lebozec K, Pini M, Hainault I, Montastier E, Denis RGP, Ancel P, Lacombe A, Ling Y, Allatif O, Cruciani-Guglielmacci C, André S, Viguerie N, Poitou C, Stich V, Torcivia A, Foufelle F, Luquet S, Langin D, Aron-Wisnewsky J, Clément K, Udalova IA and Venteclef N. *Irf5*-deficiency in macrophages promotes beneficial adipose tissue expansion and insulin sensitivity during obesity in mice. *Nature Medecine* (Accepted NMED-A67879D).

Abstract:

Accumulation of visceral adipose tissue correlates with elevated inflammation and increased risk of metabolic diseases. However, little is known about the molecular mechanisms that control its pathological expansion. Transcription factor interferon regulatory factor 5 (IRF5) has been implicated in polarizing macrophages towards an inflammatory phenotype. Here we demonstrate that mice lacking *Irf5* show no difference in growth of their epididymal white adipose tissue (epiWAT), but expansion of their subcutaneous white adipose tissue, when placed on a high-fat diet compared to wild-type mice on the same diet. EpiWAT from *Irf5*- deficient mice is marked by accumulation of alternatively activated macrophages, higher collagen deposition that restricts adipocyte size and enhanced insulin sensitivity compared to wild-type mice. In obese subjects, IRF5 expression is negatively associated with insulin sensitivity and collagen deposition in visceral adipose tissue. Genome-wide analysis of gene expression in adipose tissue macrophages highlights TGFB1 gene itself as a direct target of IRF5 inhibition. This study uncovers a novel function for IRF5 in controlling the relative mass of different adipose tissue depots and thus insulin sensitivity during obesity, while suggesting that inhibition of IRF5 would promote a healthy metabolic state during this condition.

My contribution to this complex study is related to the IRF5 full body KO mice in comparison to WT mice. I was in charge of characterizing the mRNA profile of inguinal, epididymal, retroperitoneal and mesenteric adipose tissue, liver, intestine (duodenum) and colon. I performed measurements of plasma parameters by ELISAs (glucose, adiponectin, leptin) and Millipore multiplex assays for a panel of cytokines/chemokines. I participated to the immunohistochemistry analysis of WAT infiltrating macrophages.

C. Publication 4

Hedi Soussi, Sophie Reggio, Rohia Allili, Cecilia Prado, Maria Pini, Christine Rouault, Karine Clément, Isabelle Dugail. *DAPK2* down-regulation reversibly associates with deficient adipocyte autophagic clearance in human morbid obesity. *Diabetes* (Under second review).

Abstract:

Adipose tissue dysfunction in obesity has been linked to low-grade inflammation causing insulin resistance. Transcriptomics identified *DAPK2* among the most strongly down-regulated adipose tissue gene in human obesity, but the role of this kinase is unknown. We show that mature adipocytes and not the stroma-vascular cells in adipose tissue mainly expressed *DAPK2*, and that *DAPK2* mRNA in obese

patients gradually recovered after bariatric surgery-induced weight loss. DAPK2 mRNA is also down-regulated in high fat diet-induced obese mice. Adenoviral-mediated DAPK2 overexpression in 3T3-L1 adipocytes did not affect lipid droplet size or cell viability, but increased autophagic clearance in nutrient-rich conditions, dependent on protein kinase activity. Conversely, DAPK2 inhibition in human preadipocytes by siRNA decreased LC3-II accumulation rates with lysosome inhibitors. This led us to assess autophagic clearance in adipocytes freshly isolated from subcutaneous adipose tissue of obese patients. Severe reduction in autophagic flux was observed in obese adipocytes compared to controls, inversely correlated to fat cell lipids. Following bariatric surgery, adipocyte autophagic clearance partially recovered, proportional to fat cell size reduction extent. This study links adipocyte expression of an autophagy-regulating kinase, lysosome-mediated clearance and fat cell lipid accumulation, demonstrates obesity-related attenuated autophagy in adipocytes and identifies DAPK2 dependence in this regulation.

My contribution to the study is relative to the gene expression of DAPK family members in the adipose tissue of chow- and HFD-fed mice. Body weight and blood parameters were evaluated to describe the metabolic profile of those mice.

Tableau 5:List of PCR primers (Complementary results)

Gene symbol	Gene Name	Left	Right
<i>Atf3</i>	activating transcription factor 3	gctggagtcagttaccgtcaa	cgctccttttctctcat
<i>Atf4</i>	activating transcription factor 4	atgatggcttggccagtg	ccattttctccaacatccaatc
<i>Atf6</i>	activating transcription factor 6	ggacgaggtggtgtcagag	gacagctcttcgctttggac
<i>Cldn2</i>	claudin 2	tgaacacggaccactgaaag	ttagcaggaagctgggtcag
<i>Cox1</i>	prostaglandin-endoperoxide synthase 1 (Ptgs1)	cctcttccaggagctcaca	tcgatgtcaccttacagctc
<i>Gpr43</i>	free fatty acid receptor 2 (Ffar2)	tgctctgaagaagccaatca	ttctctctggtccagtgct
<i>Gpr81</i>	G protein-coupled receptor 81	ggctgagaaaagcggtatga	tcgttaactctctccgagctaga
<i>Gpr109A</i>	niacin receptor 1	ctgctcaggcaggatcatct	ccctcttgatcttggcatgt
<i>Gpr116</i>	G protein-coupled receptor 116	cctcaaccatacttacaanaatggac	acagtgaattctgtctcattgc
<i>Gpr120</i>	G protein-coupled receptor 120	ttggtgttgagcgtcgtg	ccagcagtgagacgacaaaag
<i>Pecam1</i>	platelet/endothelial cell adhesion molecule 1	cggtgttcagcgagatcc	actcgacaggatggaaatcac
<i>Pgc1a</i>	PPAR Gamma Coactivator-1	gaaagggccaacagagaga	gtaaatcacacggcgtctt
<i>OCLN</i>	occludin	gtccgtgaggccttttga	gggtcataatgattgggtttg
<i>Tlr1</i>	toll-like receptor 1	ctgagggctctgataatgtcct	tccagctctgtgtgaatttga
<i>Tlr2</i>	toll-like receptor 2	ggggcttcacttctctgctt	agcatcctctgagattgacg
<i>Tlr4</i>	toll-like receptor 4	ggactctgatcatggcactg	ctgatccatgcattggttaggt
<i>Tlr5</i>	toll-like receptor 5	ctggagccgagtgaggtc	cggcaagcattgttctcc
<i>Tlr9</i>	toll-like receptor 9	gagaatcctcatctccaac	ccagagtctcagccagcac
<i>Vegf-a</i>	vascular endothelial growth factor A	aaaaacgaaagcgaagaaa	tttctccgctctgaacaagg
<i>ZO-1</i>	zonula occludens 1/tight junction protein 1	atgcagaccagcaaagg	tggtttgtctcatcatttctca

Table 6: List of PCR primers (Publication 1)

Gene symbol	Gene Name	Left	Right
<i>Acc1</i>	acetyl-CoA carboxylase 1	ttccctggctactatgtgacc	cctgccctgatccagta
<i>Adiponectin</i>	adiponectin, C1Q and Collagen	gagagaaaggagatgcaggt	ctttcctgccagggttc
<i>Arg1</i>	arginase type I	gaatctgcatgggcaacc	gaatcctgtacatctgggaac
<i>Atgl</i>	patatin-like phospholipase domain containing 1 (Pnpla1)	gagcttcgctcaccaac	cacatctctcgaggacca
<i>Ccl2</i>	chemokine (C-C motif) ligand 2	ggctggagagctacaagagg	tcttgagcttggtagacaaaaac
<i>Ccl5</i>	chemokine (C-C motif) ligand 5	cctactcccactcggctct	tttcttgggttctgtgtc
<i>Ccl7</i>	chemokine (C-C motif) ligand 7	ttctgtcctgctgtcata	ttgacatagcagcatgtggat
<i>Ccr2</i>	chemokine (C-C motif) receptor 2	acctgtaaatgccatgcaagt	tgcttccatttctttgattg
<i>Ccr5</i>	chemokine (C-C motif) receptor 5	gagacatccgttccccctac	gtcggaaactgacccttgaaa
<i>Ccr7</i>	chemokine (C-C motif) receptor 7	atttctacagccccagagc	agcacacctggaaaatgaca
<i>Cd40</i>	CD40 antigen	gagtcagactaatgtcatctgtggtt	accccgaaaatggtgatg
<i>Cd68</i>	CD68 antigen	gacctacatcagagcccaggt	cgccatgaatgtccactg
<i>Cd163</i>	CD163 antigen	tctcagtcctctgctgtca	cgccagtctcagttcttct
<i>Cd206</i>	macrophage scavenger receptor 1 (Msr1)	gggagtgtaggcggatca	ggagatgatagtagggtgctctg
<i>Clec10a</i>	C-type lectin domain family 10, member A	ccgggatagtttctcaactctga	aatgaggtaaagcctggaacag
<i>Col1a1</i>	collagen type I alpha 1	catgttcagctttgtggacct	gcagctgactcagggatgt
<i>Col3a1</i>	collagen type III alpha 1	ctggaccccagggtcttc	catctgatccagggtttcca
<i>Col4a1</i>	collagen type IV alpha 1	ttaaaggactccagggaccac	cccactgagcctgtcacac
<i>Col6a1</i>	collagen type VI alpha 1	ctgggcatcaaggtcttttc	gtgtggtctgtggcaatgat
<i>Ctgf</i>	tissue growth factor	tgacctggaggaaaacattaaga	agccctgtatgtcttcacactg
<i>Cxcl2</i>	chemokine (C-X-C motif) ligand 2	aaaatcatccaaaagatactgaacaa	ctttggttctccgttgagg
<i>Cxcl3</i>	chemokine (C-X-C motif) ligand 3	ccccaggctcagataatca	tctgattagaatgcaggtcctt
<i>Cxcl10</i>	chemokine (C-X-C motif) ligand 10	gctgccgtcattttctgc	tctcactggcccgtcatc
<i>Dgat2</i>	diacylglycerol O-acyltransferase 2	ggcgctactccgagactac	tggtcagcaggtgtgtgtc
<i>Fasn</i>	fatty acid synthase	agctatgaagcaattgtggatg	gtgttcgttctcggagtgag
<i>Hsl</i>	hormone sensitive (Lipe)	agcgctggaggagtgtttt	ccgctctccagttgaacc
<i>IL-1r alpha</i>	interleukin 1 receptor antagonist (Il1rn)	ggcagtggaagacctgtgt	catcttgagggtcttttcc

<i>Il1 beta</i>	interleukin 1 beta	gtaatgaaagacggcacacc	tcttctttgggtattgcttgg
<i>Il10</i>	interleukin 10	gctcctagagctgctggact	tgttgccagctgcttcttt
<i>Inhba</i>	inhibin beta-A	atcatcacctttgccgagtc	tcactgccttccttggaaat
<i>iNOS</i>	nitric oxide synthase	ggagccttagacctcaacaga	aaggtagctgaacgaggag
<i>Lep</i>	leptin	caggatcaatgacatttcacaca	gctggtgaggacctgttgat
<i>Lgals3</i>	lectin, galactose binding, soluble 3	gcctaccccagtgctcct	ggtcatagggcaccgtca
<i>Lgals12</i>	lectin, galactose binding, soluble 12	acccgattcctgacagctt	gtgacctcttgctgcata
<i>Lox</i>	lysyl oxidase	caggctgcacaatttcacc	caaacaccaggtagcgcttt
<i>Loxl2</i>	lysyl oxidase-like 2	ggagaacaaggcatcacat	gttggggttaatgacaacctg
<i>Mmp12</i>	matrix metalloproteinase 12	ttgtggataaacactactggaggt	aaatcagcttgggtaagca
<i>Opn</i>	secreted phosphoprotein 1 (Spp1)	ggaaaccagccaagtaagc	tgccaatctcatggtcgtag
<i>Plin1</i>	perilipin 1	gctgctttctcggtgttacag	gagcacgttctcctgctca
<i>Rbp4</i>	retinol binding protein 4, plasma	agacacggaggctggtga	agggcctgcttgacagtaa
<i>Retn</i>	resistin	tgccagtgtgcaaggataga	tggaaaccagctcacttc
<i>Tfeb</i>	transcription factor EB	gagctgggaatgctgatcc	gggacttctgcaggtcctt
<i>Tgf beta</i>	transforming Growth Factor beta 1	tggagcaacatgtggaactc	gtcagcagccggttacca
<i>Timp1</i>	tissue inhibitor of metalloproteinase 1	gcaaagagctttctcaaagacc	agggatagataaacagggaaact
<i>Tnf alpha</i>	tumor necrosis factor alpha	cgccccagctctgtatcctt	ggtcactgtcccagcatcct
<i>Ucp2</i>	uncoupling protein 2		
<i>Ym1</i>	lectin Ym1 (Chitinase 3)	ctgggaactgccaatatggt	gctatgtttctaagaagtgggtatttc

Cathepsin S inhibition lowers blood glucose levels in mice

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Received: 17 January 2014 / Accepted: 9 May 2014 / Published online: 3 June 2014
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Abstract

Aims/hypothesis Cathepsin S (CatS) belongs to a family of proteases that have been implicated in several disease processes. We previously identified CatS as a protein that is markedly overexpressed in adipose tissue of obese individuals and downregulated after weight loss and amelioration of glycaemic status induced by gastric bypass surgery. This prompted us to test whether the protease contributes to the pathogenesis of type 2 diabetes using mouse models with CatS inactivation.

Methods CatS knockout mice and wild-type mice treated with orally active small-molecule CatS inhibitors were fed chow or high-fat diets and explored for change in glycaemic status.

Results CatS deletion induced a robust reduction in blood glucose, which was preserved in diet-induced obesity and with ageing and was recapitulated with CatS inhibition in

obese mice. In vivo testing of glucose tolerance, insulin sensitivity and glycaemic response to gluconeogenic substrates revealed that CatS suppression reduced hepatic glucose production despite there being no improvement in insulin sensitivity. This phenotype relied on downregulation of gluconeogenic gene expression in liver and a lower rate of hepatocellular respiration. Mechanistically, we found that the protein ‘regulated in development and DNA damage response 1’ (REDD1), a factor potentially implicated in reduction of respiratory chain activity, was overexpressed in the liver of mice with CatS deficiency.

Conclusions/interpretation Our results revealed an unexpected metabolic effect of CatS in promoting pro-diabetic alterations in the liver. CatS inhibitors currently proposed for treatment of autoimmune diseases could help to lower hepatic glucose output in obese individuals at risk for type 2 diabetes.

Jean-Charles Lafarge and Maria Pini contributed equally to this study.

Electronic supplementary material The online version of this article (doi:10.1007/s00125-014-3280-2) contains peer-reviewed but unedited supplementary material, which is available to authorised users.

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Keywords Cathepsin inhibitors · Glucose homeostasis · Hepatic glucose production · Obesity · Type 2 diabetes

Abbreviations

CatS	Cathepsin S
CatS-KO	Cathepsin S knockout
FCCP	Trifluorocarbonyl cyanide phenylhydrazone
HFD	High-fat diet
MEF	Mouse embryo fibroblast
OCR	Oxygen consumption rate
REDD1	Regulated in development and DNA damage response 1
RQ	Respiratory quotient
WT	Wild type

Introduction

We previously identified the gene encoding cathepsin S (CatS) as being one of the most commonly upregulated genes in the adipose tissue of obese individuals [1]. CatS belongs to a large family of cysteine proteases that have been implicated in several disease processes [2, 3]. Adipose tissue contributes to the circulating levels of CatS, which vary in parallel to fat mass modification with obesity or weight loss [4, 5]. These human data point out CatS as a potential molecular link between enlarged adipose tissue and obesity comorbidities.

Pioneering studies relying on CatS inhibition or deletion in mice revealed the role of the protease in cleaving the invariant chain of MHC II, thereby allowing proper antigen presentation [6–9]. These preclinical results led to the development of small-molecule inhibitors of CatS proposed for the treatment of autoimmune and inflammatory diseases (reviewed in [10, 11]). Several compounds were efficient at reducing disease scores in models of autoimmunity [12, 13]. Additionally, CatS has been reported to promote neoplastic progression via matrix-derived angiogenic factors [14]. Tumour growth and neovascularisation were attenuated upon antibody-mediated CatS blockage in human xenograft mouse models [15, 16]. Mouse and human studies have also uncovered a pro-atherogenic effect of CatS, mediated by its elastin and collagen proteolytic activity in atheroma [17–19]. These detrimental effects might account for the positive association between mortality risk and serum CatS reported in two populations of older adults [20].

Along with cardiovascular and malignant diseases, to which enhanced CatS activity might contribute, one of the most devastating comorbidities of obesity is type 2 diabetes. A few clinical studies argue for a link between circulating CatS and type 2 diabetes. Serum CatS levels were elevated in diabetic individuals in a Chinese population [21] and were associated with the risk of type 2 diabetes in an elderly male

cohort during a 6 year follow-up [22]. Herein, we evaluated the contribution of CatS to obesity-induced deterioration of glucose homeostasis through detailed analysis of the metabolic phenotype of mice with CatS deletion or treated with CatS inhibitors. Our current data support an unexpected diabetogenic role for the protease and highlight the potential of CatS inhibition to improve glycaemic status in human obesity.

Methods

Mice and diets C57BL/6J CatS-deficient (CatS knockout [CatS-KO]) mice [7, 14] (provided by G-P Shi and later bred in the animal facility at Centre de Recherche des Cordeliers) were provided free access to standard chow diet (Safe, Augy, France). At 6–8 weeks of age, they were separated into three diet groups comprising CatS-KO and sex- and age-matched C57BL/6J wild-type (WT) mice (Charles River Laboratory, L'Arbresle, France). One group was maintained under chow diet (CHOW). A second group was provided with a high-fat diet (72% HFD) containing 72% kJ from fat (corn oil and lard), 28% from protein and less than 1% from carbohydrates for 8 weeks. This regimen was used to alter glycaemic status, with limited development of obesity [23]. A third group was fed a diet (60% HFD) containing 60% kJ from fat (D12492; Research Diets, New Brunswick, NJ, USA) for 20 weeks.

For tissue and blood collection, mice were anaesthetised with isoflurane and killed by cervical elongation after 3 h of food deprivation, except where otherwise stated. Inguinal, retroperitoneal and perigonadal adipose pads were weighed to calculate an adiposity index (ratio of adipose tissue weight to body weight [%]). Care of animals was within institutional animal care committee guidelines and all protocols were approved by the local ethics committee (Ce5/2009/031, Ce5/2009/032, Ce5/2012/087).

In vivo CatS inhibition Ground HFD food was supplemented with small-molecule CatS inhibitors: anti-CatSA (0.010% wt/wt) [24] or anti-CatSB (0.025% wt/wt) [25]. The weight of food consumed per cage housing five mice was determined. Blood samples were drawn from the tail between 10:00 and 11:00 hours and analysed for glucose concentration using a glucometer (Roche, Basel, Switzerland). CatS inhibition was monitored through accumulation of lip10 (BD Pharmingen, Bedford, MA, USA) vs β -actin (Abcam, Cambridge, MA, USA) in spleen extracts [7, 24]. The immune response to injection of 400 μ g ovalbumin (Sigma-Aldrich, St Louis, MO, USA) was tested by quantification of anti-ovalbumin IgGs after 3 weeks.

Glucose homeostasis Glycaemic responses to glucose, insulin or gluconeogenic substrates were investigated in unanaesthetised mice. Glucose (10 mmol/kg) was

administered by gavage after the mice had been deprived of food for 6 h. For insulin tolerance tests, insulin (Actrapid, 100 U/ml; Novo Nordisk, Copenhagen, Denmark) was administered i.p. (0.75 U/kg) after the mice had been deprived of food for 3 h. Pyruvate and L-alanine (Sigma-Aldrich) were given i.p. (2 g/kg) after mice had been fasted for 24 h. Blood samples were drawn from the tail at 0, 15, 30, 60 and 120 min and analysed immediately for glucose concentration.

In vivo glucose utilisation An indwelling catheter was introduced into the femoral vein of mice under anaesthesia. Mice were allowed to recover for 4–6 days and the test was performed after 6 h of fasting. In the basal state, D-3-[³H]glucose (NEN Life Science Products, Boston, MA, USA) was continuously infused at a rate of 3.7×10^5 Bq kg⁻¹ min⁻¹ for 3 h. Under hyperinsulinaemic conditions, mice were infused with insulin (24 pmol kg⁻¹ min⁻¹) and D-3-[³H]glucose (1.11×10^6 Bq kg⁻¹ min⁻¹). Blood glucose was assessed with a glucometer from samples (3.5 µl) collected from the tip of the tail. Euglycaemia was maintained by periodically adjusting a variable infusion of 16.5% glucose. Plasma glucose concentrations and D-3-[³H]glucose specific activity were determined in 5 µl of blood sampled every 10 min during the 60 min of infusion at steady-state conditions and used for calculation, as described in detail elsewhere [26].

Indirect calorimetry Mice were analysed for food intake, whole energy expenditure, O₂ consumption and CO₂ production during 5 consecutive days in calorimetric cages with bedding, food and water (Labmaster, TSE Systems, Bad Homburg, Germany). Energy expenditure was calculated according to the Weir equation [27]. Lean tissue and fat mass were obtained by whole-body composition analysis (EchoMRI, Houston, TX, USA). Respiratory quotient (RQ) was calculated as the ratio of CO₂ output over O₂ consumption.

Gene expression analysis Total RNA was extracted using the RNeasy total RNA Mini kit (Qiagen, Courtaboeuf, France). For microarray analysis, 200 ng of RNA was amplified and transcribed into fluorescent cRNA using Agilent's Low RNA Input Linear Amplification kit (Agilent Technologies, Santa Clara, CA, USA). Agilent 4×44 K whole mouse genome microarrays were used, according to the manufacturer's recommendations. Differential gene expression between WT and CatS-KO mice was determined with a standard analysis of microarray analysis using a false discovery rate of approximately 0.5%, followed by gene-set enrichment analysis as described in [28]. For RT-PCR analysis, SYBR Green real-time PCR was performed on 25 ng cDNA. Data are shown as fold of induction by the comparative C_t method using WT mice as reference. Primers for mouse genes are shown in ESM Table 1.

Liver oil red O staining, triacylglycerol and ATP content Liver was stained for neutral lipid detection using the oil red O technique. Triacylglycerol (Randox, Crumlin, UK) and ATP (BioVision, Milpitas, CA, USA) were determined by colorimetric assays on liver lysate.

Serum measurements Except for NEFA determination, plasma samples were stored at -20°C before use. Leptin and adiponectin concentrations were measured using immunoassay kits (R&D Systems, Minneapolis, MN, USA). NEFA concentrations were determined by enzymatic reaction (Wako Chemical, Neuss, Germany). Triacylglycerols and total cholesterol were determined using a commercial kit (Sigma-Aldrich).

In vitro oxygen consumption rate The rate of O₂ consumption was measured using the OxoPlate OP96C (PreSens, Regensburg, Germany) using liver explants (10–20 mg) or mouse embryo fibroblasts (MEFs) derived from WT and *Redd1* (also known as *Ddit4*)-deficient (*Redd1*^{-/-}) embryos kindly provided by F. Bost [29]. PO₂ was measured in a FlexStation3 microplate reader (Molecular Devices, Sunnyvale, CA, USA) supplied with SoftMax ProMicroplate Data Acquisition and Analysis Software. PO₂ values were plotted over time to calculate the oxygen consumption rate (OCR) from the slope. Addition of the uncoupler trifluorocarbonyl cyanide phenylhydrazone (FCCP) (10 mmol/l) was used to assess maximum OCR.

Statistical analysis Data are shown as mean±SEM. Comparison between groups was performed with two-tailed unpaired or paired Student's *t* tests using the GraphPad Prism 5.0 Software (GraphPad Software, La Jolla, CA, USA). A *p* value of <0.05 was considered statistically significant.

Results

Low-glucose phenotype in mice lacking CatS Blood glucose was systematically lower in mice lacking CatS compared with WT controls whatever the diet (Fig. 1a). Under HFD, the fold increase in blood glucose was similar in both genotypes (Fig. 1b), indicating that CatS-KO mice were not protected against HFD-induced hyperglycaemia. A glucose-lowering effect of CatS deletion was also observed in 1-year-old mice, when the reduction of blood glucose was $15 \pm 3.7\%$ and $14 \pm 2.0\%$ (*n*=9) in CHOW-fed male and female mice, respectively. These data show that CatS deficiency produced a low-glucose phenotype despite any other stress such as high-fat feeding or ageing.

CHOW-fed CatS-KO mice displayed increased body weight and fat mass, in line with a relative deficiency in

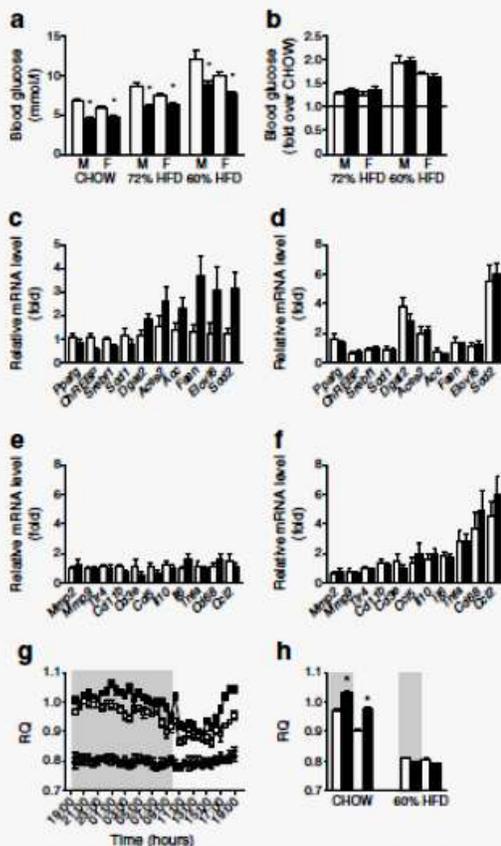


Fig. 1 (a, b) Effect of CatS deletion on blood glucose (a) and the fold increase in blood glucose over CHOW values (b) produced by a 72% or 60% HFD as indicated in male (M) and female (F) mice. White bars, WT; black bars, CatS-KO. (c–f) Adipose tissue gene expression in WT (white bars) and CatS-KO (black bars) mice fed CHOW (c, e) or 60% HFD (d, f). (g, h) RQ measured in metabolism chambers during 24 h (g) or 5 consecutive days (h) in WT (white symbols and bars) and CatS-KO (black symbols and bars) female mice of the CHOW (squares) or 60% HFD (circles) group. In (g, h) the grey shading indicates the dark period. Data are means \pm SEM for $n=6$ –12 mice. * $p<0.05$ vs WT

energy expenditure normalised to lean mass without reduction in food intake (Table 1). Accordingly, circulating leptin (Table 1) and genes related to lipid biosynthetic processes in adipose tissue (Fig. 1c) were upregulated. On an HFD, mice of both genotypes developed obesity (Table 1). Despite reduced energy expenditure, CatS-KO mice reached the same degree of adiposity, leptin and lipogenic gene expression as WT controls (Table 1, Fig. 1d), likely due to a compensatory reduction in food intake. Circulating factors related to glucose and lipid homeostasis (Table 1) and inflammatory gene

expression in adipose tissue (Fig. 1e, f) were unaltered by CatS deletion in both diet groups.

A higher RQ in CatS-KO than in WT mice indicated that CatS deletion promotes preferential glucose oxidation in CHOW-fed mice (Fig. 1g, h). Under 60% HFD, RQ decreased to the same values in both genotypes, suggesting that mice lacking CatS reduced carbohydrate usage as expected in response to HFD.

All together, these data show that the low-glucose phenotype of CatS-KO mice cannot be ascribed to learness, resistance to HFD-induced obesity or reduced adipose tissue inflammation. Additionally, this phenotype did not rely on beneficial changes in adiponectin, leptin or circulating lipids, or preferential glucose use as an energy source.

Pharmacological CatS inhibition reduces blood glucose

Small-molecule CatS inhibitors were tested for their capacity to lower blood glucose in 60% HFD-induced obese WT mice. A first compound, anti-CatSA, produced a rapid reduction of glycaemia, which was reversible upon discontinuation of treatment (Fig. 2a). This effect was sustained over 1 month of treatment, and glycaemia was reduced in WT mice to the same level as in CatS-KO mice (Fig. 2b). These short- and long-term effects of CatS inhibition were reproduced by a distinct anti-CatSB inhibitor (Fig. 2d, e). Both compounds had no significant effect on body weight (Fig. 2c, f) or food intake (Fig. 2g) and, importantly, were without significant effects on blood glucose in CatS-KO mice (Fig. 2b, d). Substantial lip10 accumulation in the spleen provided evidence of efficient CatS inhibition by anti-CatSA (Fig. 2h). Finally, production of specific IgG after ovalbumin injection was markedly reduced in CatS-KO mice, as previously reported [7], but not after 3 weeks of anti-CatSA administration (Fig. 2i). These data highlight the benefit of orally active CatS inhibitors to lower blood glucose without significant side effects on food intake, body-weight gain and immune response in diet-induced obese mice.

CatS deletion ameliorates glucose tolerance Glucose tolerance tests revealed enhanced glucose tolerance in CatS-KO mice, as shown by reduced glycaemic response to glucose load (Fig. 3a). Improved glucose clearance persisted under HFD and with ageing (ESM Fig. 1a–c). Increased insulin production contributed to this phenotype, especially in male CatS-KO mice (Fig. 3b, ESM Fig. 1d, e). A series of observations suggest that CatS deficiency had little influence on insulin sensitivity. First, plasma insulin was not significantly different in CatS-KO and WT CHOW-fed mice (Fig. 3c). In response to 60% HFD, mice became hyperinsulinaemic regardless of CatS deletion or inhibition, with a larger effect in male than in female mice (Fig. 3d). Second, the glucose infusion rate during euglycaemic-hyperinsulinaemic clamp was not significantly changed in CatS-deficient mice

Table 1 Characteristics of WT and CatS-KO female mice

Characteristic	CHOW		60% HFD (20 weeks)	
	WT	CatS-KO	WT	CatS-KO
Body weight (g)	19.3±0.38	22.9±0.33*	31.2±1.67	32.8±1.59
Fat mass (%)	10.7±0.49	13.5±0.27*	33.4±2.54	31.5±2.77
Adiposity index (%)	1.52±0.06	2.40±0.15*	8.84±1.00	8.91±0.49
Lean mass (g)	15.6±0.30	18.1±0.25*	19.5±0.22	20.3±0.36
Food intake (kJ day ⁻¹ [kg lean mass] ⁻¹)	3,430±257	3,404±180	3,479±144	2,739±392
Energy expenditure (kJ day ⁻¹ [kg lean mass] ⁻¹)	2,094±24.2	1,855±27.6*	1,690±45.0	1,365±113*
Leptin (ng/ml)	2.60±0.27	9.10±2.42*	34.9±7.57	47.0±14.6
Adiponectin (µg/ml)	15.2±0.76	14.8±1.21	16.1±0.85	15.6±1.51
Triacylglycerol (g/l)	0.66±0.07	0.62±0.06	0.62±0.2	0.65±0.03
NEFA (mmol/l)	0.85±0.11	0.92±0.08	0.46±0.06	0.59±0.04
Cholesterol (mmol/l)	1.42±0.06	1.80±0.13	2.95±0.26	2.14±0.17

Data are expressed as means ± SEM (n=6–12 mice)

*p<0.05 vs WT

whatever the diet and sex, except for a decrease in CHOW-fed CatS-KO males (ESM Table 2). Third, insulin was equally potent in inhibiting endogenous glucose production in WT

mice and mice lacking CatS, with reduced efficiency in 72% HFD insulin-resistant mice as expected (ESM Table 2). Finally, hypoglycaemic responses to a bolus insulin

Fig. 2 Effect of CatS pharmacological inhibition in 60% HFD-induced obese mice. (a–f) Short-term and long-term effects of anti-CatSA (a–c) or anti-CatSB (d–f) on blood glucose and body weight. WT (white bars and symbols) and CatS-KO (black bars and symbols) mice were treated with anti-CatS (grey bars and symbols, WT; dark-grey bars and symbols, CatS-KO) for the indicated periods of time. (g) Food consumption was recorded in WT mice untreated (white squares) or treated with anti-CatSA (grey squares) or anti-CatSB (grey circles). (h) Western blot detection of Ii p10 in spleen extracts from untreated WT mice, WT mice treated with anti-CatSA for 3 weeks and CatS-KO mice. (i) Immune response to an ovalbumin challenge in WT (white circles), WT treated with anti-CatSA for 3 weeks (grey circles) and CatS-KO (black circles) male mice. Data are means ± SEM, n=5–9 mice. *p<0.05 vs untreated WT at day 0

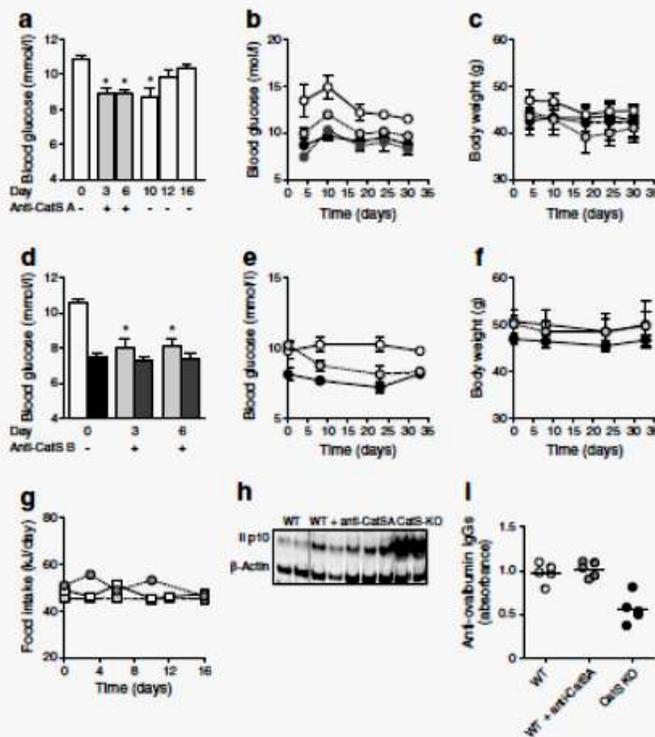
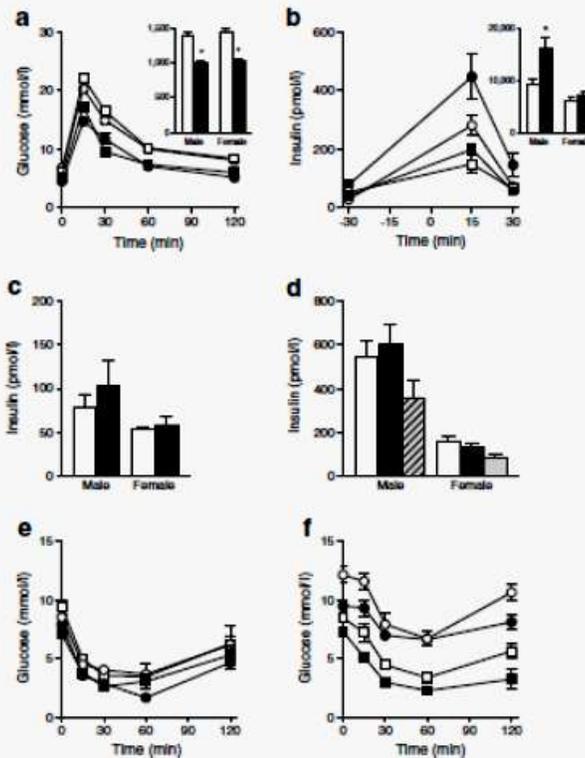


Fig. 3 Effect of CatS deletion on glucose tolerance and whole-body insulin sensitivity. **(a, b)** Glycaemic **(a)** and insulin **(b)** responses to an oral glucose load in WT male (white circles), WT female (white squares), CatS-KO male (black circles) and CatS-KO female (black squares) CHOW-fed mice. Areas under glucose curves are shown in inset in WT (white bars) and CatS-KO (black bars) mice of both sexes, as indicated. **(c, d)** Fasting insulin in WT (white bars), CatS-KO (black bars) and WT mice treated with anti-CatSA (grey bar) or anti-CatSB (hatched grey bar) for 3 weeks. **(e, f)** Glycaemic response to intraperitoneal insulin administration in WT male (white circles), WT female (white squares), CatS-KO male (black circles) and CatS-KO female (black squares) mice. **(e, e)** CHOW-fed mice and **(d, f)** 60% HFD-fed mice. Data are means \pm SEM for $n=6-10$ mice. * $p<0.05$ vs WT



administration were of the same magnitude in WT and CatS-KO mice (Fig. 3e, f). Thus, abrogation of CatS improved glucose tolerance without causing major change in whole-body or hepatic insulin sensitivity.

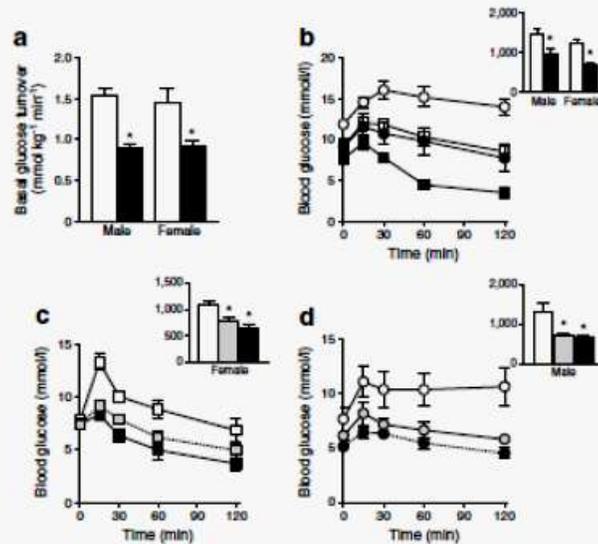
Reduced endogenous glucose production in mice lacking CatS Glucose turnover in the basal state was decreased by half in 72% HFD-fed CatS-KO mice (Fig. 4a), reflecting markedly reduced endogenous glucose production. Lower glycaemic responses to pyruvate substantiated this phenotype in 60% HFD obese mice lacking CatS (Fig. 4b). In this diet group, CatS deletion/inhibition also blunted the glycaemic response to L-alanine (Fig. 4c, d), suggesting reduced hepatic gluconeogenesis [30]. These data support the idea that CatS deficiency targets liver metabolism to promote lower glucose production.

CatS deletion alters gene expression in liver Downregulation of gluconeogenic genes was observed in the liver of mice with CatS deletion (Fig. 5a). CatS inhibition exerted a similar, although less marked, effect. By contrast, lipogenic gene expression (Fig. 5b) and neutral lipid accumulation

(Fig. 5c, d) remained unchanged in CatS-KO mice, suggesting that CatS deletion has pathway-specific effects in the liver. In a large-scale comparative analysis of hepatic gene expression in 72% HFD-fed mice, 245 genes were significantly upregulated and 315 genes were downregulated by CatS deletion. Among the upregulated genes, *Redd1* was one of the most highly expressed genes in CatS-KO liver (Fig. 5e). Increased hepatic *Redd1* expression in CatS-KO mice was confirmed in all other diet groups and in obese WT mice treated with CatS inhibitors (Fig. 5f).

Reduced respiratory chain activity in liver of CatS-KO mice Regulated in development and DNA damage response 1 (REDD1) has been proposed to function as a direct regulator of mitochondrial metabolism [31]. Since mitochondrial respiratory chain activity has a high flux-control coefficient for hepatic gluconeogenesis, we sought to determine whether REDD1 affected this metabolic process. Indeed, OCR was increased twofold in MEFs deficient for *Redd1* (Fig. 5g). Moreover, *Redd1*^{-/-} cells used their maximal respiratory capacity, as suggested by a lack of stimulatory effect of the uncoupler FCCP. These observations identify REDD1 as a

Fig. 4 Effect of CatS deletion on gluconeogenic response. **(a)** Basal glucose turnover in 72% HFD-fed WT (white bars) and CatS-KO (black bars) mice of both sexes. **(b–d)** Glycaemic response to pyruvate **(b)** or L-alanine **(c, d)** in 60% HFD-fed WT male (white circles), WT female (white squares), CatS-KO male (black circles), CatS-KO female (black squares) and WT mice treated with anti-CatSA (grey squares) or anti-CatSB (grey circles) for 3 weeks. Areas under glucose curves are shown in insets in WT (white bars), CatS-KO (black bars) and WT mice treated with anti-CatS (grey bars). Data are means \pm SEM for $n=5-9$ mice. * $p<0.05$ vs WT



negative regulator of the respiratory chain, notwithstanding that the molecular mechanisms implicated remain to be resolved.

This prompted us to test whether hepatocellular respiration was reduced in the liver of mice lacking CatS. We found that hepatic ATP content (Fig. 5h) and OCR (Fig. 5i) were substantially reduced in CatS-KO mice. Nevertheless, OCR remained responsive to the stimulatory effect of FCCP, suggesting that CatS deletion did not alleviate the maximal functional capacity of the respiratory chain. In line with this, OCR diminution occurred without downregulation of mitochondrial genes (Fig. 5j). These results suggest a potential mechanism by which increased *Redd1* expression in the liver of CatS-KO mice mediates the partial reduction of respiratory chain activity, thereby contributing to reduced ATP production and, in turn, gluconeogenic flux.

Discussion

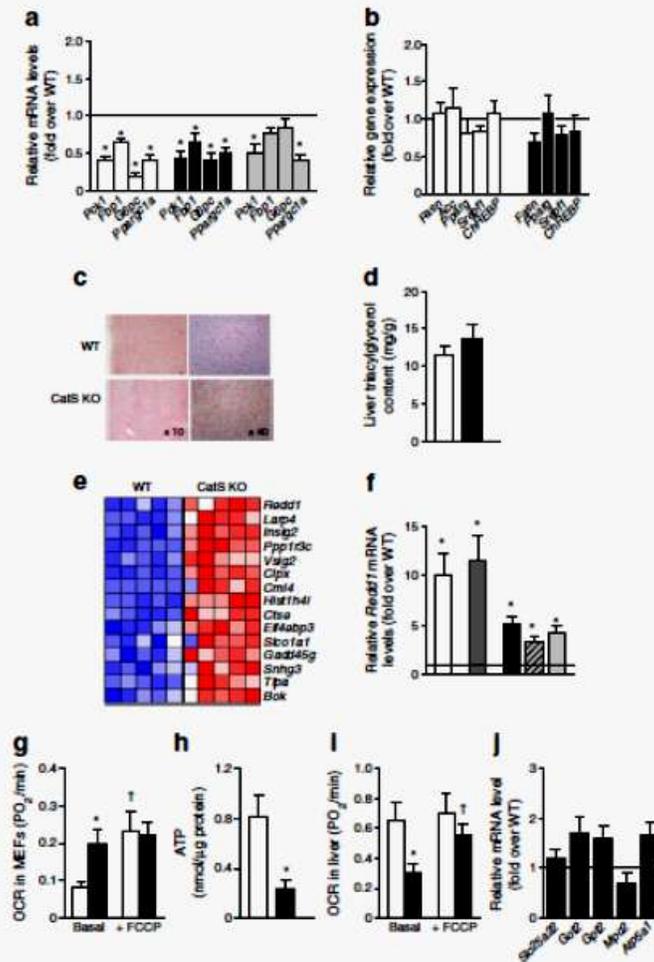
The aim of this study was to assess the pathological relevance of high levels of CatS in obesity. CatS has been implicated in diseases frequently associated with obesity, including asthma, cancer and atherosclerosis [32, 33]. Although type 2 diabetes is a major life-threatening complication of obesity, the implication of CatS in glucose homeostasis has been scarcely documented. Here, a detrimental effect of CatS on glycaemic status was substantiated by the low-glucose phenotype produced by CatS deletion/inhibition in mice. A major finding was that CatS inhibition was able to recapitulate the glucose-

lowering effect of CatS deficiency in WT mice administered with orally active CatS inhibitors.

Previous studies by our team and others had addressed the role of cathepsins in obesity. In mice, Cathepsin L or Cathepsin K deficiency induced resistance to weight gain and, in turn, amelioration of glycaemic status under HFD [34–37]. Mechanistically, inhibition of these cathepsins reduced adipogenesis and lipid storage in adipocytes [34–36]. We show here that CatS suppression led to a strikingly different phenotype, characterised by improved glucose tolerance despite increased adiposity and diet-induced obesity. Predominant measured changes were in hepatic glucose production, which was consistently reduced in diet-induced obese mice lacking CatS or treated with small-molecule inhibitors.

One of the few classes of therapeutics effective in reducing endogenous glucose production has been the biguanides, which includes metformin, the most frequently prescribed drug for type 2 diabetes [38]. Although metformin is widely used as a first-line glucose-lowering drug, its mechanisms of action remain unclear. This drug has been proposed to reduce gluconeogenesis by transcriptional repression of key gluconeogenic genes [39, 40]. Other studies suggest that partial inhibition of the mitochondrial respiratory chain is a major mechanism of action for metformin [41–43]. Similar mechanisms might account for the glucose-lowering effect of CatS deletion, which relies on both gluconeogenic gene repression and downregulation of cellular respiration in the liver. Our data further suggest the involvement of REDD1 in reducing respiratory chain activity in the liver upon CatS inhibition, through a mechanism yet to be elucidated.

Fig. 5 Effect of CatS deletion/inhibition on liver gene expression. (a, b) Expression of gluconeogenic (a) and lipogenic (b) genes in the liver of CHOW-fed (white bars) and 60% HFD-fed (black bars) CatS-KO mice and in 60% HFD-fed WT mice treated with anti-CatSA for 3 weeks (grey bars). Data are shown as fold change over WT controls. (c, d) Liver Oil Red O staining (c) and neutral lipid content (d) in 60% HFD-fed WT (white bar) and CatS-KO (black bar) mice. (e) Heat map of the 15 most upregulated transcripts in the liver of 72% HFD-fed CatS-KO mice. (f) Hepatic *Readd1* gene expression in CatS-KO mice of the CHOW (white bar), 72% HFD (dark-grey bar) or 60% HFD (black bar) group and in 60% HFD-fed WT mice treated for 3 weeks with anti-CatSA (light-grey bar) or anti-CatSB (hatched light-grey bar). Data are shown as fold change over WT controls. (g) OCR in *Readd1*^{-/-} (black bars) and WT (white bars) MEFs. (h) Liver ATP content in WT (white bar) and CatS-KO (black bar) CHOW-fed mice. (i) OCR in liver explants of WT (white bar) and CatS-KO (black bar) CHOW-fed mice after 48 h fasting. (j) Mitochondrial gene expression in liver of CHOW-fed CatS-KO mice shown as fold change over WT. Data are means \pm SEM for $n=5-8$ mice. * $p<0.05$ vs WT; † $p<0.05$ vs without FCCP



One limitation of this study is that the current knowledge on CatS protease activity does not give a hint of how the enzyme influences liver gene expression and metabolism. Since CatS ablation did not significantly affect circulating levels of leptin, adiponectin or blood lipids in diet-induced obese mice, these factors are unlikely to contribute to the mediation of CatS action in the liver. Which substrate(s) mediate(s) the effect of CatS in activating metabolic pathways that promote hepatic glucose output remains an open question.

Increased endogenous glucose production is a major and early defect in the setting of type 2 diabetes and relies on the inability of insulin to suppress hepatic glucose output [44, 45]. Here, the efficiency of CatS suppression in attenuating diet-induced hyperglycaemia was unaffected by insulin resistance.

Moreover, two CatS inhibitors were efficient at reducing blood glucose in insulin-resistant HFD-fed mice. This suggests that pharmacological inhibition of the protease holds promise for attenuating postprandial and, eventually, fasting hyperglycaemia, independent of insulin resistance aggravation in obese individuals. Strategies exist to target hepatic glucose production in type 2 diabetes [46] but individual responses to treatment and associated side effects vary. CatS inhibitors might represent an additional pharmacological tool in patients with type 2 diabetes whose glycaemia remains difficult to normalise with conventional interventions. A clinical study showing that elevated circulating CatS levels increased the risk of developing type 2 diabetes [22] supports the relevance of CatS inhibition in delaying diabetes

conversion in humans. Additionally, since CatS inhibition ameliorates glucose homeostasis (present study) and shows a strong atheroprotective activity [24], small-molecule anti-CatS therapy might be effective in attenuating cardiometabolic risks in obese individuals. Hence, the therapeutic field of anti-CatS molecules currently proposed for the treatment of autoimmune diseases [12, 13] might be extended to cardiometabolic complications of obesity.

In conclusion, the unexpected and favourable phenotype of mice lacking CatS argues for a detrimental role of the protease on glycaemic status, when circulating and adipose tissue levels increase as in human obesity. Our findings provide a strong incentive to evaluate the clinical relevance of CatS inhibition in reducing or delaying the occurrence of type 2 diabetes in obese individuals.

Acknowledgements The authors thank the following individuals: N. Naour (Haute Autorité de Santé, Paris, France), who contributed to the early phases of this study; S. André (Inserm U872 Team 7, Paris, France) and S. V. Kaveri (Inserm U872 Team 16, Paris, France) for their help with the ovalbumin test and C. Magnan (CNRS EAC 4413, Paris, France), C. Cruciani-Guglielmacci (CNRS EAC 4413, Paris, France), A.-F. Burnol (Inserm U1016, Paris, France), G. Lalmanach (Inserm U1100, Tours, France), F. Andreoli (Inserm U872 Team7, Paris, France), G. Midheux (Inserm U855, Lyon, France) and A. Madinoglu (Chalmers University of Technology, Gothenburg, Sweden) for helpful discussions. We also thank S. Vannucci (Weill Cornell Medical College, New York, NY, USA) for commenting on the manuscript. We are grateful to F. Bost (Inserm U1062 Team 7, Nice, France) and L. W. Ellisen (Harvard Medical School, Boston, MA, USA) for giving us the opportunity to use Redd1^{-/-} MEFs and to K. Lolmede (AdipoPhyt, Paris, France) for her crucial help in OxoPlate experiments. We thank J.-F. Bedel (Inserm U872 Team7, Paris, France) and F. Briand (Physiogenex, Toulouse, France) for their expert technical assistance.

Funding This work was supported by the National Agency of Research (ANR OB-Cat), Inserm Transfert, Région Ile de France/CODDIM (grant to MG-M), Fondation pour la Recherche Médicale/Danone, European Union (FP7, ADAPT) and the National Institutes of Health of the USA (HL60942, HL81090, HL88547 to GPS).

Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

Contribution statement MG-M, G-PS, TS and KC contributed to the conception and design of the study. J-CL and MP acquired, analysed and interpreted the data. VP, GO, NV, GH contributed to data acquisition. MG-M, J-CL and MP wrote the manuscript. All authors contributed to the review of the manuscript and approved the final version to be published. MG-M is the guarantor of this work, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of data analysis.

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Irf5 deficiency in macrophages promotes beneficial adipose tissue expansion and insulin sensitivity during obesity

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Accumulation of visceral adipose tissue correlates with elevated inflammation and increased risk of metabolic diseases. However, little is known about the molecular mechanisms that control its pathological expansion. Transcription factor interferon regulatory factor 5 (IRF5) has been implicated in polarizing macrophages towards an inflammatory phenotype. Here we demonstrate that mice lacking Irf5, when placed on a high-fat diet, show no difference in the growth of their epididymal white adipose tissue (epiWAT) but they show expansion of their subcutaneous white adipose tissue, as compared to wild-type (WT) mice on the same diet. EpiWAT from Irf5-deficient mice is marked by accumulation of alternatively activated macrophages, higher collagen deposition that restricts adipocyte size, and enhanced insulin sensitivity compared to epiWAT from WT mice. In obese individuals, IRF5 expression is negatively associated with insulin sensitivity and collagen deposition in visceral adipose tissue. Genome-wide analysis of gene expression in adipose tissue macrophages highlights the transforming growth factor β 1 (*TGFBI*) gene itself as a direct target of IRF5-mediated inhibition. This study uncovers a new function for IRF5 in controlling the relative mass of different adipose tissue depots and thus insulin sensitivity in obesity, and it suggests that inhibition of IRF5 may promote a healthy metabolic state during this condition.

Obesity and its common comorbidities, including glucose intolerance and type 2 diabetes (T2D), are frequently associated with the accumulation of adipose tissue in the intra-abdominal region, (so-called visceral obesity)¹. On the contrary, individuals with predominant storage of fat in the subcutaneous adipose tissue generally show a better metabolic profile^{2,3}. The molecular and cellular mechanisms behind these clinical observations remain poorly understood.

There has been a growing interest in the role of macrophages in shifting adipose tissue inflammation towards a pathological state versus an adaptive response. Macrophages in lean adipose tissue typically express 'alternatively activated' (M2-like) macrophage markers, including the mannose receptor (CD206), and secrete anti-inflammatory cytokines (such as interleukin (IL)-10), which

contributes to the maintenance of tissue homeostasis. During obesity, however, 'classically activated' (M1-like) macrophages that produce pro-inflammatory mediators such as tumor necrosis factor α (TNF- α) or IL-1 β accumulate in the adipose tissue and promote insulin resistance^{4,5}. Altering the expression of transcription factors, such as Klf4 and Ppar- γ , which control M2-like macrophage phenotypes, predisposes rodents to diet-induced obesity, insulin resistance, and glucose intolerance^{6,7}. No study has yet focused on genetically or pharmacologically mediated ablation of the M1-like macrophage molecular program to prevent obesity-induced complications.

Interferon (IFN) regulatory factors are a family of nine transcription factors (IRF1–9) involved in cytosolic pattern recognition receptor- and toll-like receptor-mediated signal transduction and immune

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Received 17 October 2014; accepted 20 February 2015; published online 4 May 2015; doi:10.1038/nm.3829

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cell differentiation. In humans, IRF5 has been associated with susceptibility to inflammatory and autoimmune diseases⁸. Our earlier work demonstrated that IRF5 has a key role in polarizing macrophages towards a pro-inflammatory M1-like phenotype by controlling expression of M1 markers and thereby promoting T helper cell type 1 (T_H1) and T_H17 responses⁹. Of interest, we and others have also shown enhanced T_H17 responses in the visceral adipose tissue of obese and diabetic individuals compared to those in non-obese controls^{10–12}. Here we reveal that whole-body and myeloid cell-specific ablation of *Irf5* in mice induces massive tissue remodeling of the intra-abdominal adipose tissue and concomitant type 2 immune responses. Consequently, *Irf5*-deficient mice showed enlarged subcutaneous fat mass and were protected from obesity-induced metabolic dysfunction. In human obese adipose tissue, *IRF5* expression negatively correlated with collagen deposition and was positively associated with insulin resistance. Mechanistically, IRF5 directly inhibits transcriptional activation of the *TGFB1* gene, which is known to have a key role in tissue remodeling¹³. Thus, we identified *Irf5* as a critical factor in adipose-tissue macrophage polarization and the control of metabolic disease during obesity.

RESULTS

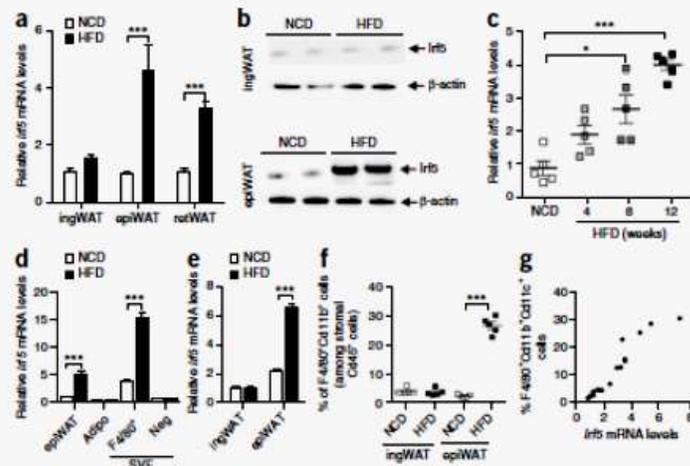
Irf5 expression and effects on adipose tissue during obesity

To explore the role of *Irf5* in adipose tissue during obesity, we analyzed *Irf5* gene expression in inguinal fat (ingWAT, defined as the subcutaneous fat depot) and in intra-abdominal adipose tissues including epiWAT and retroperitoneal (retWAT) depots from C57BL/6 mice fed with a normal chow diet (NCD) or a high-fat diet (HFD) for 12 weeks. Expression of both the *Irf5* gene and *Irf5* protein was higher in the intra-abdominal depots than in the ingWAT depots of mice on the HFD (Fig. 1a,b). *Irf5* expression in epiWAT incrementally increased over the duration of the HFD, as compared to that in epiWAT over the course of the NCD (Fig. 1c), suggesting a possible functional role of *Irf5* in the visceral fat pads. To further identify

the cellular sources of *Irf5*, we isolated adipocytes and the stromal vascular fraction (SVF), including immunoselected F4/80⁺ cells (that is, macrophages) and F4/80⁻ cells, from the epiWAT of obese mice. *Irf5* mRNA levels were higher in the F4/80⁺ cells than in any of the other cell fractions from the epiWAT of both NCD- and HFD-fed mice (Fig. 1d). We observed that *Irf5* was specifically expressed in F4/80⁺ macrophages isolated from the epiWAT compared to those from the ingWAT of mice on the HFD (Fig. 1e). Obesity also induced the accumulation of Cd11b⁺F4/80⁺ macrophages in epiWAT but not ingWAT (Fig. 1f). Accordingly, *Irf5* expression correlated with the number of Cd11b⁺F4/80⁺Cd11c⁻ M1-like macrophages infiltrating epiWAT (Fig. 1g).

Seven-week-old male C57BL/6 mice genetically ablated for *Irf5* (KO) and age- and sex-matched WT littermates were fed with either a NCD or HFD for 12 weeks. We did not observe any differences in weight between NCD-fed WT and KO mice (Fig. 2a). KO mice on the HFD, however, gained more weight and had greater magnetic resonance imaging (MRI)-evaluated fat mass without a change in lean mass, as compared to WT mice on the same diet (Fig. 2a,b and Supplementary Fig. 1a). Detailed analysis of food intake using lean body mass as a covariate revealed a difference between HFD-fed WT and KO mice (1.084 g ± 0.1853 versus 1.805 g ± 0.1853, respectively; *P* < 0.05), with the KO mice being hyperphagic (Supplementary Fig. 1b). Energy expenditure and physical activity were not different between the genotypes (Supplementary Fig. 1c,d). A specific expansion of the subcutaneous ingWAT depot probably explains the *Irf5* knockout-mediated overall weight and fat mass gain on the HFD compared to WT mice on the same diet (Fig. 2b). Histological examination revealed that epiWAT and retWAT but not ingWAT from KO mice were characterized by higher collagen deposition compared to those from WT mice, as determined by Picrosirius red staining (Fig. 2c). We also observed higher mRNA expression of collagen types I, III and IV (*Col1a1*, *Col3a1* and *Col4a1*) in obese KO mice on the HFD than in WT mice on the same diet (Fig. 2c). Adipocyte diameter

Figure 1 *Irf5* is specifically induced in visceral adipose tissue macrophages during obesity. (a) *Irf5* gene profiling in ingWAT, epiWAT and retWAT of mice fed with a NCD or HFD for 12 weeks (*n* = 5 in each group). (b) Protein levels of *Irf5* in ingWAT and epiWAT from lean and diet-induced obese mice (after 12 weeks of NCD or HFD, respectively). (c) Gene expression of *Irf5* in the epiWAT of lean mice after 12 weeks on the NCD and diet-induced obese mice subjected to 4, 8 and 12 weeks of a HFD (*n* = 5). (d) Gene profiling of *Irf5* in epiWAT fractions including adipocytes (Adipo) and the SVF composed of macrophages (immunoselected F4/80⁺ cells) and F4/80⁻ cells (Neg) (*n* = 5 in each group). (e) Measurement of *Irf5* mRNA levels in isolated adipose-tissue macrophages from the ingWAT and epiWAT of lean and diet-induced obese mice after 12 weeks of NCD or HFD (*n* = 5). (f) Flow cytometry analyses of stromal fractions isolated from ingWAT and epiWAT of mice fed a NCD or HFD for 12 weeks (*n* = 5). Macrophages were identified as Cd11b⁺F4/80⁺ cells. Differences between groups (NCD versus HFD) were determined by non-parametric unpaired Mann–Whitney comparative tests or analysis of variance (ANOVA), Bonferroni's *post hoc* test; **P* < 0.05, ***P* < 0.01, ****P* < 0.001 (from *post hoc* test). Data represent mean values ± s.e.m. (g) Correlative analysis between *Irf5* mRNA levels and percentages of pro-inflammatory M1-like macrophages identified as Cd11b⁺F4/80⁺Cd11c⁻ cells in the same epiWAT of mice after 8–12 weeks of HFD (*n* = 16). Correlation was assessed by non-parametric Spearman's test (*r* = 0.98, *P* < 0.001). mRNA levels are relative to 18S rRNA for all experiments.



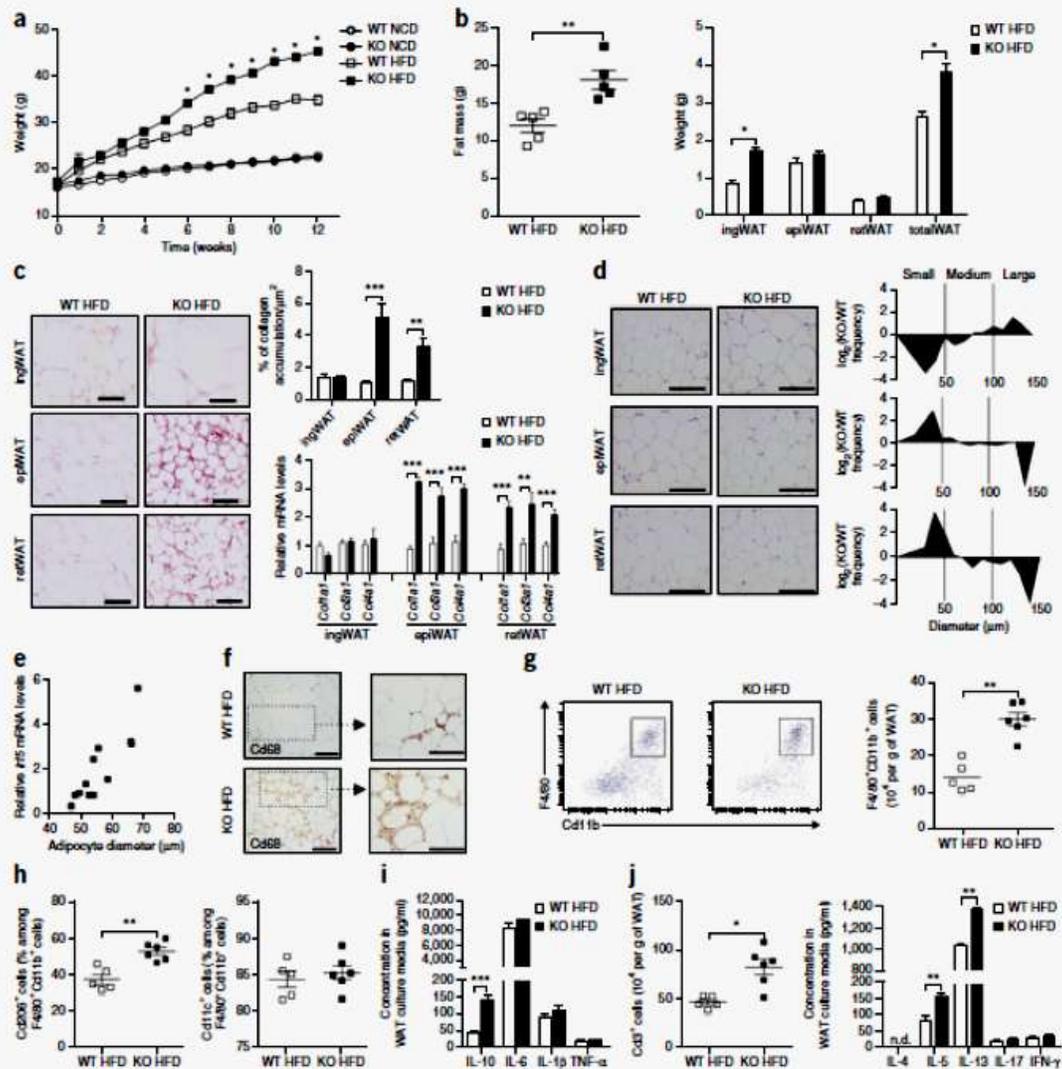


Figure 2 *Irf5* deficiency promotes intra-abdominal adipose tissue remodeling and type 2 immune responses while enhancing inguinal adiposity. (a) Weight of WT and KO mice during 12 weeks of NCD or HFD. (b) Left, fat mass in WT and KO mice. Right, weights of ingWAT, epiWAT, retWAT from WT and KO mice, and the sum of these weights (total WAT). (c) Representative images (left; $n = 3$ images per mouse) and quantification (top right) of collagen deposition in WAT from WT or KO mice. Scale bars, 100 μm . Collagen mRNA levels (*Col1a1*, *Col3a1* and *Col4a1*) in WAT from WT or KO mice (bottom right). (d) Left, representative H&E stain images ($n = 3$ images per mouse) of WAT. Right, quantification of adipocyte size in WAT of WT and KO HFD-fed mice (right). Scale bars, 100 μm . (e) Correlative analyses between *Irf5* mRNA levels and adipocyte diameters in the epiWAT of WT mice ($n = 12$). Correlations were assessed by non-parametric Spearman's test ($r = 0.87$, $P = 0.004$). (f) Representative images ($n = 3$ images per mouse) of immunohistochemical staining for Cd68 in epiWAT from WT and KO mice. Right, higher-magnification images of the boxed regions. Scale bars, 100 μm . (g) Left, representative flow cytometry plots showing frequencies of Cd11b⁺F4/80⁺ macrophages among stromal Cd45⁺ cells isolated from the epiWAT of WT (33.9% \pm 7.7) and KO (56.2% \pm 5.1) mice. Right, macrophages expressed per gram of epiWAT. (h) Quantification of Cd11b⁺F4/80⁺ Cd206⁺ (left) or Cd11c⁺ (right) cells isolated from epiWAT of WT or KO mice. (i) Concentrations of cytokines in epiWAT culture media of WT or KO mice. (j) Left, quantification of Cd45⁺Cd3⁺ lymphocytes among stromal Cd45⁺ cells isolated from the epiWAT of WT or KO mice. Right, concentrations of cytokines in epiWAT culture medium of WT or KO mice. Unless otherwise indicated, $n = 5$ for WT, 6 for KO and measurements taken after 12 weeks on the HFD. Data represent mean values \pm s.e.m. Differences between WT and KO were determined by non-parametric unpaired Mann-Whitney comparative tests or ANOVA with Bonferroni's *post hoc* test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (from *post hoc* test).

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was smaller in the epiWAT and retWAT of KO mice on the HFD than in WT mice on the HFD (Fig. 2d and Supplementary Fig. 1e), and *Irf5* expression correlated positively with adipocyte diameter in the epiWAT of obese WT mice (Fig. 2e). Adipocytes from the subcutaneous ingWAT depot were larger in KO than in WT mice when both were on a HFD (Fig. 2d and Supplementary Fig. 1e). Lipolysis-derived glycerol release and gene expression of *Atgl* (officially known as *Pnpla2*; it encodes the lipolytic enzyme adipose triglyceride lipase) were greater in the epiWAT and retWAT than in the ingWAT of obese KO mice as well as in all fat depots of HFD-fed WT mice (Supplementary Fig. 1f), which suggests lipid partitioning in favor of healthy inguinal depots. Measurement of intrahepatic triglycerides did not reveal any differences in the livers of KO and WT mice on either diet. Measurement of fibrotic gene expression and histological examination did not reveal any differences in the livers of KO mice and WT mice on the HFD (Supplementary Fig. 1g,h).

EpiWAT remodeling in *Irf5*-KO mice on the HFD was associated with a massive accumulation of macrophages compared to that in WT controls on the same diet (Fig. 2f,g). EpiWAT macrophages isolated from KO mice expressed higher levels of the typical M2 surface antigen Cd206 than those from WT controls on the HFD, but

expression of the M1 markers Cd11c and major histocompatibility complex II (MHC-II) was unchanged (Fig. 2h and Supplementary Fig. 2a). Similarly, epiWAT explants from KO mice on the HFD produced higher levels of Il-10, with no difference in Il-6, Il-1 β or Tnf- α production compared to those from WT mice on the same diet (Fig. 2i). Inflammatory gene expression did not differ between NCD-fed WT and KO mice (Supplementary Fig. 2b), suggesting a requirement of high-fat feeding for activation of the *Irf5* pathway. The M2-like expression signature of epiWAT macrophages from KO mice on the HFD was associated with a general type 2 immune response characterized by a higher number of Cd3⁺ T cells and enhanced production of adipose tissue-derived Il-4, Il-5 and Il-13 in the epiWAT of KO mice, as compared to that in WT mice on the HFD (Fig. 2j and Supplementary Fig. 2c). In contrast, concentrations of the T_H1/T_H17 prototypical cytokines Ifn- γ and Il-17 remained unchanged between HFD-fed KO and WT mice (Fig. 2j). IngWAT did not show the same fibro-inflammatory properties as epiWAT in obese KO mice, as compared to HFD-fed WT mice (Supplementary Fig. 2d-g).

To demonstrate the cellular action of *Irf5* in myeloid cells (that is, macrophages), we generated a mouse model with a macrophage cell-specific genetic deletion of *Irf5* (MacKO) by crossing *Irf5*^{flax/flax}

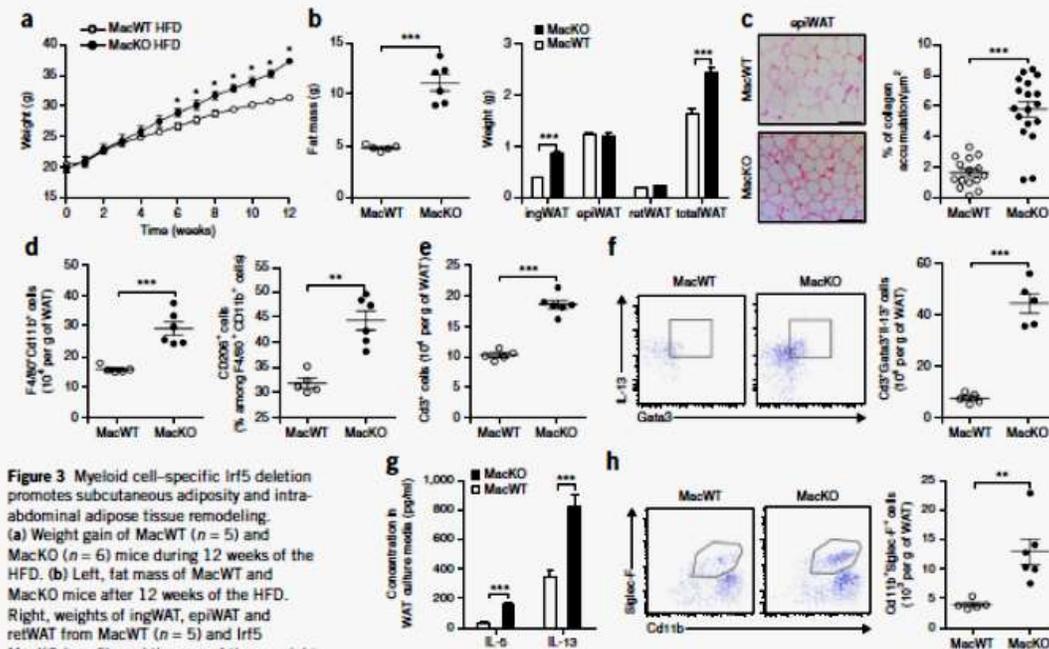


Figure 3 Myeloid cell-specific *Irf5* deletion promotes subcutaneous adiposity and intra-abdominal adipose tissue remodeling. (a) Weight gain of MacWT ($n = 5$) and MacKO ($n = 6$) mice during 12 weeks of the HFD. (b) Left, fat mass of MacWT and MacKO mice after 12 weeks of the HFD. Right, weights of ingWAT, epiWAT and retWAT from MacWT ($n = 5$) and *Irf5* MacKO ($n = 6$), and the sum of these weights (total WAT) after 12 weeks of the HFD (right). (c) Representative images (left; $n = 3$ images per KO or WT mice) and quantification of collagen deposition (right) in epiWAT from MacWT ($n = 5$) or MacKO ($n = 6$) mice after 12 weeks of the HFD. Scale bars, 100 μm . (d) Quantification of stromal Cd45⁺F4/80⁺Cd11b⁺ cells expressed per gram of epiWAT (left) and Cd206⁺ cells expressed as percentage among Cd45⁺F4/80⁺Cd11b⁺ cells isolated from epiWAT of MacWT ($n = 5$) or MacKO ($n = 6$) mice on the HFD (right). (e) Quantification of stromal Cd45⁺Cd3⁺ cells ($n = 5$ for WT, 6 for KO). (f) Left, representative flow cytometry plots showing frequencies of Gata3⁺IL-13⁺ T_H2 cells among Cd45⁺Cd3⁺ cells isolated from the epiWAT of HFD-fed WT ($8.6\% \pm 1.5$; $n = 5$) and KO ($20.8\% \pm 2.5$; $n = 5$) mice (left). Right, T_H2 cells expressed per gram of epiWAT. (g) Concentrations of IL-5 and IL-13 in epiWAT culture medium of HFD-fed MacWT ($n = 5$) or MacKO mice ($n = 6$). (h) Left, representative flow cytometry plots showing frequencies of Cd11b⁺Siglec-F⁺ eosinophils among stromal Cd45⁺ cells isolated from the epiWAT of HFD-fed WT ($0.8\% \pm 0.27$; $n = 5$) or KO ($2.05\% \pm 0.33$; $n = 6$) mice. Right, eosinophils expressed per gram of epiWAT. Data represent mean \pm s.e.m. Differences between MacWT and MacKO were determined by non-parametric unpaired Mann-Whitney comparative tests or ANOVA with Bonferroni's *post hoc* test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (from *post hoc* test).

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with *LysM-Cre* mice. *Irf5* mRNA expression and protein levels were lower in both bone marrow-derived macrophages and adipose SVF cells from MacKO mice than in those from WT littermates (MacWT) (Supplementary Fig. 3a). On a HFD, MacKO mice gained more weight than MacWT mice (Fig. 3a). The greater weight gain was mainly associated with larger subcutaneous fat mass (Fig. 3b and Supplementary Fig. 3b), mirroring the whole-body KO phenotype previously noted. Histological analyses of epiWAT and retWAT from obese MacKO mice showed enhanced tissue remodeling (Fig. 3c) concomitant with higher expression of collagen types I, III and IV (Supplementary Fig. 3c), as well as smaller adipocytes compared to those from MacWT mice on the same HFD regimen (Supplementary Fig. 3d). We did not detect any differences in liver weight and intrahepatic triglyceride accumulation between the genotypes (Supplementary Fig. 3e). Similar to that from whole-body KO mice, epiWAT isolated from obese MacKO mice was characterized by a massive infiltration of Cd206⁺ M2 macrophages (Fig. 3d) and Cd3⁺ T cells, including a specific increase in Gata3⁺IL-13⁺ T_{H2} cells, as compared to that from MacWT controls on the same diet (Fig. 3e,f and Supplementary Fig. 3f). EpiWAT explants of obese MacKO mice secreted higher concentrations of the type 2 immune cytokines IL-5 and IL-13 than the

explants from MacWT (Fig. 3g). As expected, the higher levels of IL-5 were associated with a higher number of Cd11b⁺Siglec-F⁺ eosinophils in the epiWAT of obese MacKO mice than in that of MacWT controls (Fig. 3h). IngWAT from MacKO mice showed neither structural nor fibro-inflammatory changes (data not shown). We also generated adipocyte-specific *Irf5*-KO (AdipoKO) mice and fed them a HFD. No structural or cellular changes in adipose tissue, including changes in adipocyte size, collagen accumulation, and type 2 immune response were observed in AdipoKO mice compared to WT controls when both were on the HFD (Supplementary Fig. 4a–j).

Irf5 promotes insulin resistance *in vivo*

We next examined the effect of *Irf5* deficiency on glucose homeostasis in HFD-fed mice. Fasting glycemia was not different between either of the *Irf5*-KO models (KO and MacKO) and WT littermates (Fig. 4a). Consistent with an increase in fat mass, the fasting insulin concentrations were higher in KO and MacKO mice than in controls (Fig. 4b). Oral glucose tolerance tests (OGTTs) confirmed that, despite higher total fat mass, KO and MacKO mice had improved glucose tolerance compared to WT controls (Fig. 4c). No change in OGTTs was observed in the AdipoKO mice (Supplementary Fig. 5a).

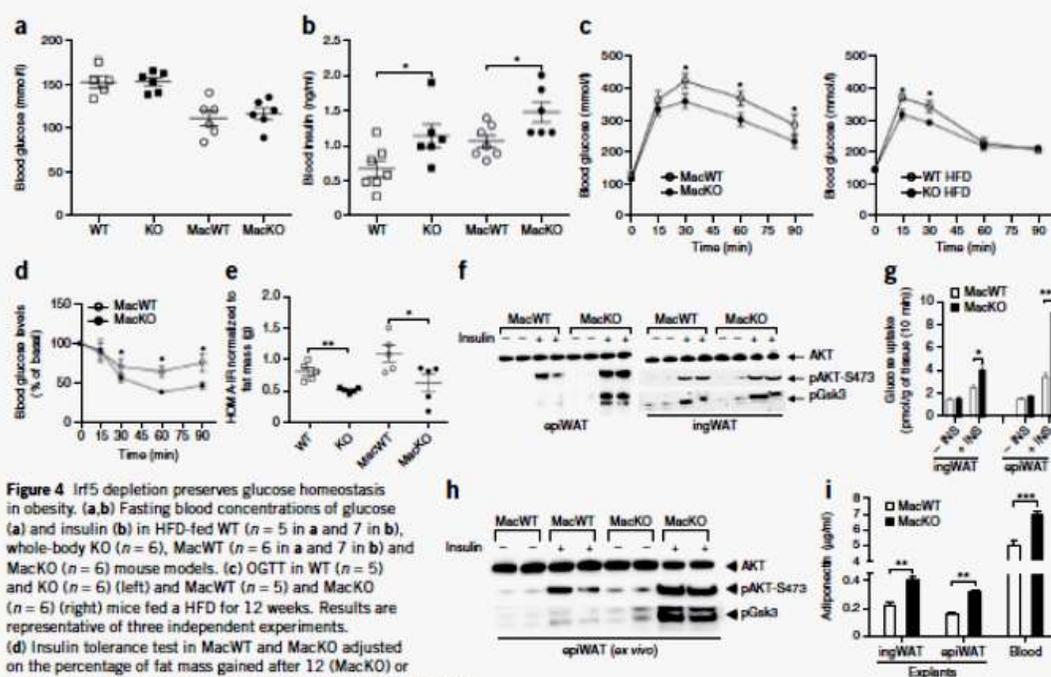


Figure 4 *Irf5* depletion preserves glucose homeostasis in obesity. (a,b) Fasting blood concentrations of glucose (a) and insulin (b) in HFD-fed WT ($n = 5$ in a and 7 in b), whole-body KO ($n = 6$), MacWT ($n = 6$ in a and 7 in b) and MacKO ($n = 6$) mouse models. (c) OGTT in WT ($n = 5$) and KO ($n = 6$) (left) and MacWT ($n = 5$) and MacKO ($n = 6$) (right) mice fed a HFD for 12 weeks. Results are representative of three independent experiments.

(d) Insulin tolerance test in MacWT and MacKO adjusted on the percentage of fat mass gained after 12 (MacKO) or 16 (MacWT) weeks of HFD ($n = 5$). (e) HOMA-IR index normalized to the fat mass of WT and KO mice and MacWT and MacKO models ($n = 5$). (f) MacWT and MacKO HFD-fed mice were injected intraperitoneally with 1 U/kg insulin. Immunoblot analyses of epiWAT (left) and ingWAT (right) tissue samples using antibodies specific to AKT, phospho-AKT (pAKT-S473) and phospho-Gsk3 (pGsk3). Results are representative of at least four independent experiments. (g,h) ingWAT and epiWAT explants from HFD-fed MacWT or MacKO mice stimulated *ex vivo* with insulin (100 nM) for 10 min. (g) Quantification of intracellular radiolabeled [³H]-2-deoxyglucose from both depots ($n = 3$). (h) Insulin signaling (AKT, pAKT-S473, pGsk3) in epiWAT under conditions similar to those described in f. Results are representative of at least four independent experiments. (i) Adiponectin concentrations in epiWAT culture medium and serum of MacWT and MacKO ($n = 5$). Data represent mean values \pm s.e.m. Differences between groups (MacWT versus MacKO) were determined by non-parametric unpaired Mann-Whitney comparative tests or ANOVA with Bonferroni's *post hoc* test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (from *post hoc* test).

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Of note, there was no modification between genotypes in either insulin secretion during OGTTs or in insulin tolerance during insulin tolerance tests (Supplementary Fig. 5b,c).

To determine the effect of weight differences, we performed insulin tolerance tests on MacKO and MacWT littermate mice matched for fat mass percentage after appropriate weeks on the HFD (Supplementary Fig. 5d). In those settings, the MacKO mice were more insulin sensitive than the MacWT mice (Fig. 4d), with a corresponding lower fasting insulinemia (Supplementary Fig. 5e). The insulin resistance index (HOMA-IR) normalized to fat mass was lower in both HFD-fed groups of tested *Irf5* KO mice (whole-body and MacKO) than in corresponding controls (Fig. 4e). EpiWAT and, to a lesser extent, ingWAT *in vivo* (Fig. 4f) as well as epiWAT explants isolated from obese MacKO mice and tested *ex vivo* (Fig. 4g,h) showed more potent insulin signaling and insulin-induced glucose uptake than epiWAT isolated from MacWT mice. Likewise, MacKO mice showed higher adipose tissue and serum adiponectin levels than MacWT mice (Fig. 4i). No differences in insulin signaling were observed in the liver or muscle between the HFD-fed MacWT and MacKO mice (Supplementary Fig. 5f-g).

IRF5 positively correlates with visceral adiposity in humans

Immunostaining IRF5 in visceral adipose tissue (viscWAT) from morbidly obese individuals confirmed that macrophages are the main cellular source of IRF5, as shown by staining for the pan and M1 macrophage markers CD68 and CD11c, respectively (Fig. 5a). The myeloid source of IRF5 was confirmed by increased *IRF5* expression in immunoselected viscWAT CD14⁺ cells (Fig. 5b), which also expressed high levels of CD68 (Supplementary Fig. 6a). *IRF5* mRNA and IRF5 protein levels were higher in viscWAT CD14⁺ cells from morbidly obese subjects compared to those from non-obese

subjects (study population 1, Fig. 5c,d and Supplementary Table 1). Expression of *IRF5* was much higher in viscWAT than in subcutaneous adipose tissue (scWAT) from morbidly obese subjects of population 1 (Supplementary Table 1 and Supplementary Fig. 6b).

Next, we measured *IRF5* expression in viscWAT isolated from lean (LE), overweight (OV) and obese (OB) subjects as well as from patients with metabolic syndrome (MS) (study population 2, Supplementary Table 1). Obese individuals of population 2 had a larger amount of visceral fat compared to lean controls and 50% of the patients with MS were diagnosed with T2D (Supplementary Table 1). *IRF5* mRNA expression in viscWAT was progressively higher in MS versus obese versus overweight subjects and all were higher compared to lean controls (Fig. 5e). *IRF5* expression also positively correlated with the percentage of fat mass (Fig. 5f; $P < 0.0001$) and with the amount of visceral fat, as determined by waist circumference (Fig. 5g; $P < 0.0001$). Moreover, glucose disposal rate (representing glucose uptake by peripheral tissues) and the HOMA-IR were negatively (Fig. 5h; $P = 0.0005$) and positively (Fig. 5i; $P < 0.0001$) correlated with *IRF5* expression, respectively. In agreement with our mouse data, the percentage of collagen accumulation, as evaluated by Picrosirius red staining, negatively correlated with *IRF5* (Fig. 5j; $P = 0.0005$), but not with *CD68* expression in viscWAT from obese subjects (population 1, Supplementary Table 1 and Supplementary Fig. 6c).

IRF5 directly inhibits *TGFβ1* expression in adipose tissue macrophages

To identify genes associated with *IRF5* expression in viscWAT macrophages of obese subjects, we conducted correlative analyses of the *IRF5* mRNA levels with the genome-wide transcriptional profiling of viscWAT CD14⁺ cells immunoselected from 21 morbidly obese

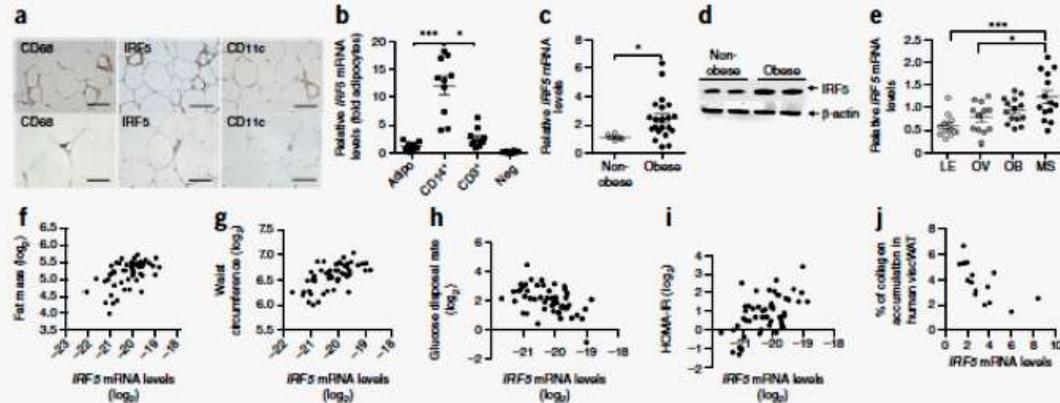


Figure 5 IRF5 expression in macrophages is associated with adipose tissue collagen deposition in human obesity. (a) Representative images of immunohistochemical staining for IRF5, CD68 and CD11c in the viscWAT of obese subjects ($n = 4$). Scale bars, 100 μ m (top) and 50 μ m (bottom). (b) *IRF5* mRNA in viscWAT immunoselected cell fractions and the double-negative fraction isolated from morbidly obese subjects ($n = 10$; population 1). Adipo, adipocytes; CD14⁺, macrophages; CD3⁺, lymphocytes; Neg, double-negative cells. (c, d) *IRF5* mRNA (c) and representative western blot analyses (d) of *IRF5* protein from immunoselected CD14⁺ macrophages isolated from the viscWAT of non-obese ($n = 4$) and morbidly obese ($n = 21$, population 1) subjects. Images are representative of two non-obese and five obese subjects. (e) *IRF5* gene profiling in viscWAT from lean (LE; $n = 12$), overweight (OV; $n = 12$), obese (OB; $n = 14$) subjects and patients with metabolic syndrome (MS; $n = 14$) (population 2). (f–j) Correlation analyses between *IRF5* mRNA levels and fat mass ($r = 0.52$, $P < 0.0001$) (f), waist circumference ($r = 0.60$, $P < 0.0001$) (g), glucose disposal rate ($r = -0.46$, $P = 0.0005$) (h) and HOMA-IR ($r = -0.77$, $P = 0.0012$) (i). (j) Correlative analysis of *IRF5* mRNA levels and percentages of collagen accumulation in viscWAT from obese subjects ($r = -0.77$, $P < 0.0001$, $n = 15$). Correlations were assessed by non-parametric Spearman's test. Data represent mean values \pm s.e.m. Differences between groups (non-obese versus obese or LE versus OV versus OB versus MS) were determined by non-parametric unpaired Mann-Whitney comparative tests or ANOVA, Bonferroni's *post hoc* test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (from *post hoc* test).

subjects of population 1 (Supplementary Table 1). We observed that *IRF5* expression was negatively associated with 135 genes that are mostly involved in the TGF- β and MAP-kinase (MAPK) pathways, and it positively correlated with 32 genes that are mainly involved in the Janus Kinase–signal transducer and activator of transcription (JAK–STAT) pathways (Fig. 6a and Supplementary Fig. 6d). In the same subjects, *IRF5* expression correlated negatively with *TGF β 1* expression (Fig. 6b; $P < 0.0001$) but positively with the expression of pro-inflammatory innate immune markers, such as *ITGAX* (encoding CD11c) and *TLR4* (encoding toll-like receptor 4) (Supplementary Fig. 6e). Moreover, *TGF β 1* expression strongly correlated with the

percentage of collagen accumulation in viscWAT of obese subjects (Fig. 6c; $P < 0.0001$).

Given these associations, we hypothesized that *IRF5* might directly control the transcription of *TGF β 1* in human viscWAT macrophages. We conducted gain (adenoviral overexpression)- and loss (siRNA depletion)-of-function experiments in CD14⁺ cells isolated from human viscWAT and human monocyte-derived macrophages (HMDMs) cultured in the presence of viscWAT adipose tissue-conditioned medium (ATCM) to mimic the fat environment, respectively^{11,14}. Ectopic expression of *IRF5* in viscWAT CD14⁺ cells from non-obese subjects strongly repressed expression of *TGF β 1* compared to

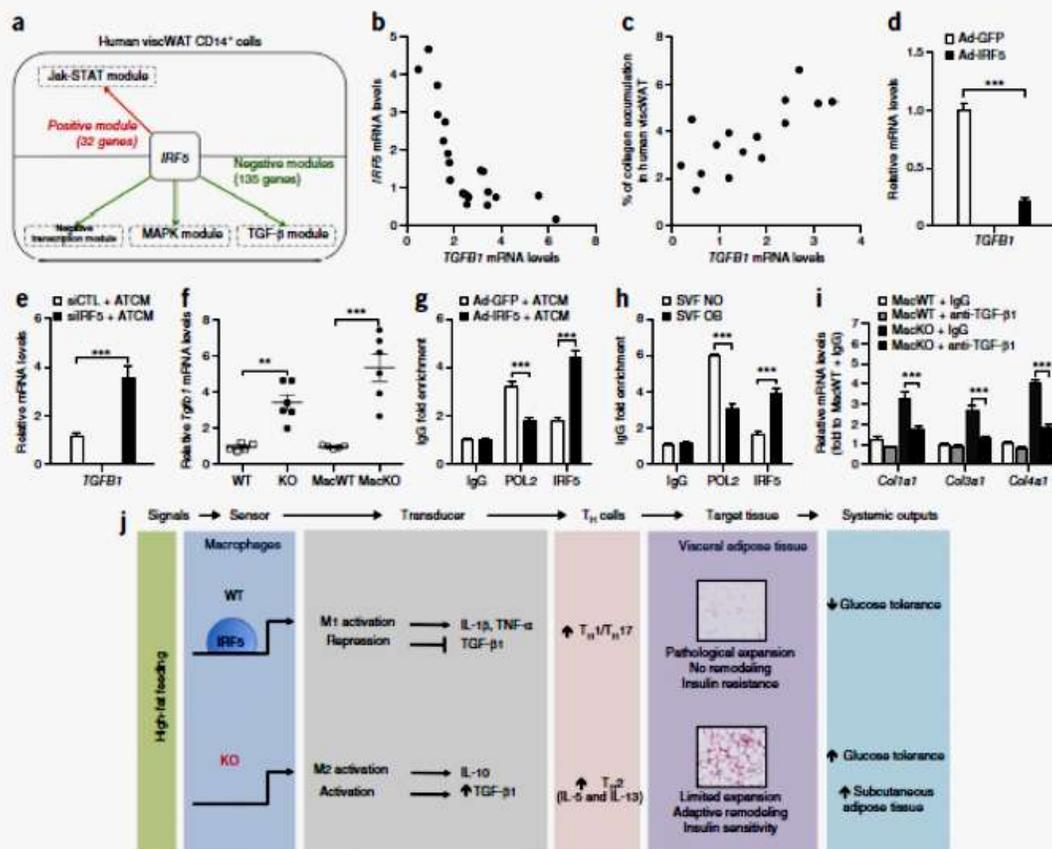


Figure 6 IRF5 controls transcription of the *TGF β 1* gene in human adipose-tissue macrophages. **(a)** Regulatory model of IRF5 activity based on pangenomic profiling of human adipose tissue macrophages isolated from the viscWAT CD14⁺ cells of obese patients ($n = 21$; population 1). **(b)** Correlative analysis between mRNA levels of *IRF5* and *TGF β 1* in viscWAT CD14⁺ cells from obese subjects ($r = -0.85$, $P < 0.0001$, $n = 21$; population 1). **(c)** Correlative analyses of *TGF β 1* mRNA levels and percentages of collagen accumulation in viscWAT from obese subjects ($r = 0.68$, $P = 0.006$, $n = 15$; population 1). **(d)** mRNA quantification of the *TGF β 1* gene with adenoviral overexpression of IRF5 or GFP in immunoselected CD14⁺ cells isolated from the viscWAT of obese subjects ($n = 5$; population 1). **(e)** mRNA quantification of the *TGF β 1* gene with *IRF5*-specific (siIRF5) or control (siCTL) siRNA in human macrophage-derived monocytes (HMDMs) stimulated by adipose tissue-conditioned medium (ATCM) for 24 h ($n = 5$ for each group). **(f)** *Tgf β 1* gene profiling in epiWAT stromal cells isolated from KO ($n = 6$), MacKO ($n = 6$) and WT littermates ($n = 5$). **(g, h)** Recruitment of IRF5 and RNA polymerase II (POL2) to the *TGF β 1* gene promoter of HMDMs overexpressing IRF5 or GFP and stimulated with ATCM **(g)** and viscWAT stromal cells isolated from obese subjects **(h)** ($n = 4$; population 1). **(i)** mRNA quantification of collagen genes in epiWAT explants from obese MacWT and MacKO treated with a neutralizing antibody against TGF- β 1 or IgG (control) for 24 h ($n = 4$). Data represent mean values \pm s.e.m. Differences between groups were determined by non-parametric unpaired Mann–Whitney comparative tests or ANOVA with Bonferroni's *post hoc* test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (from *post hoc* test). **(j)** Schematic illustration depicting the role of IRF5 in adipose tissue remodeling and glucose tolerance.

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expression of control GFP adenovirus in the same cells (Fig. 6d and Supplementary Fig. 7a). IRF5 also upregulated the expression of the ATCM-induced inflammatory markers such as *IL1B*, *IL6* and *TNF* and downregulated that of *IL10* and *MRC1* (encoding CD206), as previously reported⁹ (Supplementary Fig. 7b). Depletion of *IRF5* interfered with ATCM-induced pro-inflammatory gene expression (Supplementary Fig. 7c–e) and promoted expression of *TGFB1* (Fig. 6e). In our mouse models, *Tgfb1* expression was higher in the SVF isolated from epiWAT of both the KO and MacKO mice than that from the epiWAT of WT and MacWT controls (Fig. 6f). Expression levels of *Tgfb2* and *Tgfb3* as well as expression of genes involved in tissue remodeling such as *Lox* and *Mmp9* were also higher in the epiWAT stromal cells of the *Irf5*-KO mice (whole-body and MacKO) compared to controls (Supplementary Fig. 7f).

To further elucidate the molecular mechanisms of the IRF5-mediated inhibition of *TGFB1*, we examined IRF5 recruitment to the *TGFB1* gene promoter in the IRF5-transduced viscWAT CD14⁺ cells of non-obese subjects using chromatin immunoprecipitation (ChIP). Binding of IRF5 to the *TGFB1* promoter led to a reduction of RNA polymerase II recruitment to the same region (Fig. 6g). Moreover, IRF5 binding to the *TGFB1* promoter was enriched in viscWAT stromal cells isolated from obese subjects compared to those from non-obese controls (Fig. 6h). To further support the direct role of IRF5 in the transcriptional inhibition of TGF- β -mediated tissue remodeling, we treated epiWAT explants from MacWT and MacKO mice with either anti-TGF- β 1-neutralizing antibody or isotype control (IgG) and then quantified collagen transcripts. Collagen I, III and IV expression levels were lower in the epiWAT explants from the MacKO mice treated with anti-TGF- β 1 compared to those from MacKO mice treated with IgG control, but anti-TGF- β 1 had no effect in MacWT control mice compared to treatment with control antibody (Fig. 6i).

DISCUSSION

The expandability hypothesis states that fat expansion is required to store energy surplus efficiently and prevent ectopic lipid deposition in metabolically sensitive tissues, thus preventing metabolic complications. Indeed, not all obese subjects develop comorbidities, including T2D, despite increased adiposity. Clinical studies have demonstrated that excess accumulation of intra-abdominal adipose tissue is closely related to clustering of cardio-metabolic risk factors^{1,15,16}. Conversely, increased amounts of subcutaneous adipose tissue confer to some patients metabolic benefits such as improved insulin sensitivity and a lower risk of developing T2D^{17,18}. Our study offers a detailed analysis of a new transgenic mouse model that directly supports the concept that limiting an increase in visceral fat mass while expanding the subcutaneous fat tissue results in an improvement in glucose homeostasis. *Irf5* deficiency induced a pronounced accumulation of fat in the healthy subcutaneous depot while enhanced lipolytic activity and fibrotic tissue remodeling limited intra-abdominal adipose expansion. Both subcutaneous and intra-abdominal adipose tissue depots in *Irf5*-KO mice showed increased adiponectin secretion and insulin sensitivity, supporting metabolic improvement in these mice. This function of IRF5 was reinforced by our experiments in humans, which showed that *IRF5* gene expression was positively correlated with visceral obesity and negatively associated with visceral-fat collagen accumulation and metabolic fitness.

Adipose tissue remodeling often relates to the accumulation of interstitial extracellular matrix and is associated with modifications of adipocyte properties and adipose tissue expansion^{19,20}. In mouse models of abnormal collagen accumulation, rigidity associated with

developmental fibrosis limits adipose tissue expansion^{21,22}. In contrast, targeted deletion of matrix-involved proteins such as collagen VI (through deficiency of the *Col6a1* gene) or matrix metalloproteinase 12 (through deficiency of *Mmp12*) in obese mice results in healthy expansion of individual adipocytes^{23,24}. In humans, obese subjects with high *COL6A1* mRNA levels in the subcutaneous depot show increased visceral fat mass, supporting the adipose tissue expandability hypothesis^{25,26}. Here we observed lower *IRF5* expression in the subcutaneous adipose tissue than in the visceral depot, consistent with the subcutaneous adipose layer being the most fibrotic tissue in our cohort of morbidly obese subjects^{20,25,27}.

Over the last decade, it has been generally accepted that abnormal collagen deposition is a key feature of adipose tissue dysfunction during obesity¹⁹. However, these extracellular matrix dynamics are believed to occur in the pro-inflammatory context of hypertrophic adipocytes, local hypoxia, and pathological immunity, and they may be initiated independently of macrophage infiltration²⁸. For instance, it has been proposed that adipocyte inflammation contributes to healthy adipose tissue remodeling, supporting the concept of beneficial inflammation²⁹. Here we show that *Irf5*-deficient macrophages selectively orchestrate healthy remodeling in the epiWAT concomitantly with an insulin-sensitizing type 2 immune environment.

Inflammatory macrophages expressing *Irf5* accumulate in the intra-abdominal adipose tissue of obese mice. Macrophages, known to drive an inflammatory program within obese adipose tissue, are believed to also orchestrate fibrogenesis in several organs^{30,31}. In human adipose tissue, macrophages are often found in close proximity to collagen-producing cells such as adipocyte progenitors and inside fibrotic bundles^{25,32}. A number of recent studies demonstrated the link between TLR-signaling pathways, activation of adipose tissue macrophages, and tissue remodeling^{9,33,34}. Mice with a *Tlr4* loss-of-function mutation transplanted with WT bone marrow demonstrate increased expression of both macrophage markers and tissue-remodeling features in the epididymal adipose tissue²². In accordance with our findings, there was no evidence of fibrosis and macrophage activation in the subcutaneous fat²². Considering the absence of fibro-inflammatory changes in the epiWAT of the adipocyte-specific *Irf5*-KO mice, we believe that epiWAT remodeling observed in MacKO mice is driven by the massive accumulation of alternatively activated M2-like macrophages and subsequent increased expression of the major remodeling modulator TGF- β 1. This is consistent with other studies observing an increased expression of TGF- β 1 in adipose tissue remodeling in obesity^{22,25,35,36}.

Here we discovered an additional mechanism of IRF5-controlled macrophage polarization; that is, IRF5 can directly repress *TGFB1* expression in macrophages (Fig. 6j). Consequently, IRF5 deficiency induces TGF- β 1 signaling, which leads to collagen deposition and tissue remodeling specifically in the intra-abdominal adipose depot where macrophages preferentially accumulate during obesity. This adaptive program is characterized by a concomitant infiltration of T_H2 cells and eosinophils and the secretion of fibrokinases such as IL-5 and IL-13. Such type 2 immunity has been previously implicated in insulin sensitivity and maintenance of glucose homeostasis³⁷. In addition to its remodeling activities, TGF- β 1 has been shown to inhibit adipogenesis of 3T3-L1 mouse embryonic fibroblast cells and human preadipocytes, contributing to an overall interference of adipose tissue growth^{38,39}. Because of the relative paucity of *Irf5*⁺ macrophages in the subcutaneous fat depot in obese mice, the ingWAT was unaffected by *Irf5* deficiency and could buffer the lipid flux through healthy expansion (Fig. 6j).



In summary, our observations support the strong relationships linking inflammatory processes to extracellular matrix deposition. Depending on the adipose depot at stake, the adipocyte size restriction in the intra-abdominal or the subcutaneous area can beneficially or pathologically, respectively, affect metabolic homeostasis. Considering the key role of macrophages in adipose tissue function, it is tempting to speculate that IRF5 expression is a finely tuned balance between adaptive and pathological responses of macrophages in immunometabolic diseases such as T2D.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. Gene Expression Omnibus: Coordinates have been deposited with accession code GSE65802.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

ACKNOWLEDGMENTS

We are grateful to C. Enond and H. Foher-Ting. We thank Assistance Publique des Hôpitaux de Paris, Programs of Clinical Investigation (Contrat de Recherche Clinique Fibrota AOO759-32 PI to J.A.-W. and Programme Hospitalier de Recherche Clinique 0702 PI to K.C.), the French National Agency of Research (CONRAD to N. Venticlef, ADIPOFIB to K.C. and OBELIP to D.L.), a fellowship from Region Ile de France (to R.A., N. Venticlef and K.C.), Midi-Pyrénées (to D.L.), and the French Foundation for Medical Research (Equipe FRM DEQ20140329504 to F.F. and N. Venticlef and Equipe FRM DEQ20120323701 to K.C.). This work has benefited from a French Government grant managed by the National Agency for Research (‘Investments for the Future’ program, reference no. ANR-10-IAHU (to K.C. and F.F.) and from the Kennedy Institute Trustees’ Research Fund (to K.B., H.L.E. and I.A.U.). Metabolic analyses *in vivo* were performed on the Functional & Physiological Exploration Platform (FPE) technical platform, unit ‘Biologie Fonctionnelle et Adaptative’, (Univ. Paris Diderot, Sorbonne Paris Cité, Paris, France), and on the ICAN PRECLINIC platform (Institute of Cardiometabolism and Nutrition, Pitié-Salpêtrière Hospital, Paris, France). D.L. is a member of Institut Universitaire de France. A. Toubal is a recipient of a doctoral fellowship from the Ministère de la Recherche et de l’Enseignement Supérieur.

AUTHOR CONTRIBUTIONS

E.D., I.A.U. and N. Venticlef conceived the study and wrote the manuscript. E.D. performed part of the *in vivo* studies (human and mouse) and analyzed data. A. Toubal and F.A. performed the *in vivo* studies and data analyses, and assisted in the preparation of the manuscript. K.B., H.L.E., M.P., I.H., Y.L. and P.A. assisted with the mouse studies. S.A. and K.L. assisted with flow cytometry analyses. A.L., R.G.P.D. and C.C.-G. performed the metabolic analyses *in vivo*. E.M., N. Viguier, C.P., V.S., A. Torcivia, J.A.-W., D.L. and K.C. contributed to the human data collection, data analyses and interpretation. O.A. and K.C. performed statistical analyses in population I. F.F., S.L., J.A.-W., D.L. and K.C. interpreted and assisted in the writing of the manuscript. I.A.U. and N. Venticlef designed, analyzed and interpreted the studies.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Study populations. *Population 1.* Morbidly obese patients, candidates for bariatric surgery, were recruited in the Nutrition Division of Pitié-Salpêtrière Hospital, Institute of Cardiometabolism and Nutrition (ICAN), Paris, France. We used, as part of this study, biopsies of visceral adipose tissue (viscWAT) obtained during gastric bypass surgery (obese subjects) or during elective surgery (non-obese subjects) (**Supplementary Table 1**). In obese subjects, we evaluated body fat mass by biphotonic absorptiometry (DXA) and a series of biochemical variables were obtained after 12 h of fasting. Tissue samples were stored at -80°C until RNA extraction. The study was conducted in accordance with the Helsinki Declaration and was registered in a public trial registry (Clinicaltrials.gov; NCT01655017). The Ethics Committee of CPP Ile-de-France approved the clinical investigations for all individuals. All subjects provided written informed consent when included in the surgery program.

Population 2. Individuals scheduled to have visceral surgery (laparoscopic or laparotomic cholecystectomy, hysterectomy and gastric banding) at the Departments of Surgery and Gynecology at Kralovske Vinohrady Faculty Hospital in Prague were also included in the study. Exclusion criteria were malignancy, current inflammatory conditions as diagnosed by clinical status and laboratory findings, known endocrinopathy, chronic liver or kidney disease, psychiatric disorders, and body weight fluctuations $>2\%$ over the preceding 3 months. Each participant gave written informed consent and the study was approved by the Ethic Committee of the Third Faculty of Medicine, Charles University, Prague. According to BMI, and presence or absence of metabolic syndrome evaluated according to the International Diabetes Federation criteria, the participants were stratified into four groups: lean (LE; $n = 12$), overweight (OV; $n = 12$), obese (OB; $n = 14$) and obese with metabolic syndrome (MS; $n = 14$). Tissue samples were stored at -80°C until RNA extraction. This population has already been described^{40,41}. Requests regarding clinical studies: Karine.clement@psl.aphp.fr (population 1), Vladimir.stich@f3.cuni.cz or Dominique.langin@inserm.fr (population 2).

Animals. Male C57BL/6J *Irf5* whole-body knockout and WT littermate controls ($n = 21$) were bred at the Kennedy Institute of Rheumatology at Imperial College (London, UK) as previously described⁴². Studies were carried out according to the UK National Health and Medical Research guidelines. High-fat diets (Research Diet, 60% fat) were started at 7 weeks of age and continued for 12 weeks ($n = 5-6$ per experiment) with *ad libitum* access to water and food. The diet-induced obesity experiments were repeated three times independently.

To generate *Irf5* AdipoKO and MacKO mice, *Irf5*^{flx/flx} mice (C57BL/6-*Irf5*^{tm1Pzf}); stock no. 017311) were crossed to either LysM-Cre mice (B6.129P2-*Lyz2*^{tm1(moz)lf}); stock no. 04781) or Adiponectin-Cre mice (B6.FVB-Tg(Adipoq-Cre)1Evdrl; stock no. 010803; all from The Jackson Laboratory) for at least five generations before starting *in vivo* experiments. Male C57BL/6J *Irf5* AdipoKO or MacKO and their WT littermates were bred in the Centre Exploration Fonctionnel (CEF) at Paris University (UPMC) according to the French Research Council guidelines. High-fat diets were started at the age of 7 weeks and were continued for 12 weeks ($n = 5-8$ per experiment) with *ad libitum* access to water and food. The diet-induced obesity experiments were repeated two to three times independently. We allocated cages of mice to the experimental groups by random draw. We did blind investigators during metabolic tests.

In vivo studies. Mice were fasted overnight and tail vein blood was collected. Plasma samples were stored at -20°C until they were analyzed. Oral glucose tolerance tests (OGTTs) and insulin tolerance tests were performed as previously described⁴³. For insulin signaling assays, MacWT and *Irf5* MacKO HFD-fed mice were fasted overnight and then treated by intraperitoneal injection with 2 U/kg insulin (15 min). Tissue samples were examined by immunoblot analysis by probing with antibodies to phospho-AKT (Cell Signaling #9331S), AKT (Cell Signaling #9272S) and phospho-GSK3 (Cell Signaling #9378S).

Analysis of metabolic parameters. Blood glucose level was measured using a glucometer (Accu-Chek Performa, Roche). Plasma insulin (Crystal Chem Inc., Downers Grove, IL, USA) and adiponectin (R&D Systems) levels were

determined by ELISA. HOMA-IR in mice was calculated using the equation ((fasting glucose concentration \times fasting insulin concentration)/405) as previously reported⁴⁴. Glucose uptake of [³H]-2-deoxyglucose was assayed as described previously⁴⁵. In brief, adipose tissue explants (ingWAT and epiWAT) were preincubated in the absence or presence of 100 nmol per liter insulin and 50 mmol per liter [³H]-2-DG (NEN, Boston, USA; specific activity, 26.2 Ci/mmol). After 10 min incubation, explants were washed in ice-cold KRH buffer to stop transport and washed an additional four times in the same buffer in order to remove unbound label. Explants were blotted and then proteins were quantified to normalized β -actin. [³H] radioactivity was determined by scintillation counting using a Minaxa Scintillation counter from Packard. Basal glucose uptake was taken as 2-deoxyglucose uptake in the absence of insulin. Liver triglycerides were extracted as previously described⁴⁶ and measured with a colorimetric diagnostic kit according to the manufacturer's instructions (Randox Laboratories).

Immunohistochemical and morphometric analyses. Adipose tissue samples were fixed in formaldehyde solution and embedded in paraffin according to standard procedure. Tissue sections (7.5 μm thick) were stained with i) H&E to determine adipocyte diameters, ii) anti-human CD68 (KP1; Dako), anti-human CD11c (5D11; Novocastra) and anti-mouse/human Irf5 (ab33478; Abcam) antibodies and iii) Picrosirius red staining to quantify collagen accumulation as previously described⁴⁷. Adipocyte diameters were measured digitally in histological light-microscopy images (20 \times) of adipose tissue sections ($n = 50$ adipocytes per section, three sections per animal, five animals per group) using ImageJ software (<http://imagej.nih.gov/ij/>).

Adipose cell isolation and flow cytometry analyses. The stromal vascular fraction (SVF) containing mononuclear cells and preadipocytes was extracted from adipose tissue.

For humans, adipose tissue was digested using collagenase (Roche) as previously described⁴⁸ to obtain adipocytes and cells from the SVF. SVF cells were resuspended in endotoxin-free PBS supplemented with 2% FCS and 1 mM EDTA. Positive isolation of CD14⁺ and CD3⁺ cells from SVF cells was performed using magnetic beads (STEMCELL Technologies) according to the manufacturer's instructions. The CD14-CD3⁺ cells (Neg) were also recovered. Cells were immediately used for total RNA or protein extraction.

For mice, adipose tissue was digested using collagenase (Roche) as previously described⁴⁹ to obtain adipocytes and cells of the SVE. After 15 min incubation with Fc block, SVF cells were resuspended in fluorescence-activated cell sorter (FACS) buffer and stained with appropriate antibodies conjugated to fluorochromes or isotype controls for 30 min at 4 $^{\circ}\text{C}$ in the dark: Cd45 (30-F11; eBioscience, #47-0451-82), Cd3 (17A2; eBioscience, #11-0032-82), F4/80 (BM8; eBioscience, #25-4801-82), Cd11b (M1/70; eBioscience, #17-0112-82), Cd11c (N418; eBioscience, #48-0114-82), Cd206 (C068C2; BioLegend, #141704), MHCII (M5/114.15.2; DPharmin, #562363) and Siglec-F (BDPharmin, #552126). For intracellular staining, SVF cells were stimulated with phorbol-12-myristate-13-acetate (30 ng/ml; Sigma-Aldrich) and ionomycin (1 mg/ml; Alexis Biochemicals, San Diego, CA) at 37 $^{\circ}\text{C}$ for 6 h with GolgiStop (BD Biosciences). Cells were then stained for surface markers, permeabilized with Cytofix/Cytoperm solution (BD Biosciences), and incubated for 40 min at room temperature in the dark with anti-IL-13 (eBioscience, #25-7133-82) and anti-Gata3 (BDPharmin, #560074) antibodies. Live/Dead fixable viability dyes (Invitrogen, Paisley, UK) were used to discriminate between live and dead cells. Samples were acquired using an LSRII cytometer (Becton Dickinson) and analyzed with FACS Diva (BD Biosciences) and FlowJo (TreeStar) software programs.

Generation of monocyte-derived macrophages. Human peripheral blood mononucleated cells were isolated from enriched buffy coats of healthy volunteer donors (Etablissement Français du Sang, Hôtel-Dieu Hospital) by Ficoll density gradient centrifugation (PAA). Monocytes were purified by negative selection using the Monocyte Isolation kit II (Miltenyi Biotec). Monocytes were differentiated for 6 d in RPMI 1640 medium supplemented with 10% FCS and 100 ng/ml recombinant M-CSF (Miltenyi Biotec) to obtain human monocyte-derived macrophages (HMDMs). HMDMs were cultured for 24 h in RPMI 1640 medium



supplemented with recombinant cytokines as indicated and then processed for RNA extraction or ChIP assays.

Mouse bone marrow was isolated from femurs and tibias from *Irf5*-KO or WT control mice. Bone marrow cells were plated in RPMI 1640 medium supplemented with 10% FCS and 30% L929 conditioned medium and were allowed to differentiate for 8–10 d into bone marrow-derived monocytes (BMDMs). BMDMs were used for RNA extraction.

Adipose tissue culture. Human and mouse adipose tissue biopsies (0.1 g) were minced and incubated in 1 ml of endothelial cell basal medium (PromoCell) containing 1% bovine serum albumin, penicillin (100 U/ml), and streptomycin (100 mg/ml). Adipose tissue-conditioned medium (ATCM) were recovered after 24 h and stored at -80°C until analysis. Tissue viability was checked using the lactate dehydrogenase (LDH) assay according to manufacturer's instructions (BioVision).

Mouse epiWAT was cultured in the presence of a neutralizing antibody against TGF- β 1 (1 $\mu\text{g}/\text{ml}$) or corresponding immunoglobulin G (IgG) isotype control for 24 h (R&D Systems). At the end of the incubation period, epiWAT was frozen in liquid nitrogen, stored at -80°C and used for RNA extraction.

Analytic measurement. Cytokine concentrations from ATCM and serum were analyzed using a Milliplex Pro mouse Cytokine assay (Millipore). ATCM and serum concentrations of adiponectin and glycerol were determined using commercial kits (R&D Systems and Randox Laboratories, respectively).

Real-time PCR. Adipose tissue, freshly isolated cells from adipose tissue, and HMDMs were processed for total RNA extraction using the RNeasy RNA Mini Kit (Invitrogen). Complementary DNAs were synthesized from and prepared with SuperScript II reverse transcriptase (Invitrogen). SYBR green primers were designed by the manufacturer (Roche or Qiagen) and used for qRT-PCR using the StepOne real-time PCR system (Applied Biosystems). 18S was used for normalization and for relative quantification of gene expression.

Western blot analysis. Samples for western blot analyses were diluted to a concentration of 20 μg of protein per 15 μl and heated at 70°C for 10 min. Membranes were imaged using a LiCor Odyssey scanner. Boxes were manually placed around each band of interest, which returned near-infrared fluorescent values of raw intensity with intra-lane background subtracted using Odyssey 3.0 analytical software (LiCor). The following antibodies were used: IRF5 (ab21689, Abcam), anti- β -actin (ab8226, Abcam), phospho-AKT (#93315, Cell Signaling), AKT (#92725, Cell Signaling) and phospho-GSK3 (#93785, Cell Signaling).

Chromatin immunoprecipitation (ChIP). ChIP assays were performed as described in detail previously⁵⁰ on HMDMs stimulated with recombinant cytokines as indicated. Quantification of the precipitated DNA regions was performed by PCR. Primer sequences are listed for TGF β 1: 5'-TTGT TCCAGCCTGACTCTC-3' (forward) and 5'-TGTGGGTCACCAGA GAAAGAG-3' (reverse). The following polyclonal antibodies were used: anti-polymerase II (H224, Santa Cruz Biotechnology), IRF5 (2932, Abcam) and normal rabbit IgG (Santa Cruz Biotechnology).

Human gene expression microarray analysis. The Illumina (San Diego, CA) RNA amplification kit (NuGEN, BiotinIL Module) was used according to the manufacturer's instructions to obtain biotin-labeled cDNA from 50 ng of total RNA extracted from adipose tissue CD14⁺ immunoselected cells. Hybridization was performed onto Illumina Human HT-12 v3.0 Expression BeadChips containing 48,000 probes for 28,688 coded transcripts. ReadChips were read with an iScan Illumina scanner. Hybridization probe intensities were then detected by GenomeStudio. Among 48,000 probes, intensities were detected for 9,990 probes coding for 9,810 annotated genes in the 21 samples (Supplementary Table 1, obese subjects, population 1). We then performed a correlation analysis between *IRF5* gene expression and this set of 9,810 annotated genes enriched in omental adipose tissue macrophages (that is, visWAT CD14⁺ cells) isolated from 21 obese patients (BMI, $46.3 \pm 2.3 \text{ kg}/\text{m}^2$). The correlation was considered

significant when q -value < 0.05 . The q -value is the adjusted P value for multiple testing using an optimized false discovery rate (FDR) approach (as defined by John D. Storey's method⁵¹). q -value was provided by SAMr package in R statistical software (<http://statweb.stanford.edu/~tibs/SAM/Rdist/index.html>). Using this method, we found that 167 genes correlated to *IRF5* gene expression; 35 correlated positively and 132 negatively with this gene. These genes were further annotated using FunNet⁵² with biological annotations from Gene Ontology and KEGG databases. Gene Expression Omnibus (GEO, database repository) accession number is GSE65802.

Indirect calorimetric measurements. Mice were analyzed for whole energy expenditure (EE), oxygen consumption and carbon dioxide production, respiratory exchange rate (VCO_2/VO_2), food intake (in grams) and locomotor activity (beam breaks/hour) using calorimetric tides cages with bedding, food and water (LabMaster, TSE Systems, Bad Homburg, Germany). Ratio of gases is determined through an indirect open circuit calorimeter^{53,54}. Monitored O_2 and CO_2 concentration of each cage, in which a flow of air (0.45 liters/min) was being ventilated, were compared to a reference empty cage. Whole energy expenditure was calculated according to the Weir equation respiratory gas exchange measurements⁵⁵. The flow was previously calibrated with an O_2 and CO_2 mixture of known concentrations (Air Liquide, France). The instrument combined a set of highly sensitive feeding and drinking sensors for automated online measurements and each cage was embedded in a frame with an infrared light beam-based activity monitoring system, allowing measurement of total locomotion in both light and dark phases. Data collection was performed every 15 min. Mice were individually housed and had free access to food and water *ad libitum* with lights on from 07:00 to 19:00 h and an ambient temperature of $22.5 \pm 1^{\circ}\text{C}$. All animals were acclimated for 48 h before experimental measurements. Whole analysis was performed for 4 d and then food was removed at 12:00 h for 24 h and retrieved for 24 h.

Whole-body composition analysis. Lean tissue mass, fat mass, free water and total water content were measured using an Echo Medical systems' EchoMRI 100 (Whole Body Composition Analyzers, EchoMRI, Houston, USA). Non-anesthetized mice were weighed before being placed into a mouse holder and inserted in the MR analyzer. Readings of body composition were given within 3 min. Analysis was performed using O_2 consumed, CO_2 production (express in ml/h), and energy expenditure (kcal/h). Subsequently, each value was expressed either by total body weight or whole lean tissue mass extracted from the EchoMRI analysis. Displayed values are expressed as mean \pm s.e.m. Testing for homogeneity of variance (F -test) was performed prior to analysis. Comparisons between groups were carried out using non-parametric Mann-Whitney test (Minitab). A P value < 0.05 was considered statistically significant.

Statistical analyses. No statistical method was used to predetermine sample size. Gaussian distribution was tested using Kolmogorov-Smirnov test. Skewed data were log transformed and tested again (for the human data). The differences between groups were assessed using parametric tests such as Student's t -test (comparison of two groups) or one-way or two-way ANOVA (more than two groups) with Tukey's *post hoc* test and Bonferroni's test to correct for multiple comparison testing (after variance homogeneity hypothesis assessment using Levene tests). In contrast, when the Gaussian distribution was not fulfilled, we examined differences between groups using non-parametric Mann-Whitney test (comparison of two groups) or Kruskal-Wallis (more than two groups) test with a Dunn's *post hoc* test and a Bonferroni test to correct for multiple comparison testing. Significance code is now added in each figure legends: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ in agreement with multiple-testing correction (Bonferroni).

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DAPK2 down-regulation associates with attenuated adipocyte autophagic clearance in human obesity.

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Keywords : Adipose tissue - lipid storage- weight loss- bariatric surgery- Lipophagy-

Running title: DAPK2 down regulation in human obesity

Abstract:

Adipose tissue dysfunction in obesity has been linked to low-grade inflammation causing insulin resistance. Transcriptomics identified DAPK2 among the most strongly down-regulated adipose tissue gene in human obesity, but the role of this kinase is unknown. We show that mature adipocytes rather than the stroma-vascular cells in adipose tissue mainly expressed DAPK2, and that DAPK2 mRNA in obese patients gradually recovered after bariatric surgery-induced weight loss. DAPK2 mRNA is also down-regulated in high fat diet-induced obese mice. Adenoviral-mediated DAPK2 overexpression in 3T3-L1 adipocytes did not affect lipid droplet size or cell viability, but increased autophagic clearance in nutrient-rich conditions, dependent on protein kinase activity. Conversely, DAPK2 inhibition in human preadipocytes by siRNA decreased LC3-II accumulation rates with lysosome inhibitors. This led us to assess autophagic clearance in adipocytes freshly isolated from subcutaneous adipose tissue of obese patients. Severe reduction in autophagic flux was observed in obese adipocytes compared to controls, inversely correlated to fat cell lipids. Following bariatric surgery, adipocyte autophagic clearance partially recovered, proportional to fat cell size reduction extent. This study links adipocyte expression of an autophagy-regulating kinase, lysosome-mediated clearance and fat cell lipid accumulation, demonstrates obesity-related attenuated autophagy in adipocytes and identifies DAPK2 dependence in this regulation.

Adipose tissue function includes the deposition of nutrient-derived energy excess as lipids and the integration of energy balance signals to produce leptin and adiponectin required for metabolic homeostasis. Chronic energy balance disruption as seen in obesity leads to adipose tissue lipid overload and adaptive stress responses towards ultimate dysfunction and metabolic inflexibility (1). Large-scale transcriptomic studies of adipose tissue from obese patients or obese rodent models pointed to inflammatory pathways as a prominent response to lipid overload (2-4). In obesity, inflammation results from tissue remodeling by immune cell infiltration/proliferation within the adipose fat depot (5). Indeed, whereas adipocyte is the predominant cell type in healthy lean adipose tissue, immune cells can equal or even exceed fat cell number in obesity. Metabolic inflammation is now recognized a key factor in obesity-related insulin resistance, and the subject of current investigations of immune cell subpopulations (6) towards novel therapeutic approaches.

Besides identification of expression networks and associated biological functions, transcriptomic approaches also bring attention to single genes unrelated to annotated pathways. In the present study, we focus on DAPK2 (Death Associated Protein Kinase 2, also called DRP-1), which hits among the most down-regulated genes of adipose tissue transcriptome in human morbid obesity (7). DAPK2 belongs to a family of Ser/Thr calmodulin-regulated protein kinases which consists of five members, all displaying high sequence homology in their N-terminal kinase domains, while C-terminal regions highly diverge. DAPK1, the founding family member, is involved in apoptosis (8) and suppressed in many tumors (9). Unlike DAPK1, DAPK2 and other DAPKs are devoid of a C-terminal death domain, ankyrin repeats and cytoskeleton-interacting region (9). Therefore, DAPK2 is a small protein which only contains a kinase domain, a calmodulin binding region and a short tail with undefined structural motifs. DAPK2 is ubiquitously expressed, but its physiological functions are still largely unknown. Interestingly, large-scale RNA interference kinome screens identified DAPK2, suggesting a role in autophagy (10), secretory pathway (11), or TGF β signaling by protein-protein interaction (12).

In the present study we investigated the significance of DAPK2 down-regulation in human obesity. We first identified adipocytes, and not other non-lipid filled adipose tissue cells as targets of DAPK2 down regulation. Then, the effects of DAPK2 gain/loss of function in cultured adipose cells revealed a regulatory role of the kinase in lysosome-mediated remodeling, which led us to further investigate adipocyte autophagic clearance in human obesity and weight loss. We demonstrated attenuation of adipocyte autophagic activity in human obesity, partially reversible after weight loss following bariatric surgery. Thus these results link DAPK2 down-regulation and defective autophagy to fat cell dysfunction in human obesity.

Research design and methods

Human adipose tissue samples:

All subjects gave their informed consent in an accepted protocol related to the physiopathology of obesity (Assistance Publique/Hôpitaux de Paris, Clinical Research Contract). Clinical investigations were performed according to the Declaration of Helsinki and approved by local ethics committees. Middle-aged obese subjects candidates for bariatric surgery were not on a restrictive diet and their weights were reported stable. All subjects met usual eligibility criteria for bariatric surgery. Follow up of patients after gastric bypass included monitoring of weight loss and subcutaneous adipose tissue biopsies from the abdominal region. All tissue samples were processed immediately for adipocyte isolation or frozen for mRNA extraction.

Controls were non-obese subjects undergoing plastic surgery or programmed surgery for gall bladder ablation or hernia. None had diabetes or metabolic disorders and were taking medication. Main characteristics of patient groups are summarized in Table 1.

Adipocyte isolation: Floating adipocytes were obtained by collagenase digestion essentially as described by Rodbell (13) except that DMEM was used instead of Krebs-Ringer buffer. Floating adipocytes were separated from stromavascular cell fraction by aspirating the infranatant, and rinsed 3 times with DMEM. Minor contamination of the adipocyte fraction by stromavascular cells was checked on morphological images of adipocyte suspensions used for fat cell size determination.

Cell culture: 3T3-L1 cells were maintained and differentiated in standard conditions. Adenovirus were obtained by recombination of viral backbones with shuttle vectors containing DAPK2 cDNAs and packaging in complementing 293 cells as described previously (14). GFP-LC3 3T3-L1 stable transfectants were obtained as described (15). Human preadipocytes were isolated from subcutaneous adipose tissue, cultured as described (16). siRNA-mediated knockdown was performed with 20 nM siRNAs using lipofectamine RNAi MAX (Invitrogen) according to the manufacturer's instruction. DAPK2 siRNA sequence was from Abgent (5'UGUCUGGAGGAGAGCUCUUTTAAGAGCUCUCCUCCAGACATT3'). Scrambled siRNA were used as controls. 16h post-transfection, fresh medium supplemented with antibiotics and 10% SVF was added and cells were incubated for further 24h.

Mice studies: Male mice were studied. ob/ob and ob/+ were maintained on a normal chow diet with ad libitum feeding and sacrificed at 12-16 weeks of age (body weights 30.1+/-3.1 vs 58.2+/-3.4g). C57/B16J male mice were fed a high fat diet (60% calories from fat) for 16 weeks from weaning onwards and sacrificed (body weights 37.9+/-1.8 vs 48.2+/-3.5g).

mRNA quantification: mRNAs were reversed transcribed and cDNA were quantified by real time RT-PCR using qPCR MasterMix Plus for SYBER Green (Eurogentec). PCR efficiency of primer pairs was checked in standard curves, and expression data were expressed using $\Delta\Delta C_t$ method. 18S RNA was used for normalization. Sequence of primer pairs for DAPKs were as followed. human DAPK2 : 5' - ACGTGGTGCTCATCCTTGA -3' and 5' - TGGCCTCCTCCTCACTCA - 3' . Mouse DAPK2 : 5'- GACGTGGTGCTCATCCTTG -3' and 5'-GGCTTCCTCCTCACTTAACGA- 3'. Mouse DAPK1: 5'- CCTGATTCCAGGACAAGG-3' and 5'-CTTAGCCACGGAGTAATCAGCC-3'. Mouse DAPK3: 5'- ATTTGTACCGGAGGTTCTCG-3' and 5'- TCTGAAGGATTCTGGGGACA-3'. A complete list of other primer sequences is provided in supplemental material.

Autophagic flux measurement: Experimental design followed state of the art guidelines (17). Freshly isolated floating adipocytes or differentiated cells lines were rinsed with high glucose DMEM and incubated for 2 hours in the presence of either 100nM Bafilomycin A1, 100 μ M leupeptin/20mM NH₄Cl or 25 μ M Chloroquine at 37°C in a humidified 95% air/5% CO₂ atmosphere. Cells were collected in lysis buffer (50mM Tris, pH 7.4, 0,27M sucrose, 1mM Na-orthovanadate, 1mM EDTA, 1mM EGTA, 10mM Na β -glycerophosphate, 50mM NaF, 5mM Na pyrophosphate, 1% (w/v) Triton X-100, 0.1% (v/v) 2-mercapto ethanol supplemented with CompleteTM protease inhibitors and stored frozen at -20°C before further processing.

Western blotting: Cell lysates were processed as previously described (18). Commercial antibodies against LC3, Akt and β -actin were from Cell Signaling, DAPK2 was from Pro-Sci and p62 from Progen.

Lipolysis: Lipolytic rates were assessed in cells maintained for 2 hours in phenol-red free medium containing 2%BSA with or without 10⁻⁹M isoproterenol for lipolytic stimulation. Glycerol release was measured (Glycerol colorimetric assay kit, Cayman, USA) and normalized to cell protein content.

Cell imaging: 3T3-L1 stable transfectants expressing GFP-LC3 were differentiated and processed for fluorescent imaging as previously described (19). Intracellular lipid droplets and floating adipocytes were sized using Perfect image (ClaraVision) from phase contrast images. Immunofluorescence on paraffin-embedded adipose tissue sections was performed as described (20).

Statistical analysis: Results are expressed as mean \pm s.e.m. Mann-Whitney test was used in all analysis except for paired data which were tested with Wilcoxon or paired t test. Spearman analysis was used for correlations.

Results

Down-regulation of adipocyte DAPK2 expression in human obesity.

Our attention on DAPK2 was brought by pangenomic transcriptome analysis comparing adipose tissue gene expression in lean and obese subjects (7). We confirmed a 3-fold decrease in DAPK2 mRNA (fig1A) in subcutaneous adipose tissue samples from 65 severely obese ($34 < \text{BMI} < 79 \text{ kg/m}^2$) compared to 10 non-obese subjects ($20 < \text{BMI} < 23 \text{ kg/m}^2$), not influenced by type2 diabetes (fig 1B) or by sex (not shown). Low DAPK2 mRNA expression was also independently confirmed in an subgroup of 24 severely obese compared with 10 overweight ($24 < \text{BMI} < 28 \text{ kg/m}^2$) subjects (fig1C), pointing to DAPK2 down-regulation as a feature of severe obesity but not of simple overweight. Relative DAPK2 mRNA levels in obese subjects positively correlated with HDL cholesterol and inversely associated with triglyceridemia, and serum IL6. A trend for negative association to subcutaneous fat cell size ($p=0.057$) was also observed (Table 2). Since bariatric surgery (i.e. gastric bypass) is the most efficient intervention to induce weight loss in morbid obesity, we examined DAPK2 mRNA in 10 patients over time at 0, 3, 6, and 12 months post- surgery. Gradual time-dependent recovery, almost complete after 12 months (fig1D) indicated that DAPK2 loss was not irreversible in obese adipose tissue. We also found decreased DAPK2 mRNA in subcutaneous adipose tissue of mice with diet-induced obesity, indicating common obesity-related DAPK2 regulation in humans and rodents (fig1E). Adipose tissue digestion by collagenase was performed to determine relative DAPK2 mRNA expression in the isolated adipocyte and the non-lipid filled stroma-vascular cell compartment of human fat tissue. In non-obese as well as in obese subjects DAPK2 mRNA was more abundant in adipocytes than in stroma-vascular cells (fig1F), and major reduction with obesity was found in the adipocyte fraction (fig 1F). Thus adipocyte-enriched DAPK2 expression is suppressed in human obesity, and reversibly recovers after bariatric surgery-induced weight loss in humans. Immunofluorescence labelling on adipose tissue sections confirmed adipocyte DAPK2 suppression in obese patients, at the protein level (Fig 1G).

DAPK2 modulates lysosome-mediated remodeling and constitutive autophagy in adipocytes.

The bulk of human fat cells is renewed every ten years, consistent with function in long-term energy storage, and adipocyte is considered a long-lived cell type (21). In this context, the conserved process of intracellular organelle remodeling by lysosomes i.e. autophagy, is crucial to maintain homeostasis (22), especially in oxydative-proned conditions linked to lipid-rich adipocyte environment. Because DAPK2 was suggested a potential autophagy regulator (10;23;24), we focused on constitutive autophagy in the presence of nutrients. We generated 3T3-L1 adipocytes stably expressing GFP-LC3 which retained full differentiation capabilities, as judged by presence of large multilocular cytoplasmic lipid droplets. Fluorescent LC3-GFP distribution identified autophagosome vesicles, visualized as punctuated labelled structures (fig2A), also detectable as LC3-II fast-migrating bands on western blots (fig2B) which accumulated in the presence of lysosomal inhibitors such as chloroquine,

leupeptin/NH₄Cl or bafilomycin A1, indicating ongoing autophagic clearance. As expected, nutrient withdrawal from culture medium increased autophagy flux (fig2C) in agreement with well-known induction of autophagy by cell starvation. In some cell types such as hepatocytes (25) or macrophages (26), intracellular lipid droplet organelles can end up in lysosomes for lipid degradation through so called lipophagy. Lipophagy is poorly documented in fat cells, but the importance of cytoplasmic neutral lipases activation for lipid droplet degradation is clearly established (27). We observed no increase in autophagic flux during acute lipolytic stimulation of 3T3-L1 adipocytes with the β -adrenergic agonist isoproterenol (fig2D), and lysosomal inhibitors only marginally affected lipid mobilization as judged by cell glycerol release into the medium (fig2E). Thus, autophagic clearance and lipid store mobilization by catecholamines are distinct processes in adipocytes.

DAPK2 mRNA expression in terminally differentiated 3T3-L1 adipocytes was 2 orders of magnitude lower than the two other related DAPkinases (fig3A) pointing to 3T3-L1 model as a suitable system to explore the impact of exogenous DAPK2. Adenoviral based expression of DAPK2 (Ad-DAPK2) or a kinase-dead mutant version (Ad-DAPK2-K52A) co-expressed with an eGFP reporter indicated high proportion of transduced fat cells after 24h (not shown). Compared to cells transduced with a null virus, DAPK2 expression did not produce overt metabolic effects. Cells continued to respond to acute insulin stimulation by massive Akt phosphorylation (fig3B) and still contained lipid droplets with normal size distribution (fig3C). Metabolic (leptin, adiponectin, fatty acid synthase or perilipin1) and inflammatory (IL6, CCL2) gene expression was also unaltered (fig3D). Furthermore, we observed no sign of cell leakage by cytoplasmic LDH release (fig3E) indicating unaffected cell viability. Considering a trend (+20%) towards increased ability to release glycerol upon adrenergic lipolytic stimulation (fig3F), DAPK2 expressing adipocytes showed no evidence for dysfunction or death. Adipocyte clearance by autophagy, i.e. autophagic flux, as measured by LC3-II accumulation in the presence of lysosomal inhibitors, was higher in DAPK2 expressing cells than in cells transduced with viral backbone alone (Fig3G). Furthermore, DAPK2-mediated stimulation of autophagic clearance was abolished with a kinase-dead mutant version, indicating dependence upon kinase activity for autophagy modulation (Fig3H). We next evaluated the impact of DAPK2 inhibition by siRNA in human preadipocytes obtained from adipose tissue stroma-vascular fraction. siRNA sequences decreased DAPK2 protein by half (fig4A), increased cell contents of the p62 autophagic substrate (fig4B), and decreased autophagosome accumulation with lysosome inhibitors, indicating reduced autophagic flux (fig4C). All together these data on gain or loss of function demonstrate that modulation of DAPK2 expression in adipose cells associates with basal autophagic tone regulation.

Adipocyte autophagic clearance is impaired in obesity and partially reversed after weight loss.

Above results suggest that down-regulated adipocyte DAPK2 might be linked to attenuation of autophagic clearance in obesity. We therefore prepared isolated adipocyte cell fractions from obese and lean subcutaneous fat biopsies and evaluated steady state levels of the autophagic substrate p62 which were elevated 2-fold in obese versus lean adipocytes (fig5A-B) indicative of lower autophagic degradation. Nevertheless, p62 mRNA was also higher in obese adipocytes compared to controls (fig5C) precluding any definite conclusion on autophagic flux based on p62 protein content. We next set up an experiment in which freshly isolated adipocytes were incubated in a standard nutrient-rich medium in presence or absence of lysosome inhibitors to evaluate rates of autophagosome accumulation i.e. adipocyte autophagic flux. LC3-II accumulation in the presence of lysosome inhibitors was obvious in non-obese adipocytes, but only barely detected in obese fat cells. (fig5D). Quantitative analysis of LC3-II accumulation rates demonstrated reduced adipocyte

autophagic clearance in obesity (fig5E). Furthermore, adipocyte autophagic flux inversely correlated with fat cell size (Fig5F), which is primarily determined by lipid amounts within the unilocular lipid droplet. Thus, attenuated autophagic flux in obesity is linked with adipocyte lipid overload. Similarly, adipocytes isolated from obese (ob/ob) mice also exhibited lower autophagic activity than non-obese (ob/+) controls (fig5G-H), indicating common reduction in obese rodents and humans. Reversibility of autophagy attenuation was investigated by assessing adipocyte preparations obtained from obese patients after bariatric surgery-induced weight loss. In 9 patients, adipose tissue samples were obtained pre- and post-surgery in one occasion within the 3 to 12 months following intervention. All patients lost weight and reduced subcutaneous adipocyte cell size and lipid contents (fig6A), although at different degrees, because of the large time frame in post-surgery sample collection. Interestingly, total adipocyte LC3 protein content pre- vs. post- surgery changed proportionally to the extent of adipocyte size reduction (fig6B). Pre- versus post-surgery comparisons indicated ameliorated adipocyte autophagic clearance in all patients (fig6C) and significant recovery, regardless the use of chloroquine or leupeptin to evaluate autophagic flux (fig6D-E). Thus, adipocyte lysosome-mediated remodeling is compromised in obesity, and partial recovery can be obtained by weight loss.

Discussion

Increasing evidence points to critical role of autophagy in metabolic diseases, linked to adaptive response to chronic metabolic stress (28). In almost every tissue participating in energy homeostasis (liver, pancreas, muscle, and even hypothalamus) inactivation of autophagy by tissue specific gene knockout was found associated with metabolic dysfunction (25;29-33). However, adipocyte responses are still poorly understood because ATGs inactivation alters normal fat tissue differentiation (34;35). Our study brings new evidence that autophagy attenuation associates with adipocyte dysfunction in obesity. Interestingly, previous reports showed autophagy activation in adipose tissue in human or mice obesity (36-38). Noteworthy, the study of total adipose tissue takes account of obesity-associated inflammatory cells, in which autophagy is linked to immune function, so that it remains unclear from these reports in which cell type autophagy is modulated. Here, specific focus on adipocytes and direct measurement of autophagosome clearance show that obesity is associated with down-regulation of adipocyte autophagic turnover, partially recovering after bypass surgery-mediated weight loss. Our data are in line with a negative role of lipids on autophagosome dynamics (39;40), and with high fat diet-induced autophagy defects previously demonstrated in the liver (41;42), kidney (43) and hypothalamus (44).

Autophagic degradation of lipid droplets also called lipophagy can decrease intracellular lipid burden in many cell types, but is shown here to only marginally modulate *in vitro* adipocyte lipolysis. This is in good agreement with prominent role of neutral cytoplasmic lipases in fat cell lipid mobilization (27) and suggests that autophagy might control other adipocyte phenotypes. Indeed, autophagy inhibition potentially induces cell inflammation (45) including fat cell inflammation (37;46). Furthermore, mice with systemic haplo-insufficiency for the Atg7 autophagy gene overproduce ROS in adipose tissue when obese (47). Thus, we favor the possibility that autophagy serves in the long-term to dampen adipocyte inflammation linked to metabolic dysfunctions.

The present study links obesity-related down-regulation of a kinase, DAPK2, with attenuation of adipocyte autophagy. DAPK2 was reported pro-apoptotic when transiently overexpressed (48;49). In the context of adipose cells lines with low endogenous expression, we found no evidence for cell death induction after adenoviral-mediated DAPK2 protein expression. Furthermore, by retroviral-mediated gene transfer, we could obtain viable 3T3-L1 adipocyte clones stably expressing wild type

DAPK2, but not the constitutively active form. Thus, we believe that DAPK2 is not pro-apoptotic in the adipocyte cell environment, likely because of fine tuning of kinase activation by appropriate mechanisms. Our present data rather indicate that DAPK2 is linked to constitutive autophagy, and provide evidence that kinase activity is required for this regulation. Molecular mechanisms by which DAPK2 sustains autophagic clearance are not yet elucidated, but might involve the targeting of autophagy proteins still to be defined. Recently, several DAPK2 interacting partners or substrates have been identified in the autophagy protein network, including inhibitory interaction with 14-3-3, interference with mTOR complex inhibition or participation in the beclin interactome.(50); (51) Interestingly, DAPK2 silencing was also found to impact TRAIL signaling and NF-KB activation (52), reinforcing connections to cell inflammation. Clearly, more understanding is needed before a unified view on DAPK2 molecular action emerge but, in the context of obesity-related meta-inflammation, our present data identify the loss of DAPK2 expression and establish a link to attenuated autophagic clearance of adipocytes, revealing a potential novel actor in metabolic diseases.

Acknowledgments

We thank A. Kimchi (Weizmann Institute, Israel) for providing bacterial clones containing human DAPK2 cDNA sequences and K52A kinase-dead mutant version, Xavier Le Liepvre and Françoise Lasnier (INSERM, Paris) for virus construction, and Joan Tordjman for providing paraffin-embedded adipose tissue sections. Financial support from CORDDIM (Ile de France region) to HS is acknowledged. HS, SR, RA, CP, CR, MP, SM performed experiments. HS, KC, ID designed study and wrote the manuscript.

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Figure legends

Figure 1: Down-regulation of adipocyte DAPK2 expression in obesity.

A: DAPK2 mRNA (relative to 18S) was quantified by RT-PCR in subcutaneous adipose tissue of 10 non-obese healthy subjects (mean BMI 23) and 65 massively obese patients (BMI range 34-79).

B: Stratification of the obese group for diabetes (33 subjects among 65). C: DAPK2 mRNA in a subgroup of subjects with overweight (n=10, -BMI range 25-28) or with massive obesity (n=24).

D: Time course of DAPK2 mRNA recovery after obesity treatment by gastric bypass. 9 patients undergoing surgery were followed overtime and adipose tissue biopsies collected. Significant changes pre versus post surgery were assessed by Wilcoxon test.

E: DAPK2 mRNA expression in inguinal adipose tissue of mice fed a control chow diet or high fat diet for 16 weeks. Bars are mean +/- sem values from 5 individual mice.

F: Floating adipocyte fraction was separated from stroma-vascular cell fraction (SVF) by collagenase digestion of adipose tissue biopsies obtained from 9 lean and 10 obese subjects. DAPK2 expression (relative to 18S) was determined in paired cell fractions by quantitative RT-PCR. Statistically significant differences between groups are indicated (** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$).

G: DAPK2 immunolabeling of subcutaneous adipose tissue sections. DAPK2 fluorescence intensity was quantified using Image J, and background values obtained by omitting primary antibody were subtracted. A total of 50 adipose tissue fields from 3 lean and 3 obese donors was quantified.

Figure 2: Constitutive lysosome-mediated autophagy in cultured adipocytes.

A: Terminally differentiated 3T3-L1 stably expressing LC3-GFP. Growing 3T3-L1 cells were transfected with a retroviral pBabe construct expressing LC3-GFP and stably expressing cells were selected with puromycin. Differentiation was induced under standard conditions. Terminally differentiated cells (day12) were left untreated (basal) or incubated for 2 hours with indicated lysosome inhibitors chloroquine (CLQ), leupeptin/ NH_4Cl (LEU), bafilomycin A1 (BAF) in serum containing medium. Confocal Fluorescent images are shown after fixation and DAPI staining of nuclei. Circular black areas in cells are lipid droplets (LD). B: Terminally differentiated 3T3-L1 were treated or not with lysosomal inhibitors as in A and lysed for LC3 analysis by western blotting. Bars represent inhibitor-induced accumulation of LC3-II/actin relative to medium with no inhibitor. Values are mean +/- sem from 10 independent experiments. C: 3T3-L1 adipocytes were incubated for one hour with Hanks Balanced Salt Solution (starvation) or complete medium. D-E: 3T3-L1 adipocytes lipolytic stimulation by isoproterenol (10^{-6}M , 2h) in the presence of lysosomal inhibitors. Values are mean +/-sem in 6 experiments. D: LC3-II accumulation as in C. E: Glycerol release into the medium was expressed as nmol/mg cell protein. Values obtained in isoproterenol stimulated cells with no inhibitor were set to 1.

Figure 3: DAPK2 exogenous expression increases adipocyte autophagic clearance in 3T3-L1.

A: Relative expression of the three DAPKinases in terminally differentiated 3T3-L1 adipocytes. Expression is normalized to 18S RNA. At least 3 independent mRNA preparations were used for quantification. B: Insulin response in Ad-DAPK2 expressing 3T3-L1 cells. Terminally differentiated cells were transduced with indicated adenoviruses and left in standard medium for 24 h (in the absence of insulin). Stimulation with the hormone was for 15 min with 100nM in DMEM. DAPK2 and

Akt were assessed on separate membranes using lysates from same cell batches. phosphorylation of Akt (on indicated amino acid residues) was probed with phospho-specific antibodies. C: 24 h after virus transduction, terminally differentiated cells were imaged by phase contrast to measure lipid droplet size using PerfectImage Software. 4 images from random fields were quantified, representing a total number of >500 individual lipid droplets in each group. Lipid droplet size distributions in a representative experiment are shown. D: Differentiated 3T3-L1 cells were transduced with adenovirus and used for mRNA extraction after 24h. Indicated mRNA were measured by RT-PCR, normalized to 18S mRNA and expressed relative to control group. RNA was obtained from at least 3 independent experiments. E: LDH release into the medium was assessed 24h after virus transduction. A representative experiment out of 2 is shown. F: Lipolytic activity of terminally differentiated 3T3-L1, 24h after transduction with indicated viruses. Glycerol release into the medium was measured and normalized to cell protein content. Values for maximal isoproterenol stimulation are set to 1, bars are means from 2 independent experiments. G: . Cells expressing DAPK2 or a kinase-dead mutant version were incubated in complete medium for 2 hours in the presence or absence of lysosomal inhibitors and lysed for western blot analysis. H: Autophagic flux quantification. (LC3-II/actin accumulation in the presence (versus absence) of lysosome inhibitors) is calculated from 5 independent experiments. * indicates statistically significant difference $p < 0.05$.

Figure 4: siRNA-mediated DAPK2 inhibition reduces autophagic flux.

Effect of DAPK2 inhibition by siRNA on endogenous DAPK2 (A) and p62 (B) content. C: cells were incubated in complete serum-supplemented medium for 2 hours in the presence or absence of lysosome inhibitors and lysed for western blot analysis. D: Bars represent quantification of LC3-II accumulation with lysosome inhibitors (autophagic flux) as mean values \pm s.e.m of 3 independent experiments with different cell donors in which LC3-II/ actin signals with no inhibitor were arbitrarily set to 1. Significant difference ($p < 0.05$) is indicated *.

Figure 5: Decreased adipocyte autophagic clearance in human and rodent obesity.

A: Freshly isolated adipocytes obtained by collagenase digestion of 24 obese patients and 12 non-obese controls were lysed and proteins (15 μ g) analyzed by western blotting. B: A representative blot is shown with quantitative analysis of p62. C: p62 mRNA quantification by RT-PCR, performed on adipocyte RNAs preparation (10 non-obese and 14 obese patients). Values were normalized to 18S. Bars are means \pm sem of signal intensity relative to non-obese controls values set to 1. D-E: Autophagic flux in adipocyte freshly obtained from adipose tissue biopsies as in A. Adipocytes were incubated in DMEM medium for 2 hours with or without lysosomal inhibitors as indicated (left panel :chloroquine, middle: leupeptin/ NH_4Cl , right: bafilomycin A1). Each point represents inhibitor-induced LC3-II accumulation relative to LC3 content with no inhibitor. F: Spearman correlation between autophagic flux and adipocyte diameter.

G-H: LC3-II/actin signals in adipocytes isolated from lean (ob/+) or obese (ob/ob) mice epididymal adipose tissue, incubated with or without lysosome inhibitors. Representative blots from individual mice of each genotype are shown, a total of 6 mice in each group were studied. H: Bars represent quantifications of autophagic flux i.e. LC3-II/actin accumulation in the presence (versus absence) of lysosome inhibitor as mean values \pm sem, *** indicate significant difference ($p < 0.001$).

Figure 6: Recovery of autophagy by weight loss after bariatric surgery.

Adipose tissue needle biopsies were obtained from 9 obese patients pre-and post-surgery (once after intervention, within 3 to 12 months). Adipocytes were isolated from biopsies and used to determine adipocyte size (A). B: Spearman correlation between change in LC3 expression and fat cell size reduction post surgery. C-D: Changes in adipocyte autophagic flux after surgery. Data obtained in individual cell preparations irrespective of lysosomal inhibitor are plotted in C. Mean values +/- sem of pre versus post surgery autophagic flux are shown in D.

Table 1: Description of patients

	Lean	Overweight	Obese	Obese (for surgery follow up)
Number of subjects	22	10	65	9
Female/male	20/2	4/6	38/27	9/0
BMI (kg/m ²)	23,6 ± 3,2	26,35 ± 0,19	47,56 ± 0,04	42,55 ± 3,14
Age (years)	38 ± 11	43 ± 2	47 ± 0,05	33 ± 8
Subcutaneous fat cell diameter (µm)	87,4 ± 12	-	121,16 ± 0,04	119,9 ± 3,8

Table 2: Association of adipose tissue DAPK2 mRNA expression with clinical parameters in obese patients.

	Spearman coefficient	P value
Pre-surgery evaluation		
Triglyceridemia	-0,36216	0,0056
Serum Interleukin 6	-0,28225	0,0448
Subcutaneous fat cell surface	-0,24069	0,0574
HDL cholesterol	0,35756	0,0063
12 months post-surgery evaluation		
Glycemia	-0,69007	0,0397

Impact de l'inflammation sur les altérations métaboliques associées à l'obésité: dialogues entre le tissu adipeux et l'intestin

Le tissu adipeux blanc (TA) joue un rôle central dans la physiopathologie de l'obésité et la résistance à l'insuline. Durant ma thèse, j'ai étudié la réponse cellulaire et moléculaire du TA au stress métabolique induit chez la souris par 1) l'administration d'un isomère de l'acide linoléique (CLA) et 2) un régime riche en graisses (HFD) associé à l'administration de probiotiques. Dans l'étude 1, nous avons utilisé l'isomère *trans10,cis12* de l'acide linoléique (t10,c12-CLA) pour promouvoir un syndrome lipoatrophique chez la souris C57BL/6J. La dynamique de cette réponse a été explorée dans le TA, l'intestin et au niveau systémique, sur deux périodes consécutives de 7 jours d'administration de t10,c12-CLA puis d'interruption du traitement. Nos résultats montrent que t10,c12-CLA induit une dérégulation des gènes métaboliques et un dépôt de matrice extracellulaire (ECM) dans le TA. Le profil immunologique est perturbé, avec l'accumulation de macrophages M2 anti-inflammatoires. Ces altérations métaboliques, immunitaires et structurelles sont inversées, mais pas toujours normalisées, après 7 jours de récupération. La réversibilité des modifications observées dans le TA indique que cette réponse est plus adaptative que pathologique. Cependant, les altérations induites par t10, c12-CLA dans le TA sont temporellement liées à l'hyperinsulinémie, ce qui renforce l'idée que l'intégrité du TA est cruciale pour l'homéostasie glucidique. Bien qu'aucune réponse immunitaire n'ait été observée dans l'intestin, l'expression des gènes codant pour des protéines de jonctions serrées (claudine et occludine) est diminuée. Une partie des effets de t10,c12-CLA pourrait être relayée par l'augmentation de la perméabilité intestinale. L'étude 2 a été conçue sur la base de l'hypothèse que la modification de la microflore intestinale *via* l'administration quotidienne de probiotiques pourrait alléger altérations métaboliques associées à un régime obésogène chez la souris. L'effet de 2 souches de probiotique a été évalué sur le poids corporel, l'adiposité, la glycémie, la sensibilité à l'insuline, la tolérance au glucose et sur des variables métaboliques. Nos résultats montrent qu'une des souches a généré un phénotype défavorable, avec gain de poids et augmentation de la masse grasse, en association avec une élévation de la leptine circulante et des triglycérides du foie et une tendance à la résistance à l'insuline. Aucune des deux souches n'a pu atténuer l'expansion du TA ni la détérioration glycémique induite par le régime gras. Ces études montrent que, en réponse à un stress nutritionnel de courte durée, le TA regagne rapidement son homéostasie. Lorsque la contrainte est plus chronique, comme dans l'obésité, la plupart des interventions, y compris l'administration de probiotiques, ne parviennent pas à inverser l'état dysfonctionnel du TA. Le grand défi dans les années à venir reste de trouver des stratégies thérapeutiques à long terme dans les maladies métaboliques.

Mots clés: Tissu adipeux, Intestin, Macrophages, Fibrose, Métabolisme, Isomères conjugués de l'acide linoléique

Title: Impact of inflammation on the metabolic changes associated with obesity : dialogues between adipose tissue and the intestine.

White adipose tissue (WAT) plays a central role in the physiopathology of obesity and insulin resistance. During my PhD, I studied the cellular and molecular response of WAT to metabolic stress induced in mice by 1) administration of an isomer of linoleic acid (CLA) and 2) a high fat diet (HFD) associated with specific probiotics. In Study I, we used *trans10,cis12* isomer of linoleic acid (t10,c12-CLA) as a tool to promote a lipoatrophic syndrome in C57BL/6J lean female mice. The short-term dynamics of this response was explored in WAT, gut and at systemic level, over two consecutive 7-day periods of t10,c12-CLA administration and withdrawal. Our results show that t10,C12-CLA-induced alterations in WAT included metabolic gene deregulation and extracellular matrix deposition. The immunological profile of WAT was markedly disturbed, with anti-inflammatory M2-polarized macrophage accumulation. Metabolic, immune and structural alteration were fully reversed, but not always normalized after 7 days without treatment. The reversibility of WAT alterations indicates a more adaptive than pathological response to t10,C12-CLA. However, t10,c12-CLA-induced WAT deregulation is temporally linked to hyperinsulinemia supporting the primacy of WAT integrity for glucose homeostasis. No immune alteration was observed in response to t10,C12-CLA in the gut, where genes encoding tight junction proteins (claudin and occludin) were down regulated. Thus, part of the effects of t10,c12-CLA could be mediated by increased intestinal permeability. Study II was designed based on the hypothesis that altering gut microflora through daily administration of probiotic in C57BL/6J obese mice could alleviate metabolic alterations. The respective effects of two specific strains of probiotic were evaluated over time on body weight, adiposity, blood glucose, insulin sensitivity and glucose tolerance and after sacrifice on circulating metabolic variables. Our results show that one strain over two promoted an unfavorable phenotype, with increased weight gain and fat mass, in association with elevated circulating leptin and liver triglycerides. Moreover, a trend toward systemic insulin resistance was found. None of the two strains tested was able to attenuate WAT expansion and glycemic deterioration induced by HFD. These studies show that in response to an acute nutritional stressor, WAT and glucose homeostasis could be rapidly regained. When the stress is more chronic, as in obesity, most of the interventions, including probiotic supplementation, fail to reverse WAT dysfunctional state, underlining the big challenge in the years ahead of finding long-term therapeutic strategies in metabolic diseases.

Key words : Adipose tissue, Intestin, Macophages, Fibrosis, Metabolism, Conjugated linoleic acids