



**HAL**  
open science

# Obésité: inflammation du tissu adipeux HMGB1, une nouvelle adipokine ?

Manojkumar Gunasekaran

## ► To cite this version:

Manojkumar Gunasekaran. Obésité: inflammation du tissu adipeux HMGB1, une nouvelle adipokine?. Endocrinologie et métabolisme. Université de la Réunion, 2013. Français. NNT: 2013LARE0040 . tel-04093734

**HAL Id: tel-04093734**

**<https://theses.hal.science/tel-04093734>**

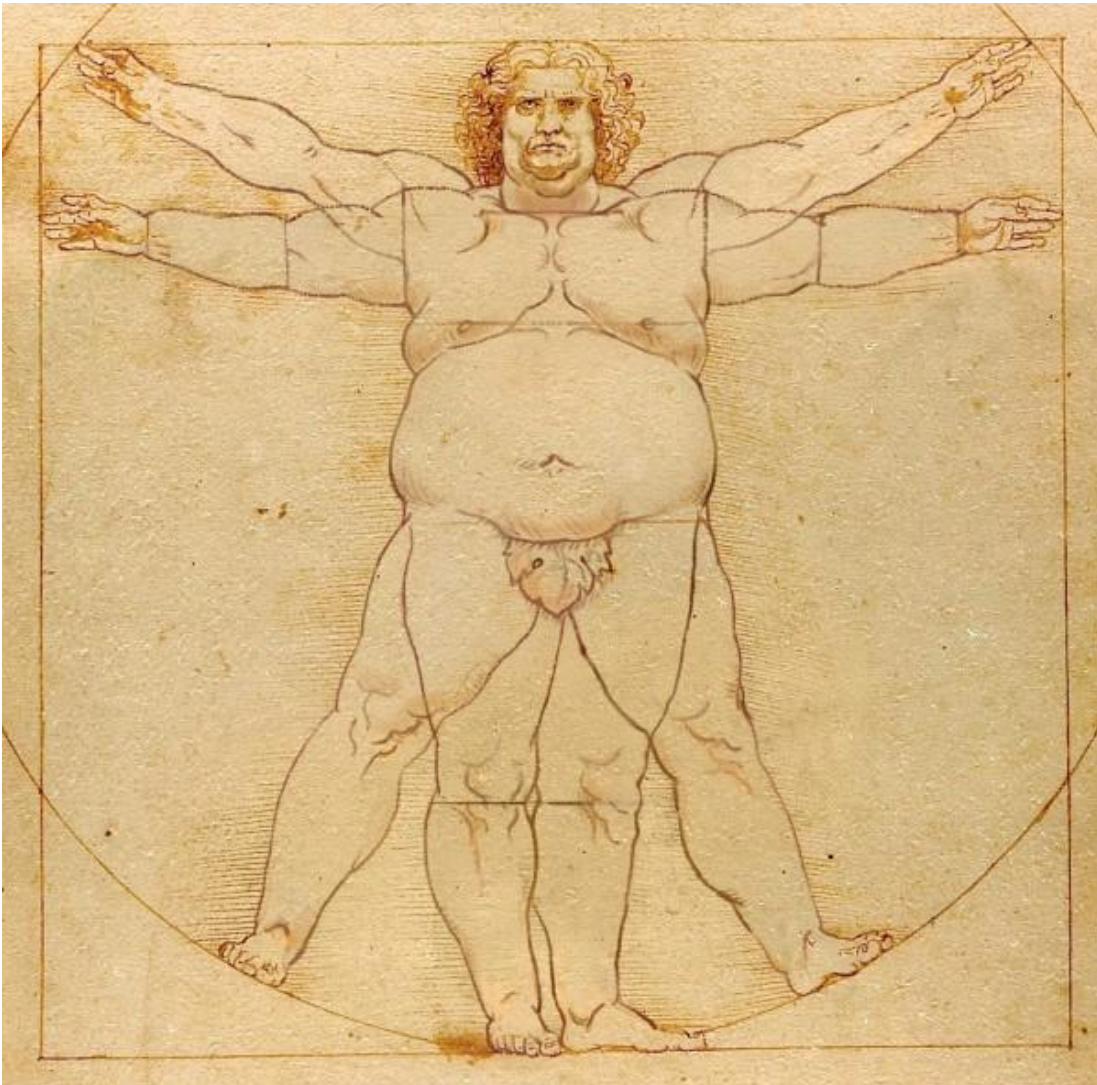
Submitted on 10 May 2023

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

# **OBESITY**

## **ADIPOSE TISSUE INFLAMMATION**



**As a matter of fat, HMGB1 is an adipokine**

**Manoj kumar Gunasekaran**

La Réunion, 2013



*To my parents,*





## Thèse

Soutenue à l'Université de La Réunion pour obtenir le grade de Docteur en Sciences

Discipline : Biologie cellulaire et moléculaire

par

**Manoj Kumar GUNASEKARAN**

**Obésité : Inflammation du tissu adipeux**

**HMGB1, une nouvelle adipokine?**

**30 Octobre 2013**

<b>Pr. Ulf ANDERSSON</b>	Unité de Rhumatologie, institut Karolinska, (Stockholm, Suède)	Rapporteur
<b>Dr Isabelle DUGAIL</b>	DR UMRS872 - INSERM (Paris, France)	Rapporteur
<b>Pr. Theeshan BAHORUN</b>	Centre de recherche biomédicale, Univ. de Maurice	Examinateur
<b>Dr. Gérard LE FUR</b>	Membre de l'Académie des Science, France	Examinateur
<b>Dr Maya CESARI</b>	GEICO – EA 4516, Univ. La Réunion (Réunion)	Examinateur
<b>Dr Laurence HOAREAU</b>	GEICO – EA 4516, Univ. La Réunion (Réunion)	Examinateur
<b>Pr. Marie Paule GONTHIER</b>	GEICO – EA 4516, Univ. La Réunion (Réunion)	Directeur
<b>Dr Regis ROCHE</b>	Directeur, Stemcis/ADIP'sculpt	Co- Directeur





## Thesis

Submitted to Université de La Réunion for the award of Doctorate degree in science

**Molecular and Cellular biology**

by

**Manoj kumar GUNASEKARAN**

**Obesity: Adipose tissue inflammation**

**As a matter of fat, HMGB1 is an adipokine**

**30 October 2013**

<b>Pr. Ulf ANDERSSON</b>	Rheumatology unit, Karolinska institute, (Stockholm, Sweden)	Referee
<b>Dr. Isabelle DUGAIL</b>	DR UMRS872 - INSERM (Paris, France)	Referee
<b>Pr. Theeshan BAHORUN</b>	Center for biomedical research, Uni of Mauritius	Examiner
<b>Dr. Gérard LE FUR</b>	Member, the Academy of Science, France	Examiner
<b>Dr. Maya CESARI</b>	GEICO – EA 4516, Univ. La Reunion (Reunion)	Examiner
<b>Dr. Laurence HOAREAU</b>	GEICO – EA 4516, Univ. La Reunion (Reunion)	Examiner
<b>Pr. Marie Paule GONTHIER</b>	GEICO – EA 4516, Univ. La Reunion (Reunion)	Director
<b>Dr. Régis ROCHE</b>	Director, Stemcis/ADIP'sculpt	Co – Director



All previously published papers were reproduced with permission from the publisher.

© Manoj kumar Gunasekaran, 2013

Front cover page: Art Direction by Clay Nowak (<http://v1tal.com>)

## Résumé

L'obésité est aujourd'hui considérée comme une pandémie majeure et 2,8 millions d'adultes meurent chaque année de maladies associées à cette pathologie. Plus que l'augmentation de la masse adipeuse, c'est l'inflammation du tissu adipeux qui représente la première complication. Cette inflammation résulte de la sécrétion de cytokines pro-inflammatoires, comme le TNF $\alpha$  ou l'IL-6, qui vont jouer un rôle majeur dans la survenue de l'insulino-résistance, du diabète, des maladies cardio-vasculaires, des désordres articulaires et du cancer. Le fait que les adipocytes répondent à des stimuli pro-inflammatoire par la sécrétion active de ces cytokines renforce d'autant plus l'idée qu'il faut désormais considérer le tissu adipeux comme un organe endocrine. De plus, les adipocytes expriment les TLR2 et TLR4, de façon similaire aux macrophages, et il est donc probable que l'inflammation du tissu adipeux soit, de ce fait, majoritairement véhiculée par l'activation de ces récepteurs, et plus particulièrement du TLR4.

Les stimuli physiologiques qui activent l'inflammation véhiculée par TLR4 dans l'adipocyte ne sont pas encore tous mis à jour, cependant le LPS bactérien, les HSP60 et les acides gras libres sont connus pour induire l'inflammation adipocytaire via TLR4. Dans ce contexte, une précédente étude de notre laboratoire a détaillé la voie de signalisation du LPS dans l'adipocyte. Par ailleurs, d'autres études mettent en doute la capacité des acides gras libres à activer TLR4 et de ce fait à provoquer une inflammation. Ainsi nous avons confirmé qu'en l'absence d'endotoxine, les acides gras saturés ainsi que les polyinsaturés ne peuvent pas activer TLR4, ou TLR2, ni même se lier à ces récepteurs, et n'induisent donc pas la sécrétion de TNF $\alpha$ , d'IL6 ou de MCP1 dans les adipocytes et le tissu adipeux humains (*Article 1*).

Récemment, HMGB1 a été redécouverte en tant que cytokine, en plus de son rôle dans le maintien et la transmission du génome, et son implication a été démontré dans de nombreuses maladies métaboliques associées à l'obésité. Identifiée comme un médiateur tardif de l'inflammation dans les cellules immunitaires, HMGB1 se lierait à TLR4 afin de provoquer l'inflammation. Ces données nous ont conduits à étudier si HMGB1 pouvait être libérée pendant l'inflammation du tissu adipeux et si oui, de quelle façon cette molécule pouvait réguler cette inflammation. Nous avons ainsi montré pour la première fois qu'HMGB1 est exprimée à des taux supérieurs chez le sujet obèse comparé au sujet normo-pondéral. De plus, nos expériences *in vitro* montrent que pendant l'inflammation adipocytaire, HMGB1 est effectivement sécrétée comme un médiateur tardif. Les cellules dérivées de la fraction stromale-vasculaire du tissu adipeux (ASC)

sont responsables de cette sécrétion, alors que les adipocytes matures et les ASC différenciées *in vitro* en adipocytes ne sécrètent pas HMGB1 (**Article 2**).

Par ailleurs, nous avons également démontré l'implication d'HMGB1 lors de l'inflammation induite par le LPS, par son action autocrine sur les ASC. De plus, nous avons mis en évidence le rôle pro-inflammatoire d'HMGB1 sur les adipocytes matures humains, par la sécrétion d'IL-6 et de MCP-1, alors que le TNF $\alpha$  n'est pas détectable. Nous poursuivons ces travaux dans le but de déterminer les récepteurs impliqués ainsi que la voie de signalisation (**Article 3 en préparation**).

En conclusion de ce travail, nous avons donc confirmé que les acides gras n'étaient pas des ligands pour TLR2 et TLR4 qui pourraient déclencher l'inflammation du tissu adipeux humain. Par contre, nous démontrons que les ASCs soumises à une inflammation sécrètent HMGB1, qui agit en retour de façon autocrine et paracrine sur les adipocytes, exacerbant l'inflammation du tissu adipeux. De fait, bien qu'HMGB1 ne soit pas sécrétée par les adipocytes différenciés, il faut probablement la considérer tout de même comme une nouvelle adipokine.

## Abstract

Obesity is now considered as a major pandemic and leading risk of global death with an average of 2.8 million adults each year as a result of being overweight or obese. More than increase in fat deposition, the real complication rises due to inflammation in adipose tissue. This inflammation which results in the secretion of proinflammatory cytokines such as TNF $\alpha$  and IL-6 plays major role in the development of insulin resistance, diabetes, cardiovascular diseases, joint disorders and cancer. The fact that adipocytes respond to pro-inflammatory stimuli by active secretion of these cytokines reinforces the idea to consider adipose tissue as an endocrine organ. In addition, adipocytes expression of TLR2 and TLR4 in a similar manner to macrophages, suggests that inflammation in adipose tissue is therefore mainly mediated by the activation of these receptors, particularly TLR4.

The physiological stimuli that activate inflammation mediated by TLR4 in adipocytes are not yet known completely, however few possible stimuli such as bacterial LPS, Hsp60 and FFA are suggested to activate TLR4 and induce inflammation in adipocytes. In this context, earlier work from our laboratory has shown detailed LPS mediated inflammatory signaling pathway in adipocytes. Moreover, other studies have questioned the ability of free fatty acids to activate TLR4 and thereby cause inflammation. However we have confirmed that in an endotoxin free condition none of the SFA and PUFA are able to bind or activate neither TLR4 nor TLR2 and induce inflammation in human mature adipocytes and adipose tissue (*Paper 1*).

Recently HMGB1 has been rediscovered as a cytokine in addition to the nuclear role and its involvement has been demonstrated in many metabolic diseases associated with obesity. Identified as a late mediator of inflammation in immune cells, HMGB1 is found to act through TLR4 to induce inflammation. This led us to investigate whether HMGB1 could be secreted during adipose tissue inflammation and if so how it can regulates inflammation. We have reported for the first time that HMGB1 is expressed more in adipose tissue of obese compared to lean persons. By *in vitro* studies, we have shown that during adipose tissue inflammation, HMGB1 is effectively secreted as a late mediator. The cells derived from the stromal vascular fraction of adipose tissue (ASC) are responsible for this secretion, whereas mature adipocytes and *in vitro* differentiated adipocytes from ASCs do not secrete HMGB1 (*Paper 2*).

Further, we have also demonstrated the involvement of HMGB1 in inflammation induced by LPS, through its autocrine action on ASCs. In addition we have shown the pro-inflammatory action

of HMGB1 on human mature adipocytes which induced the IL-6 and MCP-1 secretion while TNF $\alpha$  was not detectable. We are continuing our investigation in order to determine through which receptor and signaling pathways, HMGB1 could induce inflammation in human adipocytes (*Paper 3 – in preparation*).

To conclude this work, we therefore confirmed that fatty acids were not ligands for TLR2 and TLR4 that could trigger inflammation in human adipose tissue. Instead we demonstrated that ASCs subjected to inflammation secrete HMGB1, which acts back in an autocrine and paracrine manner on adipocytes, exacerbate inflammation in adipose tissue. In fact, although HMGB1 is not secreted by differentiated adipocytes, we should probably still consider it as a novel adipokine.

## List of publications

---

- 1) Murumalla RK\*, **Gunasekaran MK\***, Padhan JK, Bencharif K, Gence L, Festy F, Cesari M, Roche R, Hoareau L: **Fatty acids do not pay the toll: effect of SFA and PUFA on human adipose tissue and mature adipocytes inflammation.** *Lipids in health and diseases* 2012, 11:175 (PMID: 23259689).
- 2) Girard AC, Atlan M, Bencharif K, **Gunasekaran MK**, Delarue P, Hulard O, Lefebvre-d'Hellencourt C, Roche R, Hoareau L, Festy F: **New insights into lidocaine and adrenaline effects on human adipose stem cells.** *Aesthetic plastic surgery* 2013, 37 (1):144 (PMID: 23238646).
- 3) **Gunasekaran MK**, Viranaicken W, Girard AC, Festy F, Cesari M, Roche R, Hoareau L: **Inflammation triggers High Mobility Group Box 1 (HMGB1) secretion in adipose tissue, a potential link to obesity.** *Cytokine*, 2013 (PMID: 23938155, Epub ahead of print).
- 4) Nativel B, Marimoutou M, Thon-Hon V, **Gunasekaran MK**, Andries J, Stanislas G, Planesse C, Robert Da Silva C, Cesari M, Iwema T, Gasque P, Viranaicken W: **Soluble HMGB1 is a novel adipokine stimulating IL-6 secretion through RAGE receptor in SW872 cell line: Contribution to chronic inflammation in fat tissue.** *Plos One*, 2013 (*In press -Accepted*).
- 5) Girard AC\*, **Gunasekaran MK\***, Denizot M, Neyret A, Bencharif K, Delarue P, Hulard O, Lefebvre-d'Hellencourt C, Roche R, Hoareau L, Festy F: **Lidocaine effects on human adipose stem cell: The phantom menace?** (*Manuscript ready for submission*).

\* These authors contributed equally

## Conference abstracts

---

- 1) Anne Claire GIRARD, **Manoj kumar GUNASEKARAN**, Regis ROCHE, Laurence HOAREAU, Franck FESTY: "Lidocaine effects on human adipose derived stem cells: The phantom menace", IFATS, New York, 2013. Oral presentation.
- 2) **Manoj kumar GUNASEKARAN**, Wildriss VIRANAICKEN, Maya CESARI, Franck FESTY, Regis ROCHE, Laurence HOAREAU: "HMGB1, a novel biomarker of adipose tissue inflammation in obesity", Obesity and diabetes - International symposia, EMBL, Heidelberg, Sep 2012. Poster presentation.



# Contents

---

General Introduction.....	1
1. Adipose tissue .....	4
1.1 Introduction .....	5
1.2 Brown adipose tissue .....	5
1.3 White adipose tissue .....	6
1.3.1 WAT Cellular components .....	7
1.3.1.1 Mature adipocytes .....	8
1.3.1.2 Stromal vascular fractions .....	9
1.3.2 WAT Distribution .....	9
1.3.2.1 Visceral adipose tissue .....	9
1.3.2.2 Subcutaneous adipose tissue .....	10
1.3.3 Functions .....	11
1.3.3.1 Lipid storage .....	11
1.3.3.2 Endocrine organ .....	12
1.4 Adipose tissue – dysfunctions .....	15
1.4.1 Hypoxia .....	17
1.4.2 AT stresses .....	18
1.4.2.1 ER stress .....	18
1.4.2.2 Mitochondrial and oxidative stress .....	19
1.4.3 Autophagy .....	20
1.4.4 Chronic inflammation .....	21
1.4.4.1 Macrophages infiltration and polarization .....	21
1.4.4.2 Paracrine crosstalk between macrophages and adipocytes .....	23
1.5 Metabolic diseases .....	24
1.5.1 Insulin resistance and type II diabetes .....	24
1.5.2 Vascular diseases .....	25
1.5.3 Cancer .....	25

2	TLR regulates inflammation .....	27
	2.1 Adipocytes TLR expression and inflammation .....	30
	2.2 Adipose tissue inflammation: What pays the toll???? .....	32
	2.2.1 Bacterial LPS .....	33
	Gut microbiota – the source of LPS .....	33
	2.2.2 Hsp60 – the mediator of adipose tissue inflammation .....	34
	2.2.3 Free fatty acids .....	35
	<b><i>Fatty acids do not pay the toll: effect of SFA and PUFA on human adipose tissue and mature adipocytes inflammation</i></b> (Paper 1). .....	37
3	HMGB1 .....	47
	3.1 Introduction .....	48
	3.2 Structure of HMGB1 .....	49
	3.3 Post translational modifications .....	49
	3.4 HMGB1 location and functions .....	50
	3.4.1 Nuclear HMGB1 .....	50
	3.4.2 Intra-cellular HMGB1 .....	51
	3.4.3 Extra-cellular HMGB1 .....	51
	3.4.3.1 Active secretion .....	53
	3.4.3.2 Passive release .....	54
	3.5 HMGB1 recognizing receptors and pathways .....	54
	3.5.1 RAGE .....	55
	3.5.2 TLR4 .....	56
	3.5.3 TLR2 .....	56
	3.5.4 TLR9 .....	57
	3.6 HMGB1 role in sterile inflammation and auto-immune diseases .....	57
	3.6.1 Atherosclerosis .....	57
	3.6.2 Diabetes .....	57
	3.6.3 Rheumatoid arthritis .....	58

3.7	HMGB1 expression in adipose tissue .....	58
	<i>Inflammation triggers high mobility group box 1 (HMGB1) secretion in adipose tissue, a potential link to obesity</i> (Paper 2) .....	60
	<i>HMGB1 – an inflammatory adipokine</i> (Paper in process) .....	70
4	General summary.....	74
5	General discussion and conclusion. ....	75
6	Acknowledgements.....	79
7	References.....	81

## List of figures

---

Figure 1	Global prevalence of obesity, 2008 (WHO).....	2
Figure 2	Brown adipose tissue (BAT), location and distribution.....	6
Figure 3	Structure of white adipose tissue (WAT).....	7
Figure 4	Structure of mature adipocytes and their cellular components.....	8
Figure 5	White adipose tissue - visceral and subcutaneous adipose tissue distribution in lean and obese human (MRI scan from unknown resource).....	10
Figure 6	Primary role of adipose tissue – TAGs (Triacylglycerols) synthesis, storage and energy supply during starvation and shortage.....	12
Figure 7	Endocrine role of adipose tissue.....	13
Figure 8	Adipose tissue dysfunctions and potential progression of inflammation.....	16
Figure 9	Adipose tissue under inflammation, dying adipocytes and infiltrating macrophages forming “crown like structures”.....	22
Figure 10	The paracrine crosstalk between adipose tissue macrophages and adipocytes.....	23
Figure 11	Toll like receptors, ligands and their signaling pathways.....	29
Figure 12	Possible TLR4 ligands for adipose tissue inflammation .....	32
Figure 13	Protein structure of HMGB1.....	48
Figure 14	HMGB1 nuclear, intracellular and extracellular functions.....	50
Figure 15	Extracellular HMGB1 - types and functions.....	52
Figure 16	Passive release of HMGB1 and its redox status.....	54
Figure 17	HMGB1 recognizing receptors.....	55

## List of tables

---

Table 1	Adipokines and its functions.....	14
---------	-----------------------------------	----

## List of abbreviations

---

ANGPTL-2	Angiopoietin-related protein 2
ATG7	Autophagy-related protein 7
Bcl-2	B cell lymphoma -2
BAT	Brown adipose tissue
BMI	Body mass index
BSA	Bovine serum albumin
CCL2	Chemokine (C-C motif) ligand 2 (CCL2)
CCR5	Chemokine (C-C motif) ligand 5 (CCL5)
CD14	Cluster of differentiation 14
CEBP	CCAAT-enhancer-binding proteins
CIDE	Cell death-inducing DFF45-like effectors
CRP	C – reactive protein
CVD	Cardiovascular diseases
CXCL-1	Chemokine (C-X-C motif) ligand 1
CRM-1	Chromosome region maintenance-1
DAMP	Danger associated molecular patterns
ER	Endoplasmic reticulum
ERK	Extracellular-signal-regulated kinases
FFA	Free fatty acids
HFD	High fat diet
HMGB1	High mobility group box 1
ICAM-1	Intercellular Adhesion Molecule 1
IFN $\gamma$	Interferon gamma
IGF-1	Insulin like growth factor - 1
IL-6	Interleukin – 6
IL-8	Interleukin – 8
LA	Lauric acid
MAPK	Mitogen activated protein kinase
MCP-1	Monocyte chemoattractant protein – 1
NF $\kappa$ B	Nuclear factor kappa B
PAI-1	Plasminogen activator inhibitor type 1
PA	Palmitic acid

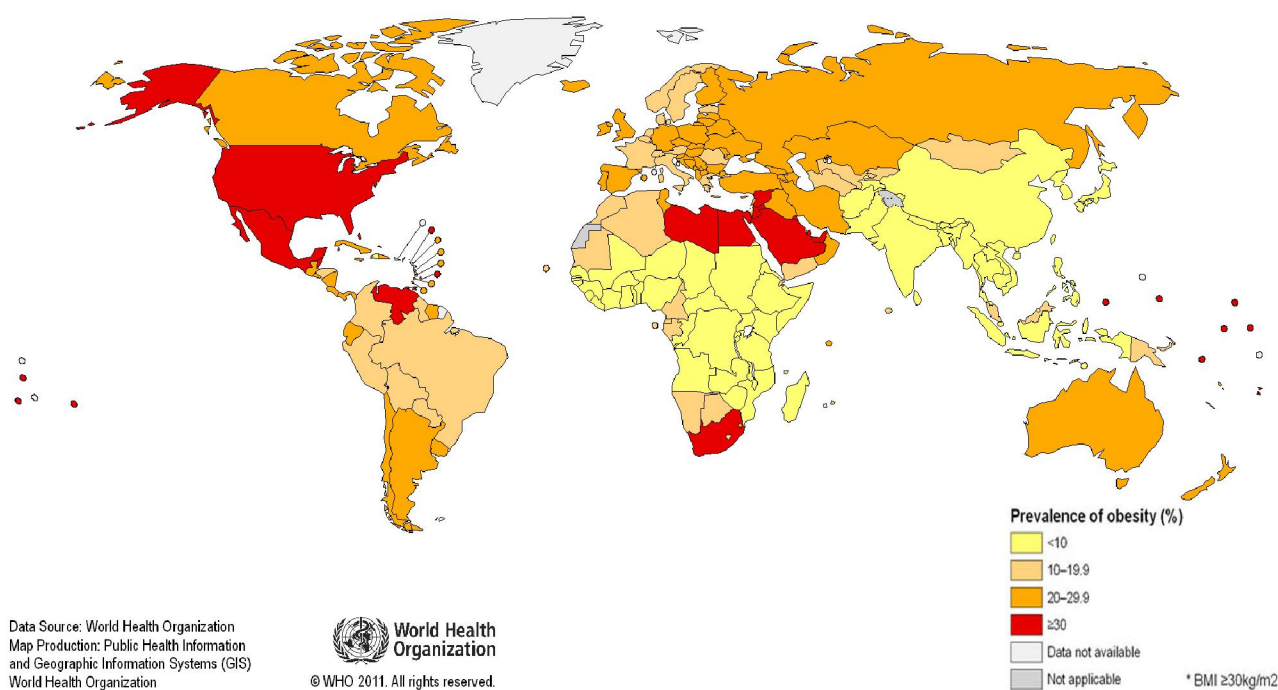
PAMP	Pathogen associated molecular patterns
PPAR	Peroxisome proliferator activated receptors
RANTES	Regulated upon activation normal T cell expressed and presumably secreted
RBP – 4	Retinol binding protein – 4
ROS	Reactive oxygen species
SFRP-5	Secreted frizzled-related protein 5
SVCs	Stromal vascular cells
T2DM	Type 2 diabetes mellitus
TGFb	Tumor growth factor - beta
TLR	Toll like receptor
TNF	Tumor necrosis factor
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
WAT	White adipose tissue
WHO	World health organization
WHR	Waist hip ratio

# 1. General introduction

---

Increase in globalization across countries developed substantial improvements in quality of life and food security. Unfortunately it also led to the development of unintended consequences by increasing the energy rich food consumption combining with less physical activity driving global obesity, an epidemic. The availability and consumption of cheap foods and sugar rich beverages without real nutritional values has increased rapidly in developed and developing countries, driving the increase in obesity and associated chronic diseases [1]. This linear increase in obesity staggers the quality of life and health care expenditure rapidly worldwide. WHO defines overweight and obesity as abnormal or excessive adipose tissue accumulation that creates risk to health. The global effect of obesity as epidemic was formally recognized by WHO during a special obesity consultation in 1997 [2]. According to WHO across the globe, it has been estimated that 10 – 14 % of total world population, about 500 million adults were obese in 2008. In addition to that, childhood obesity becomes one of the most serious public health issues of the 21<sup>st</sup> century with the increased risk of associated complications and disabilities. About 40 million children under the age of five were found to be overweight in 2011 and this is increasing in an alarming rate.

### Global prevalence of obesity



**Figure 1:** Global prevalence of obesity, 2008. Source: <http://www.who.int>



In addition to causing various physical disabilities and psychological problems, excess weight drastically increases the person's risk of developing non communicable diseases (NCDs), including cardiovascular disease (CVD), cancer and diabetes. Obesity has become a major contributor to the global burden of disease and function as an important risk factor for morbidity and mortality. About 347 million people worldwide are affected by diabetes with the increasing mortality rate in low and middle income countries. At present the CVDs are the number one cause of death globally and an estimated 17.3 million people died globally in 2008 representing almost 30% of total death. It has been predicted with this increasing rate of CVD, the number of death due to heart disease and stroke will reach 23.3 million by 2030. The mortality and the health risk due to obesity induced chronic diseases are more compared to the obesity itself.

Body mass index (BMI) is a measure of body fat based on height and weight that classifies individuals into underweight, normal, overweight and obese. BMI is a mathematical calculation as the individual's body mass (kg) divided by the square of their height (m<sup>2</sup>). The increase in BMI of a person is found to be directly proportional to the increase in risk of diabetes, cardiovascular and other chronic metabolic syndrome and diseases. A person with BMI of 30 or more is generally considered obese and a person with BMI equal to or more than 25 is considered overweight. The abdominal obesity is also defined by waist-hip ratio (WHR), where ratio above 0.9 for male and 0.85 for female are considered as obese.

# 1. Adipose tissue

---

## 1.1 Introduction

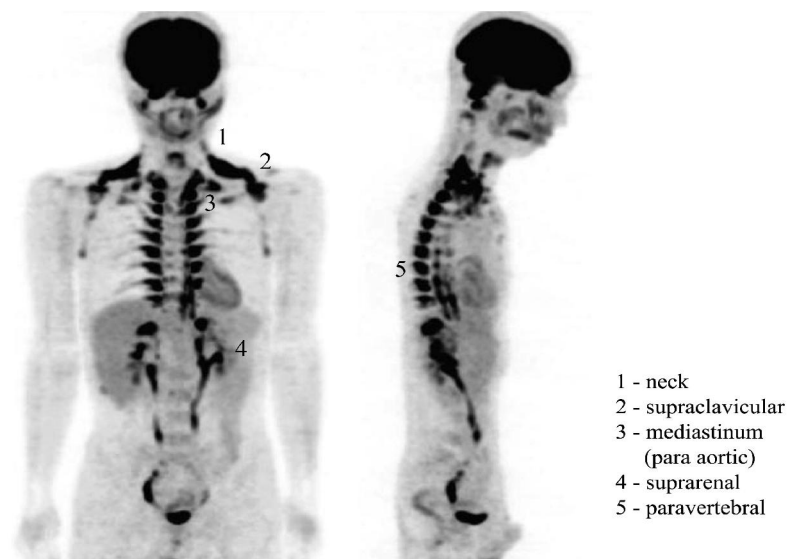
Adipose tissue is a loose connective tissue composed mostly of fat storage cells called adipocytes arranged in the net framework of collagen fibers. It is a specialized energy storage tissue that functions as the major site for lipid storage in the form of triglycerides. These stored lipids are utilized by the body during energy shortage, acts as a backup during hunger and fuel to brain and muscles. It protects the body from excess glucose and FFA by storing them as triglycerides. Apart from energy storage, it insulates and also acts as a mechanical cushion for the body. In mammals two types of adipose tissue exist, white adipose tissue (WAT) and brown adipose tissue (BAT). The distribution, amount and location vary across the species and age, however in humans most of them are WAT.

## 1.2 Brown adipose tissue:

Brown fat or BAT is more abundantly found in newborns and hibernating animals to protect from cold and acts as thermal insulator. BAT is rich of vascularisation and its adipocytes cytoplasm is occupied with multiple small lipid droplets and abundant of iron rich densely packed mitochondria that help in BAT releases energy directly as heat and protects the body by non-shivering thermogenesis. Mitochondria in brown adipocytes have a special carrier protein called Uncoupling protein 1 (UCP1) that transfers protons inside the mitochondrial matrix without the subsequent utilization or production of ATP. To produce heat, brown adipocytes are activated by sympathetic nerves acting on beta3-adrenoreceptors (beta3AR), activating lipolysis and burns fatty acids. UCP-1 activated by fatty acids uncouples oxidative phosphorylation, resulting in the energy dissipation in the form of heat [3, 4]. It is believed for many years that mitochondria in brown adipocytes disappear upon ages in infants and later functions as white adipose tissue. However, it has been recently identified that brown adipose tissue remains in upper chest and neck of adults [5].

It has been discovered that BAT are more related to skeletal muscles rather than white adipose tissue upon time. They are found to carry a same cell surface marker called myogenic factor 5 (Myf5) as skeletal muscle and derived from the same stem cells in the embryo [6]. Recently a new type of brown adipocytes is identified in WAT of adults called beige or brite cells when exposed to cold or to PPAR ligands. These “inducible” brown adipocytes possess

the morphological and biochemical characteristics of classical brown adipocytes, including the expression of UCP1 and multilocular lipid droplet [7] but they arise from non-Myf-5 lineage [8]. Importantly, increase in number of beige cells in WAT are associated well with a protection against diet induced obesity and metabolic disease [9-11]. As brite cells is found to utilize more calories from normal fat and burns it, research is going on to activate brite cells in WAT to stimulate this process for weight loss.



Modified from internet ([www.markyoungtrainingsystems.com](http://www.markyoungtrainingsystems.com))

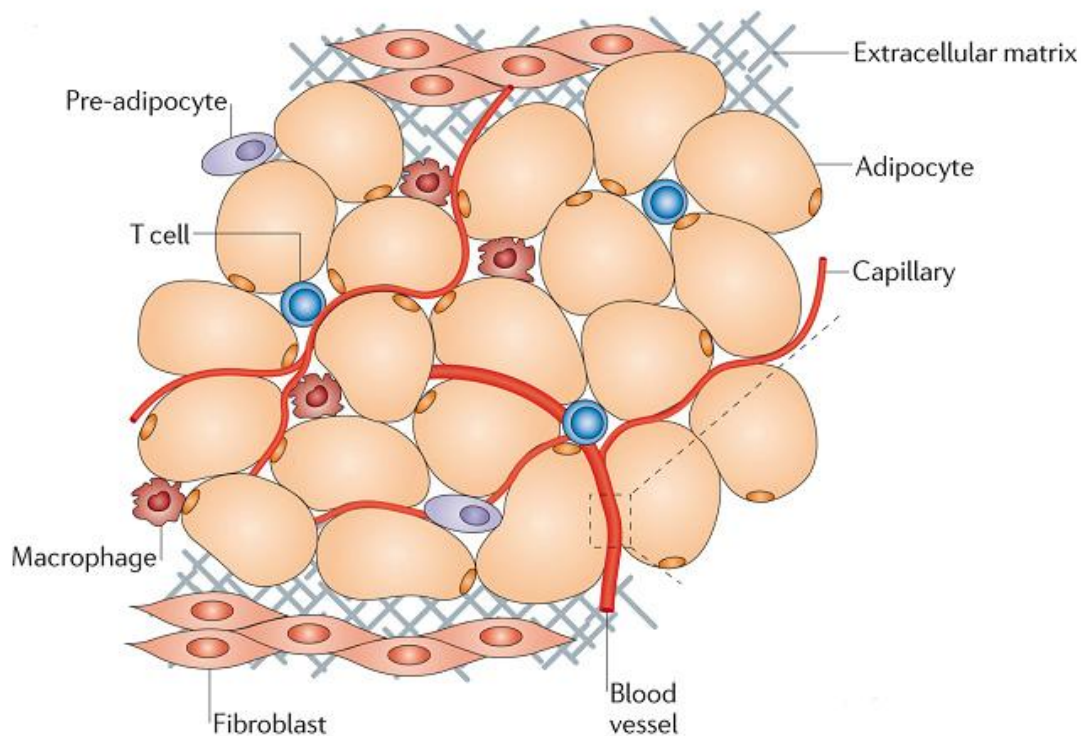
**Figure 2:** Brown adipose tissue (BAT), location and distribution.

### 1.3 White adipose tissue:

WAT is composed primarily of tightly packed large unilocular mature adipocytes supported by well vascularized mesh framework of loose connective tissue. A strong external connective tissue is necessary to protect the delegate adipocytes and to maintain the tissue structure. Adipocytes exoskeleton is composed of meshwork of collagen 1 and reticular fibers building the “collagenic peri-adipocyte basket”, which functions to protect the adipocytes from mechanical disruption. Each cell in adipose tissue presents a basal lamina of a typical

composition. Adipocytes and other cell populations present in WAT synthesize collagens type 1, 3 and 4 as well as substantial amounts of collagen type 6a1, which interact with collagen type 4 in mediating the attachment of the basal lamina to fat cells [12]. The scaffold provided by adipocytes basal laminas and a multitude of extracellular matrix (ECM) proteins and proteoglycans (e.g. fibronectin, decorin, tenascin C, osteonectin, osteopontin, matrix metalloproteinases) reduces external forces and ensures structural and functional integrity of the adipose tissue [13, 14]. Adipose tissues are facilitated with dense network of capillaries, which delivers substrates, oxygen and also it helps in the transport of hormones, cytokines and other biological agents. It also contains lymphatic vessels which help in the transport of secreted cytokines. Apart from the ECM and vascular network, the other non adipocyte component of WAT are the stromal fraction, comprises of multipotent stem cells, preadipocytes, fibroblasts, pericytes, endothelial cells of blood and lymphatic vessels, macrophages and other infiltrating immune cells [15].

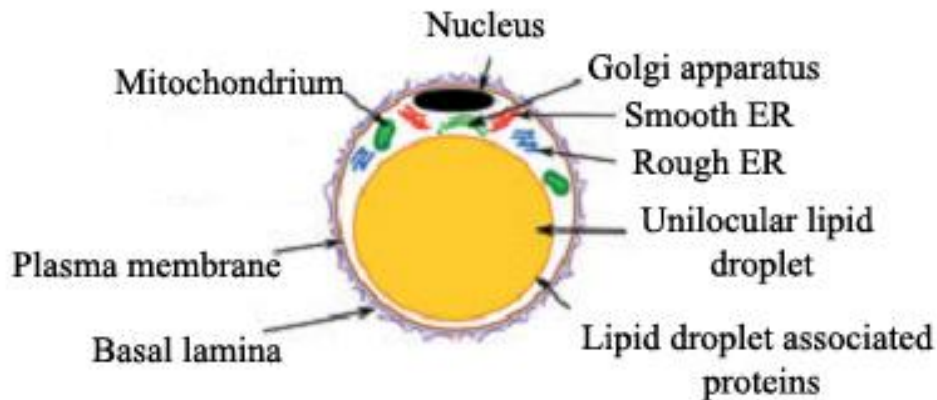
### 1.3.1 WAT cellular components



**Figure 3:** Structure of white adipose tissue (WAT), [15]

### 1.3.1.1 Mature adipocyte

In WAT, the adipocytes constitute more than 90% of tissue volume and more than 50% of the total cell number. In an average there are around 20 to 40 billion mature adipocytes in a normal person, however this number can be doubled or tripled in obese patients depending upon their adipose tissue mass [16, 17]. Adipocytes size varies from 30 – 130  $\mu\text{m}$  based on its cellular lipid droplet and its functions depend based upon size, the larger adipocytes secrete more inflammatory cytokines, chemo attractants and exhibits higher metabolic activity [18]. The human adipocyte can grow up to  $\sim 20$  fold in diameter and several thousand fold in volume [19]. In mature adipocytes, the cytoplasmic space is occupied entirely by a big lipid droplet squeezing out rest of the cellular components along the sides, with a range of structural proteins. It is surrounded by phospholipid monolayer protected by a cage like structure of vimentin network, perilipin and CIDE proteins [20]. The nucleus is along the periphery of the cell and the nuclear lamina is coupled to vimentin network [21]. Near the nucleus, lies the densely packed other cell components such as Golgi apparatus, smooth and rough endoplasmic reticulum, free ribosomes, and large elongated mitochondria [22]. Adipocytes cell membranes are occupied with different receptors to recognize hormones, neurotransmitters, DAMPs and PAMPs [15]. Adipocytes are equipped with various TLRs to recognize PAMPs contributing to the innate and adaptive immunity [23].



**Figure 4:** Structure of mature adipocyte and their cellular components, [24]

### **1.3.1.2 Stromal vascular fraction:**

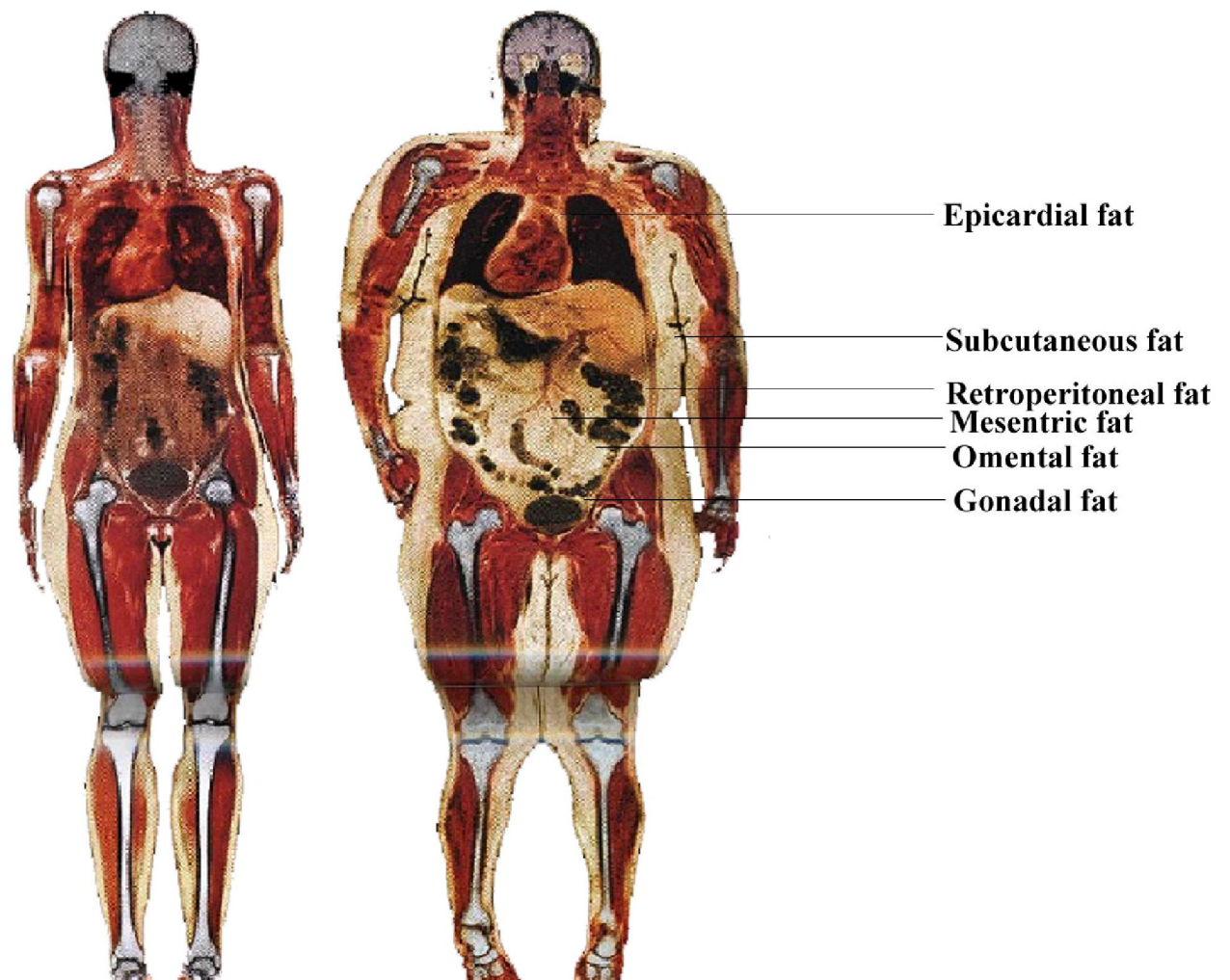
Apart from lipid filled mature adipocytes, WAT comprise of preadipocytes, mesenchymal stem cells (MSC), endothelial cells, fibroblasts and immune cells (macrophages, neutrophils, eosinophils, lymphocytes, natural killer cells, and mast cells), collectively termed as stromal vascular fraction (SVF). Preadipocytes and MSC proliferate and differentiate in to adipocytes to replace the dead adipocytes during adipose tissue remodeling and extension. Adipose derived stem cells (ASCs) from SVF has the capability to differentiate into different type of cells such as vascular-endothelial cells, adipocytes and several other cell lineages depending up on the adipose tissue expansion requirements. The preadipocytes which compose 10-15% of adipose tissue also display various proliferation capabilities based on its various depots.

### **1.3.2 WAT distribution:**

The WAT is distributed in different parts of the body with varied functions and they are classified into two major WAT depots, visceral and subcutaneous fat.

#### *1.3.2.1 Visceral adipose tissue (VAT):*

The visceral adipose tissue surrounds inner organs in the abdominal cavity and mediastinum. The visceral fat in the abdominal region are further classified into four major depots, omental fat, surrounding the intestines superficially; mesenteric fat, which is more deeply buried around the intestines; retroperitoneal fat, near the kidneys - at the dorsal side of the abdominal cavity and gonadal fat, around testis and ovary. In mediastinum, the visceral white adipose tissues are located around specific organs, such as in the heart, epicardial; in stomach, epigastric and in blood vessels, perivascular adipose tissue. The visceral fat increases with age in both genders and accounts for up to 10–20% of total fat in men and 5–8% in women [25]. VAT contains greater number of large adipocytes and undergoes rapid lipolysis and secretes large amount of FFA [26]. In visceral adipose tissue, venous blood is drained directly in to the liver through the portal vein and it gains direct hepatic access to FFAs and adipokines secreted by visceral adipocytes.



**Figure 5:** White adipose tissue - visceral and subcutaneous adipose tissue distribution in lean and obese human (MRI scan from unknown resource).

#### *1.3.2.2 Subcutaneous adipose tissue (SAT):*

The SAT is distributed in the hypodermal layer of the skin over the body's surface. In most regions of the human body, there are two anatomically and histologically distinct layers within the subcutaneous fat, 1) superficial 2) deep SAT (dSAT). Compared to visceral adipose tissue, subcutaneous fat is more cellularized, that is, it has a higher proportion of smaller cells

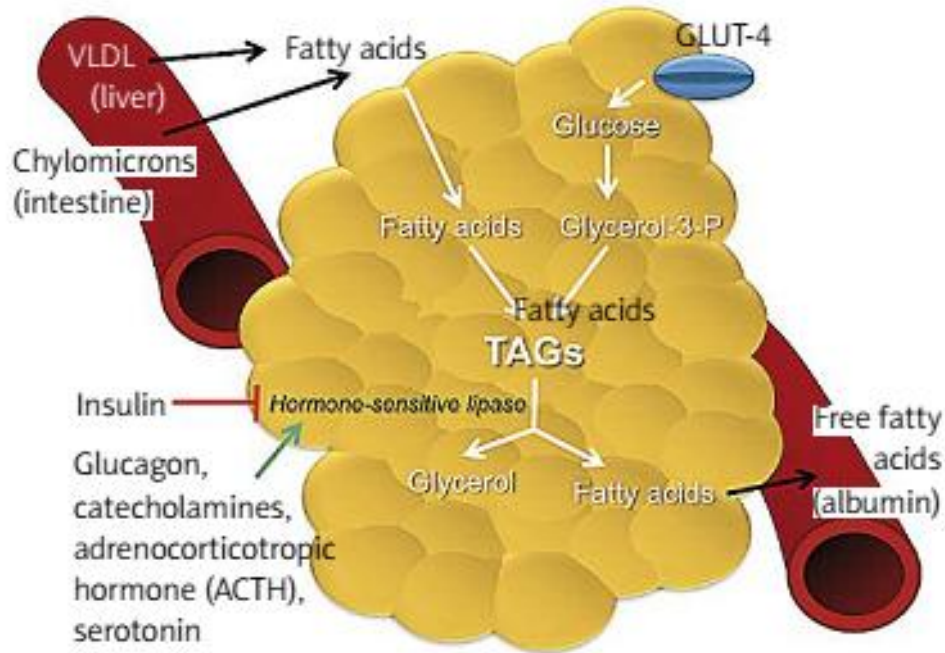


because of the higher differentiation potential of subcutaneous adipocytes [27]. Subcutaneous fat is more densely vascularized than visceral fat and it retains higher angiogenic potential [28]. SAT adipocytes are smaller in size and they are less metabolically active, secretes less pro-inflammatory cytokines and has less macrophage infiltration compared to VAT [29]. SAT serves as the long term lipid storage tissue and delivers backup for energy production during fasting, starvation and exercise. In women, the adipose tissue forming mammary fat pad plays an important role in the development of mammary gland and involves in the regulation of epithelial cell proliferation and functions.

### **1.3.3 Functions**

#### *1.3.3.1 Lipid storage*

The primary function of WAT is to regulate energy metabolism in the body and also acts as site of lipid storage and synthesis. During surplus nutrient diet or reduced physical activity, WAT stores or accumulates excessive lipid in adipocytes in the form of triglycerides (TG). Insulin regulates the uptake of glucose by adipocytes via GLUT4 transporters and utilizes it to synthesize TG for storage. Adipocytes also uptake free fatty acids by the action of lipo protein lipase (LPL) which hydrolyses TG of chylomicrons and very low-density lipoproteins (VLDL) in endothelial lining of blood vessels. WAT also recruits FFA from blood plasma lipoproteins and re-esterifies to form TG and this process has higher significance than *de nova* lipogenesis. WAT supplies energy during starvation or increased energy demands by undergoing lipolysis process by converting TG in to FFA with the help of adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL). These fatty acids are then transported to the desired location via blood or lymph vessels, where they serve as energy substrates for beta oxidation in mitochondria.



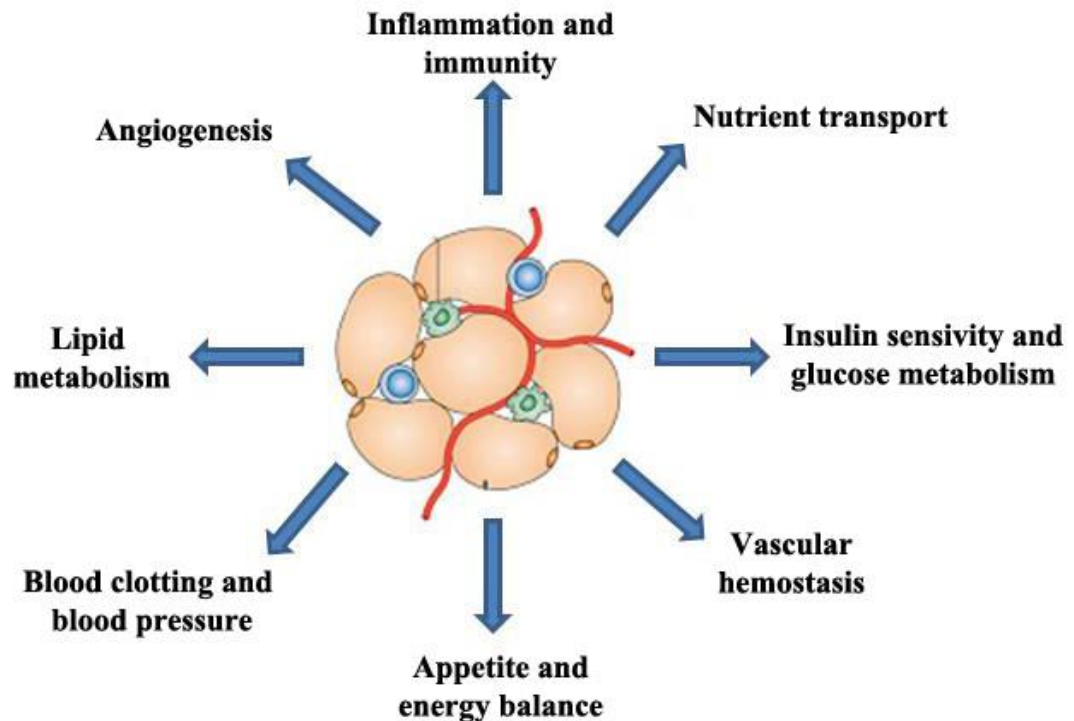
**Figure 6:** Primary role of adipose tissue – TAGs (Triacylglycerols) synthesis, storage and energy supply during starvation and shortage [30].

Insulin not only promotes the uptake and utilization of glucose in fat cells but also activates lipogenic enzymes such as acyl-CoA carboxylase and fatty acid synthase and simultaneously diminishes lipolysis by inhibiting the activity of hormone sensitive lipase (HSL). Glucocorticoids counteracts insulin effects on glucose utilization and stimulate lipolysis by increasing the activity of adipocyte's intracellular lipases (HSL, ATGL) and WAT's intra-vascular lipase (LPL), thus modulating both the release and uptake of fatty acids. Catecholamines belong to the key lipolytic agents and binding of noradrenaline to  $\beta$ 1-,  $\beta$ 2- and  $\beta$ 3-adrenergic receptors on adipocytes activates HSL, which results in increased fuel delivery to tissues.

### 1.3.3.2 Endocrine organ

The idea of WAT only as a lipid storage organ has changed after the discovery of leptin, a hormone secreted by white adipocytes which regulates the energy intake and expenditure including diet, metabolism and behavior [31]. The discovery of leptin opened up a whole field of studies into the biology of adipocytes, their metabolic and endocrine functions, and the

functional relationships between secretions of adipocytes and peripheral metabolic functions. TNF $\alpha$  is a pro-inflammatory cytokine, and it was the first adipocyte-derived factor that suggested a link between obesity, inflammation and T2DM [32]. The WAT represents the largest endocrine organ in humans, due to its majority adipocytes ability to secrete numerous hormones, cytokines, growth factors, complement factors [30]. Adipose tissue was identified as a major site for sex steroid metabolism [33] and production of adiponin, an endocrine factor that is negatively correlated with obesity in rodents [34]. Adipose tissue is dynamically involved in different functional regulation through a complex network of endocrine, paracrine and autocrine signals that influence the response of many tissues, including hypothalamus, pancreas, liver, skeletal muscle, kidneys, endothelium and the immune system.



**Figure 7:** Endocrine role of adipose tissue.

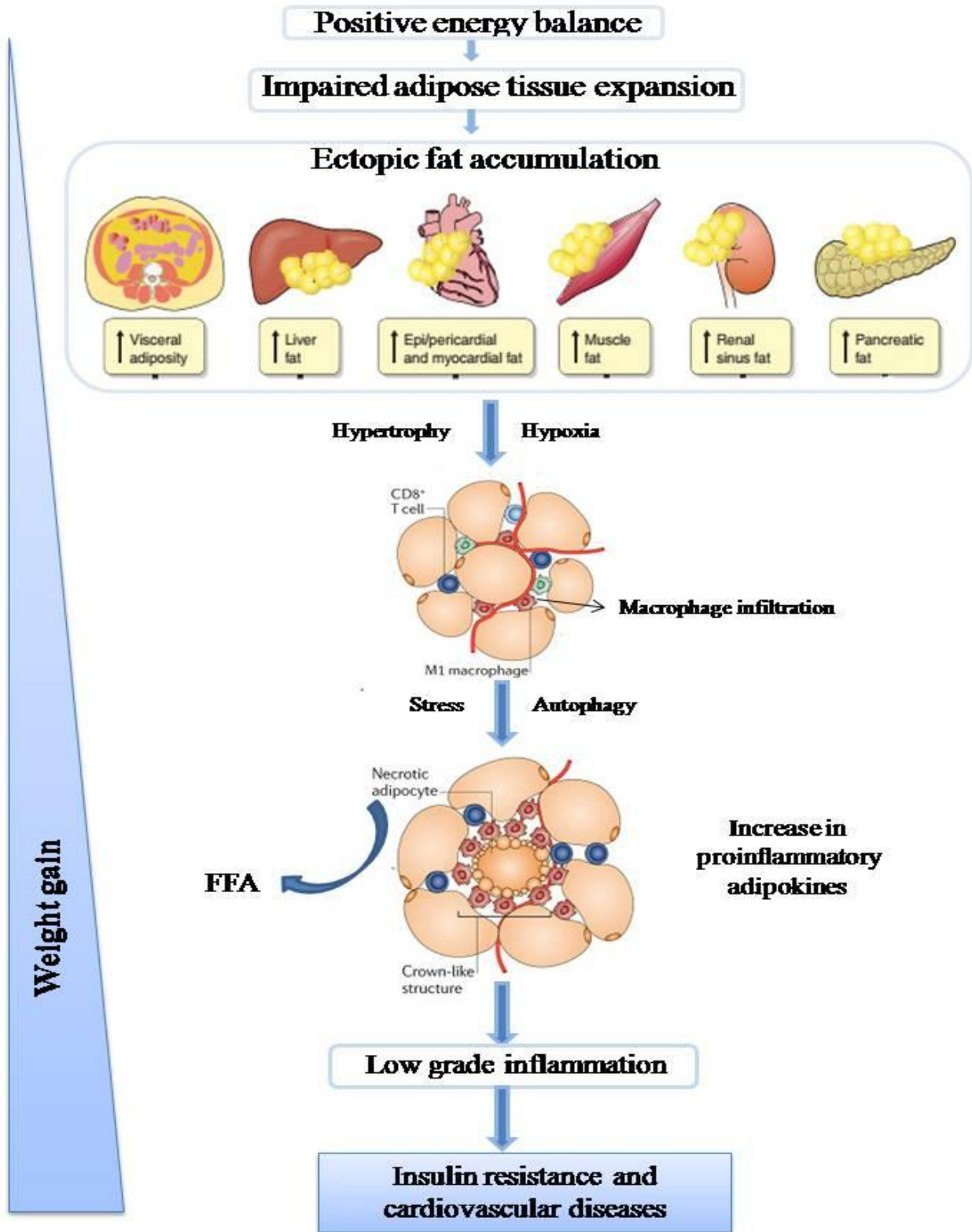
<b>Adipokines</b>	<b>Functions/effects</b>
<i>Leptin</i>	Signals to the brain about the body fat stores, regulates appetite and energy expenditure, proinflammatory
<i>Adiponectin</i>	Anti-inflammatory, Improves energy homeostasis, insulin sensitivity and glucose uptake.
<i>TNF</i>	Proinflammatory, affects insulin receptor signalling and sensitivity, reduces insulin secretion and stimulates lipolysis
<i>IL-6</i>	Proinflammatory, affects glucose and lipid metabolism. Regulates body weight
<i>MCP-1</i>	Increases insulin resistance, activates macrophages infiltration
<i>Resistin</i>	Proinflammatory cytokine affects glucose metabolism and cause insulin resistance.
<i>IL8</i>	Proatherogenic, neutrophil chemotaxis and degranulation
<i>RANTES</i>	Proinflammatory
<i>Vistafin</i>	Proinflammatory and mimicks insulin activity
<i>PAL-1</i>	Inhibitor of the fibrinolytic system by inhibition of activation of plasminogen
<i>Adipsin</i>	Potential relation between the complement pathway and adipose tissue metabolism
<i>TGFb</i>	Varied role in proliferation, differentiation, apoptosis and development
<i>Vaspin</i>	Improves insulin sensitivity
<i>VEGF</i>	Stimulation of angiogenesis
<i>IGF-1</i>	Stimulates proliferation of a wide variety of cells and mediates many cells and many of the effects of growth hormone
<i>Apelin</i>	Improves insulin sensitivity
<i>IL8</i>	Proatherogenic, neutrophil chemotaxis and degranulation
<i>Nesfatin</i>	Acts centrally to reduce apetite
<i>Omentin</i>	Increases insulin sensivity
<i>RBP4</i>	Proinflammatory, inhibits insulin-induced phosphorylation of IRS1
<i>ANGPTL2</i>	Increase inflammatory responses through integrin mediated signaling.
<i>Chemrin</i>	Mediate inflammatory responses, chemoattractant, regulate adipogenesis and adipocytes metabolism
<i>SFRP5</i>	Anti-inflammatory

**Table 1:** Adipokines and its functions.

As adipose tissue contains various cell types such as adipocytes, immune cells, endothelial cells and fibroblasts, it produces and releases diverse secretory proteins called **adipokines or adipo-cytokines** into the systemic circulation. Adipocytes and other cell members in WAT also express various receptors to recognize and respond to many of these factors and play an important role in the regulation of appetite and satiety control, fat distribution, insulin sensitivity and insulin secretion, energy expenditure, inflammation, blood pressure, blood clotting, angiogenesis, vascular hemostasis, and endothelial function [15].

#### **1.4 Adipose tissue dysfunctions:**

Adipose tissue dysfunctions develop under conditions of continuous positive energy balance with an impaired expandability of subcutaneous AT [35, 36]. This imbalance results in the excess storage of energy in the form of lipids in adipocytes, resulting in intra-cellular abnormalities like increased adipocyte size (hypertrophy) and increased number of adipocytes (hyperplasia). Although subcutaneous AT has better capability to expand its capillary network than visceral AT, this function was impaired with increasing adipose tissue accumulation [36]. After reaching its maximum storage capacity, impaired vascularization and angiogenesis, subcutaneous adipose tissue lipid accumulation comes to an end. The excess lipids are shifted to visceral fat deposition and other tissues (liver and skeletal muscle [35, 37]) developing adverse metabolic effects, leading to a state of hyper secretion of adipokines that mediate auto/paracrine and endocrine communication, mediating inflammation and infiltration of macrophage cells. Hypertrophied adipocytes undergo necrosis and release free fatty acids which are suggested to activate macrophage infiltration and inflammation in adipose tissue. In the end, AT dysfunction is characterized by predominant visceral (ectopic) fat accumulation, changes in the cellular and intracellular matrix composition of adipose tissue [38, 39], enlarged adipocytes [18], increased autophagy [40], apoptosis [41] and increased number of AT infiltrating immune cells [42, 43] which possibly leads to chronic inflammation.



**Figure 8:** Adipose tissue dysfunctions and potential progression of inflammation.

### 1.4.1 Hypoxia:

Expansion or increase in adipose tissue is not compensated with the required blood flow and proper vascularization [44, 45]. Obese persons are often observed with poor cardiac output and blood flow to the adipose tissue compared to the lean persons [46-48]. Increase in adipose tissue growth results in clustering of mature adipocytes lacking proper vascularisation and with this initiate the potential inflammation process. Capillary density is also found to be significantly lower in visceral and subcutaneous adipose tissue from obese person, accompanied by larger vessels [49]. Hypoxia in obese adipose tissue leading to initiation and progression of inflammation is directly linked with low PO<sub>2</sub> (Interstitial partial pressure) [50]. This was verified directly by measuring oxygen tension and hypoxia probe marker system. Hypoxia probing using pimonidazole by immunostaining or western blot has shown increased hypoxia in epididymal and parametrial adipose tissue depots [51-53]. In one study, the level of PO<sub>2</sub> is found to be low in obese persons compared with lean: 47 mmHG vs 55 mmHG [47]. In vitro studies on adipocytes show that experimental hypoxia stimulates secretion of number of inflammation-related adipokines, including angiopoietin-like protein 4, IL-6, leptin, macrophage migration inhibitory factor (MIF-1) and vascular endothelial growth factor (VEGF) [54]. Hypoxia induces inflammation through activation of two major transcription factors, HIF-1 $\alpha$  and NF $\kappa$ B, each of which activates transcription of a variety of angiogenic and/or pro-inflammatory cytokines.

Hypoxia inducible factor 1 (HIF-1) is a key transcription factor expressed and upregulated during O<sub>2</sub> homeostasis in the cell. It regulates different cellular response during low PO<sub>2</sub> and often described as “master regulator for hypoxia”[55]. As a transcription factor, HIF-1 is found to regulate more than 70 genes during hypoxia by binding to cis-acting response elements. HIF-1 regulate genes corresponding to some of the major functions such as inflammation, apoptosis, differentiation, glucose utilization, angiogenesis, adipogenesis and matrix remodeling [55]. In adipose tissue, HIF-1 expression is directly proportional to adiposity, with increased HIF-1 level observed in obese mice [56]. The increase in HIF-1 transcription factor during hypoxia upregulates the expression of leptin, VEGF, macrophage migration inhibitory factor (MIF), IL-6 and downregulates haptoglobin, adiponectin in human adipocytes [54]. High fat diet with over expression of HIF-1 modulates increase in obesity, accompanying with larger adipocytes [57]. The adipose tissue hypoxia not only explains the origin of

inflammation in adipose tissue but it also provide the mechanism for pathological responses in the adipose tissue, such as ER stress [51, 58], oxidative stress [59], adipocyte death [60], adiponectin reduction [53, 61] and leptin induction [62].

#### **1.4.2 AT stresses:**

Due to uncontrolled AT expansion, ectopic fat accumulation and hypertrophied adipocytes, the AT experiences several forms of stresses. Adipocytes and other AT cells respond to these stresses by the activation of stress sensing pathways, which leads to cellular malfunction and contribute to the metabolic co-morbidities associated with obesity. These stress-responsive signaling pathways include kinases dedicated to transmitting stress response, like the “stress-activated protein kinases” p38MAPK and Jun N-terminal kinase (JNK). Except ERK and inhibitory-kB, p38MAPK and JNK are highly expressed and further more highly phosphorylated in omental compared to subcutaneous AT in obesity and omental fat from lean controls [63]. Increase in immune cell infiltration is also associated with increase in adipose tissue expansion and stresses. Other than adipocytes, infiltrated macrophages mediating stress link between adipose tissue and the risk of obesity induced complications [43]. In addition, systemic oxidative stress causes the imbalance between oxidant generation and antioxidant defense mechanisms, characterize the expanding adipose tissues in obese condition [64].

##### *1.4.2.1 Endoplasmic reticulum (ER) stress:*

Endoplasmic reticulum is a critical organelle which plays a crucial role in production and post translational modification of secretory and membranous proteins. With the assistance from molecular chaperones it folds the protein in proper conformation by making disulphide bonds, glycosylation and ships it to the final cellular destination. The ER maintains the cellular homeostasis and serves as the checkpoint for protein quality control. Any mistakes due to misfolding or unfolded proteins are screened out and end up in specialized ER associated degradation (ERAD) machinery and rapidly degraded by ubiquitin pathways. ER also hosts the production of neutral lipids and phospho-lipids. It also actively regulates lipid storage, including modulation of fatty acid uptake, storage of fatty acids as triglycerides and bundling of triglycerides into lipid droplets to serve as energy stores or for phospholipid synthesis.



ER experiences stress due to increase in load to its protein machinery during adipocytes hypertrophy and lipolysis. To deal with ER stress, cells activate the specialized stress sensors, PKR like ER kinase (PERK), activating transcription factor – 6 (ATF-6) and Inositol requiring ER-to-nucleus signal kinase-1a (IRE-1a) and their signaling pathways, collectively referred to as the unfolded protein response (UPR), which slows protein synthesis and promotes unfolded or misfolded protein degradation [65, 66]. In addition, the UPR also upregulates the transcription of genes encoding ER chaperones and folding catalysts to increase protein folding activity and promotes antioxidant responses to maintain tissue homeostasis. A short UPR activity reduces the ER stress and results in cell survival while prolonged UPR activities provoke complex responses by tumor necrosis factor receptor-associated factor 2 (TRAF-2) [67, 68]. TRAF-2 increases ER stress (promoting a reversal of translational attenuation) and activates inflammation (c-Jun N-terminal kinase, activator protein 1 (JNK-AP-1) [69], inhibitor kB kinase beta-nuclear factor kB (IKK $\beta$ -NFkB) and apoptotic signaling pathways [65].

ER stress is evidenced in obese adipose tissue and also it has been characterized by the increased presence of hypertrophic ER stressed adipocytes [70]. Adipocytes in response to ER stress activates inflammatory and apoptotic pathways resulting in insulin resistance (IR) in adipose tissue. In vitro, induction of the UPR in various cell types has been reported to cause increased expression of inflammatory genes, including IL-8, IL-6, MCP-1, and TNF- $\alpha$  [71]. Interestingly, several studies have demonstrated that free fatty acids have the potential to induce ER stress in various cells, including adipocytes in AT depots [72]. Adipocytes, probably through ER stress induced MCP-1 secretion which initiates the progression of macrophage infiltration into the adipose depots and thus initiating a cascade of chronic low grade inflammatory events.

#### *1.4.2.2 Mitochondrial and oxidative stress*

In state of obesity, cellular accumulation by excess triglycerides impairs cellular function and develops oxidative stress through increased ceramide formation which induces mitochondrial dysfunction and excess ROS formation and release [73]. Oxidative stress is defined as imbalance in levels of reactive oxygen species (ROS) vs the reducing substances that protect against damaging free radicals and peroxides. Mitochondria are double membrane cell organelles specialized in energy yielding macronutrients into ATP via oxidative phosphorylation.

Dysfunctional mitochondria leads to glucose and lipid handling disorders, pathological triglycerides accumulation (steatosis) ending up with lipotoxicity [74]. During excess caloric uptake, increased lipid uptake into the adipocyte produces increase in mitochondrial substrate load, subsequent increase in electron transport chain (ETC) activity and adverse ROS production.

ROS are generated during glucose or free fatty acids oxidation by mitochondria and from other metabolic process in the cell. This oxidative stress damages cellular structures and triggers an inflammatory response [75, 76]. Under hyperglycemic conditions, FFA or TNF $\alpha$  treatment has been reported to stimulate mitochondrial dysfunction, ROS accumulations [77] and enhanced proinflammatory cytokine secretion [78] and decreased insulin sensitivity in 3T3-L1 adipocytes [79, 80]. The inflammatory cytokines in turn induce and worsen the oxidative stress and more ROS release. The increase in ROS directly inhibits oxygen consumption in adipocytes and this result in enhanced lipid accumulation [81]. This develops a state of oxidative stress which further leads to down regulation of PPAR $\gamma$  which impairs adipocyte differentiation and adiponectin secretion [82]. The oxidative stress is associated with adiposity, insulin resistance and metabolic syndrome [82, 83] and further suggested that it could be an early event in the pathology of these chronic diseases.

### **1.4.3 Autophagy**

As autophagy is the cellular protective mechanism against various stresses and metabolic dysfunctions, it has been implicated in the pathophysiology of obesity and its related metabolic co-morbidities [40]. Knockdown studies of autophagy related gene ATG7 revealed that autophagy contributes to the regulation of fat mass and the balance between white and brown adipocytes [84]. Autophagy is a part of adipogenesis process, where excessive mitochondria and other cytoplasmic organelles are engulfed in to auto-phagosomes for adipocytes remodeling [85, 86]. Further to that, ATG7 knockout mice are found to be lean and insulin-sensitive, have enhanced metabolic rate and they are found to be resistant to the development of obesity [87]. Moreover increased expression of autophagy genes directly correlates with the degree of obesity, visceral fat distribution and adipocyte hypertrophy [40]. Taken together autophagy activation plays major part in AT expansion by both involving in adipogenesis and also protects the tissue from stress and inflammation as the last attempt from cell death.

#### **1.4.4 Chronic inflammation**

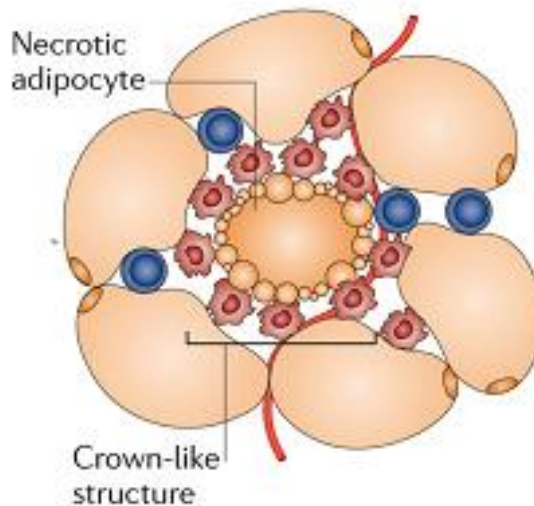
Excess fat accumulation than the physical need, impairs adipose tissue expansion and develops hypertrophy and hypoxia due to decrease in vascularization. The impairment of adipogenesis and angiogenesis along with adipocyte hypertrophy triggers vicious cycle of inflammation in subcutaneous adipose tissue and subsequent ectopic fat deposition. Hypertrophy reduces adipocytes life time due to excessive storage of lipids, leading to necrosis releasing all its cellular contents including huge amount of free fatty acids. This abnormal rupture of adipocytes is found to be more rapid in case of obesity, suggests to initiates the low grade inflammation process in adipose tissue. This metabolic dysfunction mediated inflammation in adipose tissues is perfectly orchestrated by mature adipocytes and stromal vascular cells including infiltrated macrophages.

The hypoxia along with ER stress upregulates both the expression and secretion of proinflammatory adipokines such as TNF, IL-6, MCP-1, VEGF, PAI-1, Angpt14 and decreases adiponectin [53, 88, 89]. Decrease in adiponectin develops pro-inflammation and pro-angiogenic response leading to destruction of insulin sensitivity. Additionally, macrophages are recruited to adipose tissue stress to investigate the dying adipocytes which results in the increase in inflammation.

##### **1.4.4.1 Macrophage infiltration and polarization:**

Improper adipocyte cell death with all its cellular components outside especially FFA drives macrophages to infiltrate the adipose tissue. These macrophages believed to scavenge all the free lipids and cellular components from dead adipocytes resulting in the formation of multinucleated “crown like structures”. This macrophage structural formation leading to chronic inflammation remains as the hallmark for obese adipose tissue. Macrophages infiltrate the adipose tissue by two possible mechanisms 1) macrophages can differentiate from bone-marrow-derived monocytes that reach the adipose tissue by diapedesis from the systemic circulation, 2) Macrophages can trans-differentiate from local adipose tissue preadipocytes and mesenchymal stem cells. The wide variety of chemoattractants such as CCL2, CCL5, macrophage inhibitory factor (MIF), macrophage inflammatory protein (MIP)-1a and macrophage colony-stimulating

factor (M-CSF) secreted from adipose tissue drives monocytes from the circulation into fat stores and contributes for macrophage differentiation [90-95].

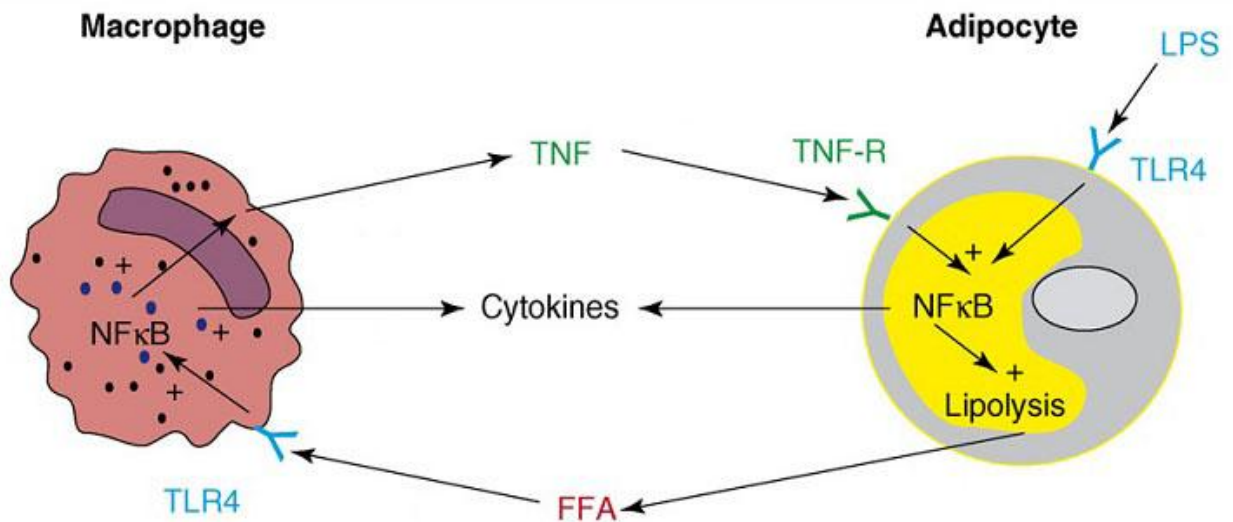


**Figure 9:** Adipose tissue under inflammation, dying adipocytes and infiltrating macrophages forming “crown like structures” [15].

More than 30% of adipose tissue genes encode similar to macrophages, giving an impression that preadipocytes and adipocytes could mimic as macrophages to induce the innate immune response [92, 96-98]. Interestingly, adipocytes and macrophages share macrophage-specific antigens and peroxisome proliferator-activated receptor (PPAR  $\gamma$ ) which controls the differentiation and function of both adipocytes and macrophages [99]. Therefore, it has been suggested that adipocytes and macrophages might be closely related and possibly interconvertible [96, 100]. Evidence demonstrated the trans-differentiation of preadipocytes into macrophages and acquired phagocytic activity [101]. The increase in HIF-1 during obesity inhibits PPAR  $\gamma$  and PPAR  $\delta$  which is an important differentiation factor for M2 polarization and for adipocyte differentiation. Interestingly in obese AT, expression of CD14, the cell marker for monocytes and macrophages observed in stromal vascular fraction evidence the presence of these kinds of cells in adipose tissue. Surprisingly the CD14 expression is also found in murine and human adipocytes, further enhance the role of adipocytes in innate immune response [98].

#### 1.4.4.2 Paracrine crosstalk between macrophages and adipocytes

WAT is also composed of immune cells such as macrophages, lymphocytes (natural killer, T helper, T regulatory and B lymphocytes) and mast cells [15]. Alternatively activated (M2) macrophages predominate in WAT of lean subjects, whereas in obesity, the adipose tissue becomes markedly infiltrated by macrophages polarized towards the classically activated, pro-inflammatory M1 phenotype [23, 102]. These immune cells participate in paracrine cross talks with adipocytes and regulated the innate and adaptive immunity in adipose tissue [23].



**Figure 10:** The paracrine crosstalk between adipose tissue macrophages and adipocytes [103].

The scavenging M1 macrophages undergo inflammation due to dying adipocytes, secreting inflammatory cytokines which induce more lipolysis. Paracrine crosstalk between adipocytes and infiltrated macrophages plays an important role in chronic inflammation process. Inflammatory cytokines released from macrophages acts in a paracrine manner by inducing inflammation in preadipocytes and adipocytes resulting in more proinflammatory cytokines secretion. Macrophages derived TNF alpha acts via TNF receptor in hypertrophied adipocyte inducing inflammation and lipolysis, resulting in cytokines and fatty acids release via NFκB dependent and independent mechanisms. It also further inhibits the preadipocyte differentiation by selectively activating TNF receptor 1 [104]. Among free fatty acids, especially saturated fatty acids are found to induce inflammation alone or in partner with other molecules in macrophages

[105, 106]. This paracrine mediated inflammation between adipocytes and macrophages are suspected to be mediated via, Toll like receptors (TLR) due to its active participation in inflammation and abundant expression in both adipocytes and macrophages [107]. This crosstalk between adipocytes and macrophages emphasize the role of adipocyte-derived saturated fatty acids as naturally available endogenous TLR4 ligands. In a clinical context, both the local release of fatty acids by adipocytes and the systemic increase in free fatty acids after a meal could activate TLR4 and subsequent signaling cascade. This mechanism provides a good explanation of why obesity has been defined as a chronic and low grade state of inflammation [108, 109].

## **1.5 Metabolic diseases**

### *1.5.1 Insulin resistance and Type II diabetes*

The low grade inflammation with varied adipokines secretion and fatty acids release disrupts insulin signaling, ending up with development of insulin resistance. As insulin is the main regulator for hormone sensitive lipase (HSL), the rate controlling enzyme for triglycerides hydrolysis, the inhibitory effect of FFA on insulin leads to enhanced lipolysis in adipocytes [110]. FFA and proinflammatory cytokines especially TNF during adipocyte lipolysis induce serine phosphorylation of insulin receptor substrate – 1 (IRS1) and insulin receptor substrate – 2 (IRS2), which reduce the capacity of insulin receptor substrate protein to be tyrosine phosphorylated and attenuates the insulin signaling cascade [111-113]. In parallel TNF also contributes to the insulin resistance by inhibiting the expression of genes such as CEBP, PPAR, glucose transporter type 4, insulin receptor substrate 1 and adiponectin, which are essential for insulin signaling and glucose uptake [32, 113, 114].

Higher concentrations of circulating free fatty acids and triglycerides are associated with lipid accumulation in multiple tissues, including liver, skeletal muscle, heart, and pancreatic  $\beta$ -cells. In skeletal muscle, accumulation of intracellular free fatty acids reduces insulin receptor substrates, with resulting skeletal muscle insulin resistance [112]. These processes lead to a cycle of increasing dysfunction: higher circulating free fatty acids induce intracellular lipid accumulation and lead to peripheral insulin resistance, resulting in increased insulin secretion [115], whereas peripheral insulin resistance results in attenuated lipolysis of circulating chylomicrons and triglycerides [116]. FFA and long chain fatty acyl CoA open potassium

channels in the  $\beta$ -cells of pancreas disrupts the insulin secretion and this dysfunction is the most important risk factor for type II diabetes [117, 118]. It also reduces the ATP production necessary for insulin secretion by enhancing the expression of uncoupling protein 2 (UCP-2) [119]. In addition, FFA induce beta cell apoptosis via ER stress [118, 120] and by inhibiting the expression of Bcl-2, an anti-apoptotic factor [120].

### *1.5.2 Vascular diseases:*

Release of FFA and proinflammatory cytokines from expanded adipose tissue mediates systemic inflammation and plays an important role in the development of vascular associated diseases such as hypertension, atherosclerosis, endothelial and myocardial dysfunction [121]. The circulatory FFA and adipokines such as IL-6, TNF- $\alpha$ , VEGF, angiotensinogen and PAI-1 increases endothelial inflammation and release of adhesion molecules, facilitating platelets aggregation, stimulating pro-thrombic state and endothelial dysfunction [122] The low plasma level of adiponectin marked the adipocyte dysfunctions leading to the development of vascular diseases [123]. The increase in adipokine secretion during AT expansion elevates CRP levels in endothelial cells and causes dysfunction in the form of inappropriate vascular constriction / relaxation. CRP has been shown to cause induction of endothelial adhesion proteins ICAM-1, VCAM-1, E-selectin, P-selectin and angiotensin type 1 receptor [124]. In addition CRP amplifies vascular inflammation by the activation of endothelial NF- $\kappa$ B and induction of IL-1 $\beta$ , PAI-1, IL-6, TNF- $\alpha$  and MCP-1 [125].

### *1.5.3 Cancer:*

Being overweight or obese increases the risk of developing different types of cancer such as colon [126], kidney, prostate, breast [127-129], endometrial [130] and in addition, obesity is associated with increased risk of death [126, 131]. The increase in proinflammatory cytokines and leptin produced by obese adipose tissue activates multiple cancer-related signaling pathways, including phosphoinositide 3-kinase/protein kinase B (PI3K/AKT), mitogen-activated protein kinase (MAPK), signal transducer and activator of transcription 3 (STAT3), and mammalian target of rapamycin (mTOR) [132-134]. The paracrine activation of cytokines pathways promotes tumor cell proliferation [135, 136]. In breast cancer, the crown-like structures of necrotic adipocytes surrounded by macrophages were increased in the mammary gland with high

levels of proinflammatory mediators and NF $\kappa$ B activation [137]. Cytokines upregulated during obesity also promotes self-renewal, survival and proliferation and differentiation of cancer stem cells (CSC) [138].



## **2. TLR regulates inflammation**

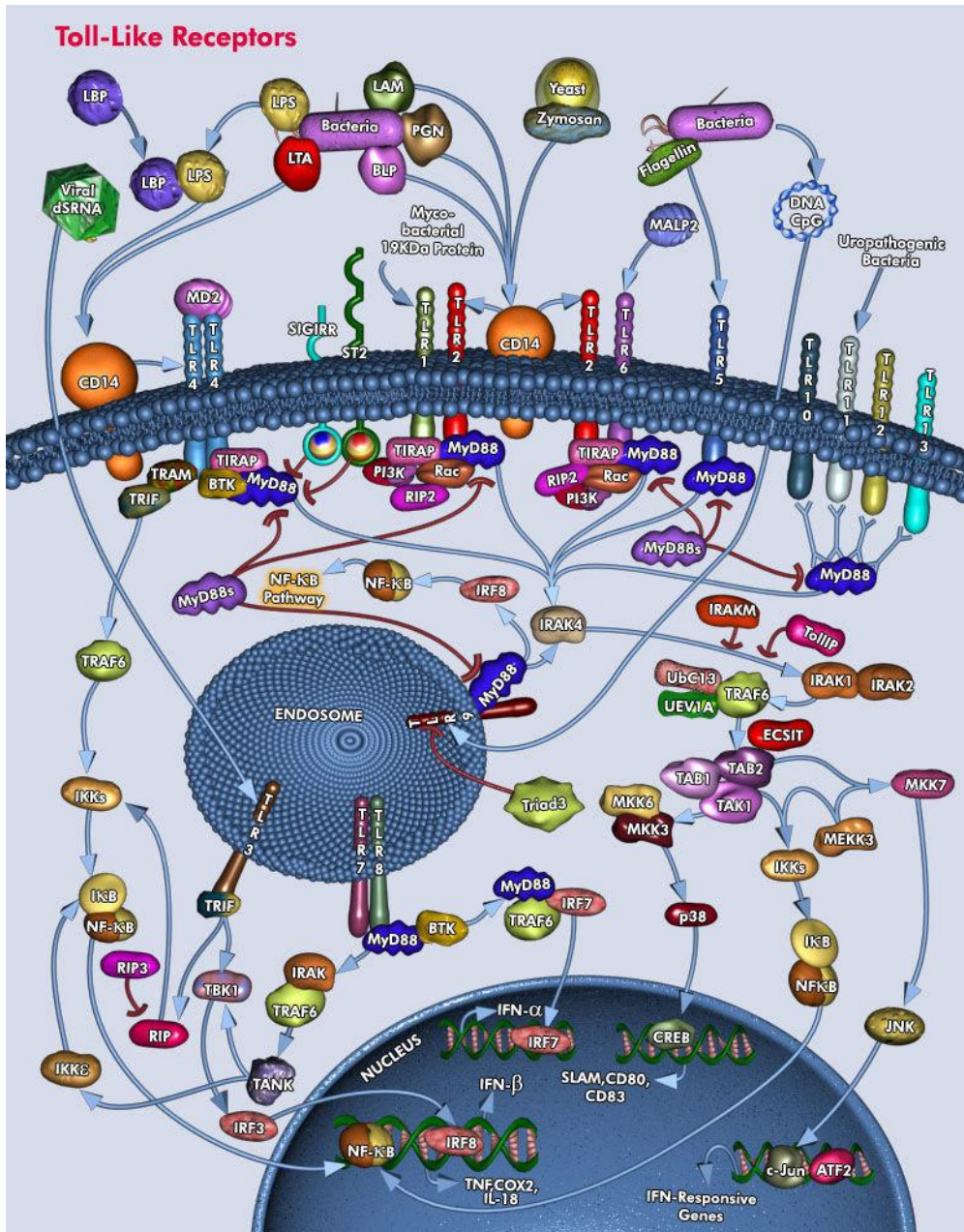
---

Toll like receptor (TLR) named after “Toll”, a homologous protein found from *Drosophila melanogaster* which regulates anti-fungal immunity in the fly [139]. It was later realized that toll activates a transcription factor called dorsal, which is a homologue of NFκB known for its inflammatory signal activation. The first TLR was reported by Normura *et al* in 1994 [140] and four more in 1998 [141]. Among that TLR4 is found to recognize bacterial component answers the innate immune response to protect against bacteria leading to sepsis. Later TLR1, 2, 4, 5 and 6 was identified to recognize various bacterial, fungal and mycobacterial products while viral nucleic acids are found to be recognized by TLR 3, 7, 8 and 9.

TLRs are type I integral membrane glycoproteins belonging to the same superfamily as IL-1 receptor. TLR and IL-1 receptor have a conserved region of ~200 amino acids in their cytoplasmic tails, which is known as the Toll/IL-1R (TIR) domain. They differs from each other through its distinct leucine rich repeats motifs (LRR) in the extracellular region. The subcellular localization of different TLRs correlates to some extent with the molecular patterns of their ligands. TLRs belong to pattern recognition receptors (PPR's), able to recognize multiple pathogens associated molecular patterns (PAMP's) such as bacterial cell wall fragments. TLR activation allows the host cell to distinguish self-molecules from pathogen-associated non-self structures or endogenous danger signals before initiating a defensive immune response. TLRs detect various PAMP's, including LPS (TLR-4), bacterial lipoproteins and lipoteichoic acid (TLR-2), flagellin (TLR-5), unmethylated CpG DNA of bacteria and viruses (TLR-9), double-stranded RNA (TLR-3), and single-stranded viral RNA (TLR-7). TLR1, TLR2 and TLR4 are located on the cell surface and are recruited to phagosomes after activation by their respective ligands. By contrast, TLR3, TLR7-9, all of which are involved in the recognition of nucleic-acid-like structures is not expressed on the cell surface. Instead like TLR9, they are expressed in endoplasmic reticulum and when get activated, gets recruited to endosomal/lysosomal compartments.

After ligand binding, the TLR/IL-1R dimerize and undergo conformational change to facilitate the recruitment of downstream signaling adaptor molecules. The adaptor molecules are myeloid differentiation primary-response protein 88 (MyD88), IL-1R-associated kinases (IRAKs), transforming growth factor-β (TGF-β)-activated kinase (TAK1), TAK1-binding protein 1 (TAB1), TAB2 and tumour-necrosis factor (TNF) - receptor-associated factor 6

(TRAF6). The TLR activation leads to sequence of intracellular signals with the help of above mentioned adaptor molecules and ends up with NFkB activation and nuclear translocation [142]. Finally NFkB activation induces the gene upregulation and secretion of pro-inflammatory cytokine such as IL-6, TNF and MCP-1. TLR signalling also ends up with MAPK pathway through TAK1 activation, including JUN N-terminal kinase, JNK.



**Figure 11:** Toll like receptors, ligands and their signaling pathways. (www.qiagen.com)

## 2.1 Adipocytes TLR expression and inflammation

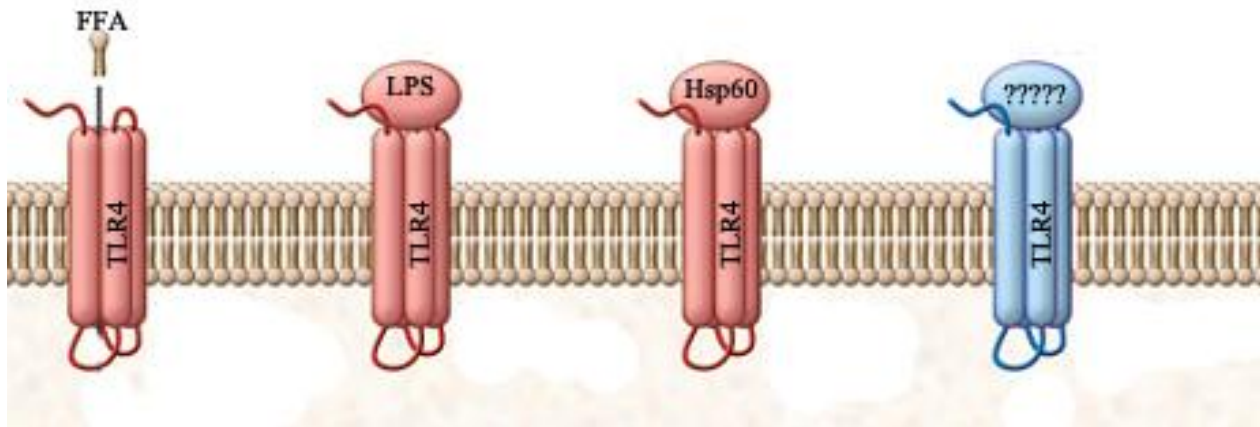
TLR4 and TLR2 knockout mice models under HFD results in less fat pad and showed no inflammation in the adipose tissue [143, 144]. Furthermore, both TLR2 and TLR4 expression are increased in adipose tissue in association with obesity and noninsulin dependent diabetes mellitus. Recent studies indicate that C3H/HeJ mice, which have a loss-of-function mutation in TLR4, are protected against the development of diet-induced obesity (DIO), insulin resistance and there is also an attenuation of obesity induced inflammation [145]. TLR-4 deficient C57BL/10ScN mice fed with diet based on saturated fats were protected against DIO [143]. All these experimental evidences strongly indicate that TLR-2 and TLR-4 play an important role in inducing inflammation, leading to insulin resistance and cardiovascular disease.

The image of adipose tissue as a fat storage organ has changed long back after its ability to secrete cytokines which induce inflammatory innate immune response, classifying as an endocrine organ. TNF $\alpha$  is the first proinflammatory cytokine found to be released from mouse adipocytes [32]. Its varied expression between fat and lean mice adipose tissue marked the importance of its role in adipose tissue inflammation and insulin resistance. Adipocytes are the descendants of pluripotent mesenchymal stem cells which are found to express TLR1 to 6 and TLR9 similar to monocytes and macrophages [146]. Preadipocytes and mature adipocytes from mice are found to express wide variety of TLRs (TLR 1 to 9), when activated results in the production of IL-6 [147, 148]. Moreover ob/ob mice (leptin deficient obese mice) and db/db mice (mice lacking the long and functional isoform of the leptin receptor) show the broadest mRNA expression profile of TLRs in preadipocytes and mature adipocytes [147]. The proinflammatory response is more rapid in preadipocytes compared to adipocytes when induced with LPS [149]. Inflammation is also found to impair the adipocytes differentiation resulting in more preadipocyte accumulation [149, 150]. The TLR expression varies during adipocyte differentiation, more expression of TLR2 at early stages and TLR9 at later stages of the differentiation process [151]. Increase in TLR2 expression is observed in murine adipocytes when stimulated with LPS, yeast cell wall zymosan or TNF [152]. TLR2 production is also found to be induced in murine adipose tissue by a high-fat diet and directly by fatty acids in murine 3T3-L1 adipocytes [153].

TLR4 and TLR2 expression in human mature adipocytes was first reported by our team in 2006 and its expression levels are comparatively higher than monocytes [154]. In that study, TLR4 and TLR2 functionality was tested by inducing with its respective ligands LPS and Pam3Cys, which results in the up regulation of TNF at both gene and protein level. Also the significance of these receptors during inflammation is tested by blocking them specifically by neutralizing antibodies that result in low inflammation. Further we identified that soluble CD14 and LBP were provided from the fetal bovine serum which is necessary for the ligand binding to TLR4 and TLR2. LPS and Pam3Cys also induced the expression of both TLR4 and TLR2 through NFkB activation in human adipocytes which further supports our study [155]. Also important clinical studies performed on humans for the first time reveals that, the injection of LPS induced both systemic and adipose tissue levels of TNF and IL-6 [156].

Later broad TLR expression profile from human subcutaneous abdominal adipocytes reveals TLR expression analysis, SVCs and mature adipocytes of human subcutaneous and visceral adipose tissue express only TLR1 to TLR4 and TLR6, whereas the total human adipose tissue expresses all TLR family members (TLR1 to TLR10) [157]. It has been suggested that the expression of TLR5 and TLR7 to TLR10 in total adipose tissue is caused by nonadipocyte cells (stromal cells, endothelial cells, immigrated monocytes). TLR4 is highly expressed in adipose tissue in comparison with other available receptors and its increase in expression correlates with inflammation and the onset of obesity. Later my team has identified that LPS induced TNF alpha production in human adipocytes is dependent upon NFkB and p38 MAPK pathways [158]. Stimulation of TLRs initiates intracellular signaling cascades resulting in downstream NF-κB and mitogen-activated protein kinase activation and production of proinflammatory cytokines and chemokines associated with metabolic dysfunctions. All these data shows the functional existence of TLR pathway in adipocytes which is involved in the innate immune response and adipocytes functions.

**2.2 Adipose tissue inflammation, what pays the toll???** As most of the metabolic dysfunctions in adipose tissue and its cells are passing through toll, mainly TLR4, the hunt for physiological trigger or stimuli that activate TLR4 is not deciphered completely. However few possible mediators like PAMPs and DAMPs are suggested from the recent studies are discussed below.



**Figure 12:** Possible TLR4 ligands for adipose tissue inflammation.

### 2.2.1 Bacterial LPS – a possible trigger

Lipopolysaccharide (LPS), from the outer cell membrane of gram negative bacteria is a well known characterized PAMPs and TLR4 ligand stimulating the innate immune response. LPS is been used to stimulate an inflammatory response, as a positive control, in many in vitro experiments. It is also well documented that endotoxin stimulates the innate immunity pathway through the activation of TLRs via several proteins, including the LPS binding protein (LBP), CD14 and myeloid differential protein 2 (MD2). This TLR mediated signaling further leads to intracellular activation of NFkB, proinflammatory genes expression and secretion. The mammalian intestine is crawling with trillion of microorganisms collectively called gut flora. Among that, the bacteria's are the most abundant one evolved within the host in a symbiotic relationship. Recently many studies considered how the gut-derived endotoxin affects metabolic function leading to systemic inflammation. Also bacterial LPS activation of TLR4 in human and mouse adipocytes results in the upregulation of TLR2, which corresponds with high fat diet induced TLR2 expression in adipocytes [159].

#### *Gut microbiota – the source of LPS*

The gastrointestinal tract is the potential source of inflammation associated with altered gut microbiota during obesity. There is an increase in plasma concentration of LPS during a fat-enriched diet defined as metabolic endotoxemia [160, 161]. The enteric bacteria's are found to participate in diet induced obesity as the germ free mouse remains lean even though when fed with high fat diet and do not become insulin resistant compared to the normal mice [162]. Gut bacteria metabolize indigestible polysaccharides to generate short-chain fatty acids and monosaccharides and promote their absorption and storage as fat. In addition, gut bacteria increase vascularization and blood flow within the mucosa, facilitating nutrient absorption. Gut bacteria also suppress the expression and release of fasting induced adipose factor (Fiaf) from small intestine, resulting in increased activity of lipoprotein lipase (LPL) in adipocytes and enhanced storage of liver-derived triglycerides [163]. Endotoxin is known to have a strong affinity for chylomicrons (lipoproteins that transport dietary lipids including long chain SFAs through the gut wall) and, as such, can cross the gastrointestinal mucosa coupled to damaging lipoproteins [164, 165]. Once in the circulation, endotoxin has been shown to mediate metabolic dysfunction in several tissues including adipose tissue, liver and muscle [166, 167].

Diet modulates the population of bacterial colonies in the intestine from gram negative (Bacteroidetes) to gram positive (Firmicutes) ratio increases during HFD [168]. HFD in association with commensal gut microbiota promotes intestinal inflammation in multiple cell types. HF diet increases the proportion of lipopolysaccharide (LPS) containing gut bacteria and increases intestinal permeability leading to elevated circulating LPS concentrations [169]. In normal circumstances, only small amounts of endotoxin cross from the intestinal lumen into the systemic circulation. Usually, the absorbed endotoxin will be rapidly removed by monocytes, particularly resident Kupffer cells within the liver. However during obese condition due to ectopic fat deposition, resulting in fatty liver has diminished its capacity to remove the endotoxin, which can directly aggravate liver disease exacerbated by weight gain. Intestinal inflammation results in proinflammatory molecules released by these cells are ideally placed to enter the hepatic circulation and develop impact on development of systemic inflammation. Onset of intestinal inflammation precedes diet-induced increases in body weight, fat mass, insulin resistance and increased TNF- $\alpha$  expression and secretion [170].

### **2.2.2 Hsp60 - the mediator of adipose tissue inflammation**

Heat shock protein (Hsp60) is a mitochondrial chaperonin protein responsible for transportation and refolding of proteins from cytoplasm into the mitochondrial matrix. Recently it has been evidenced that Hsp60 acts as a danger signal and also plays a role in innate immune response [171, 172]. Hsp60 is confirmed to be released during stress and inflammation in different cells [173]. It also has the capability to activate inflammatory response in monocytes, macrophages, T cells, B cells and dendritic cells, resulting in the secretion of various proinflammatory cytokines [174-177]. Hsp60 activation of TLRs varies from cell to cell and induces specific immune responses. It activates macrophages and B- cells inflammation and immune response via TLR4-MyD88 [177, 178], T-cells via TLR2 [174].

Hsp60 is secreted in the adipose tissue and more specifically by adipocytes, further categorized Hsp60 as an adipokine [179]. Hsp60 has been shown to induce inflammation for the first time in obese mouse adipocytes and 3T3-L1 resulting in the release of IL-6, CXCL-1 and MCP-1 [180]. Later human preadipocytes and adipocytes are identified to release Hsp60 in response to LPS, TNF and IL-1 beta mediated inflammation but not IFN $\gamma$  [179]. This gives a clue, how macrophages exhibit paracrine effect with adipocytes leading to chronic low grade



inflammation. Hsp60 is found to be higher in obese human with or without diabetes compared with lean persons suggesting adipose tissue participation in the circulating level of Hsp60 [179]. It is also defined that the activation of TLR4 but not TLR2 induces the release of Hsp60 from adipocytes. However the TLR responsible for Hsp60 mediated autocrine effects on adipocytes inflammation is not yet characterized.

### **2.2.3 Free fatty acids**

Free fatty acids (FFA) are released during lipolysis from adipocytes, where intracellular triglycerides are broken down to FFA and glycerol. These FFA enters the blood circulation and transported to liver (triglyceride synthesis) and skeletal muscle (energy production) in normal condition. During fasting, exercise and aggression, increased lipolysis supplies FFA as a source of energy for brain, liver, heart and skeletal tissues. This organized regular mechanism of lipolysis exceeds the limit during obesity, resulting in chronic release of FFA in the blood circulation. This increase in FFA flux induces and aggravates liver and skeletal muscle insulin resistance. Visceral adipose tissues are considered as the major source of FFA due to higher lipolysis as the consequence of increased fat accumulation. Among FFA, saturated fatty acids (SFA) secreted by hypertrophied adipocytes are identified to induce inflammation in macrophages with TNF secretion. These TNF acts in a paracrine manner on adipocytes through TNF receptor and undergoes inflammation which induces further lipolysis. Among FFA, the poly unsaturated fatty acid (PUFA) is found to protect against inflammation process and inhibits the insulin resistance.

TLR mediated inflammation leading to insulin resistance has been confirmed in various cells through different studies. As both adipocytes and macrophages are found to express active TLR4 and TLR2, FFA are doubted to act via these receptors. In macrophages and adipocytes cell lines, SFA such as palmitic acid (PA) and lauric acid (LA) are shown to induce NFkB mediated secretion of proinflammatory cytokines [105, 181]. Especially these SFA induced inflammation has been suggested to act via TLR4 in adipose tissue and adipocytes. In contrary, Erridge and Samani has described that SFA mediated TLR4 activation and inflammation is due to the endotoxin contaminants present in the BSA which is used to conjugate the FFA [182]. In their studies through different cell models including adipocytes, endotoxin free BSA alone or

conjugated with SFA are not able to induce inflammation. Moreover they have detected the presence of LPS contaminants in various BSA which was used in previous studies.

Their results challenged the whole idea and belief of SFA mediating inflammation through TLR4. This interests us to directly investigate the effects of SFA and PUFA on inflammation process in primary culture of human adipose tissue and adipose cells. In the following study we have described whether FFA is able to bind and activate TLR4 or TLR2 by *in vitro* studies in human adipose tissue and mature adipocytes.

RESEARCH

Open Access

# Fatty acids do not pay the toll: effect of SFA and PUFA on human adipose tissue and mature adipocytes inflammation

Ravi Kumar Murumalla<sup>†</sup>, Manoj Kumar Gunasekaran<sup>†</sup>, Jibesh Kumar Padhan, Karima Bencharif, Lydie Gence, Franck Festy, Maya Césari, Régis Roche<sup>†</sup> and Laurence Hoareau<sup>\*†</sup>

## Abstract

**Background:** On the basis that high fat diet induces inflammation in adipose tissue, we wanted to test the effect of dietary saturated and polysaturated fatty acids on human adipose tissue and adipocytes inflammation. Moreover we wanted to determine if TLR2 and TLR4 are involved in this pathway.

**Methods:** Human adipose tissue and adipocytes primary cultures were treated with endotoxin-free BSA conjugated with SFA (lauric acid and palmitic acid - LA and PA) and PUFA (eicosapentaenoic acid, docosahexaenoic acid and oleic acid - EPA, DHA and OA) with or without LPS. Cytokines were then assayed by ELISA (TNF-alpha, IL-6 and MCP-1). In order to determine if TLR2 and TLR4 are activated by fatty acid (FA), we used HEK-Blue cells transfected by genes from TLR2 or TLR4 pathways associated with secreted alkaline phosphatase reporter gene.

**Results:** None of the FA tested in HEK-Blue cells were able to activate TLR2 or TLR4, which is concordant with the fact that after FA treatment, adipose tissue and adipocytes cytokines levels remain the same as controls. However, all the PUFA tested: DHA, EPA and to a lesser extent OA down-regulated TNF-alpha, IL-6 and MCP-1 secretion in human adipose tissue and adipocytes cultures.

**Conclusions:** This study first confirms that FA do not activate TLR2 and TLR4. Moreover by using endotoxin-free BSA, both SFA and PUFA tested were not proinflammatory in human adipose tissue and adipocytes model. More interestingly we showed that some PUFA exert an anti-inflammatory action in human adipose tissue and adipocytes model. These results are important since they clarify the relationship between dietary fatty acids and inflammation linked to obesity.

**Keywords:** FFA, Human adipose tissue, Inflammation, TLR

## Background

Recent decades, overnutrition-related diseases are increasing in developed countries due to inappropriate diets. Indeed high fat diet regulates some metabolic dysfunctions which lead to low grade inflammation, long before the onset of overweight and obesity [1]. Specifically it has been shown that high fat diet leads to hypertrophy and inflammation in adipocytes [2,3], which release proinflammatory cytokines and free fatty acids

via lipolysis [4]. These circulating free fatty acids released into plasma through lipoproteins, specifically saturated fatty acids trigger inflammation within adipose tissue [5] and set the platform for the emergence of cardiovascular diseases such as atherosclerosis [6,7]. This led researchers to study the inflammatory properties of various fatty acids. It is thus well proved in numerous cell types and animal models that polyunsaturated fatty acids (PUFA) exhibit protective effect on health by diminishing the secretion of proinflammatory cytokines [8-11] while on the other hand saturated fatty acids (SFA) promote inflammation by increasing secretion of proinflammatory cytokines [12-14].

\* Correspondence: laurence.hoareau@adipsculpt.com

<sup>†</sup>Equal contributors

GEICO (Study Group on Chronic Inflammation and Obesity), Platform 'Cyclotron Reunion Ocean Indien' CYROI, 2, rue Maxime Rivière, Sainte-Clotilde, Reunion Island 97 490, France



As adipose tissue is a major reservoir for fatty acids, it is particularly interesting to know the exact effect of fatty acids specifically on this particular tissue. Moreover adipose tissue is highly involved in chronic low grade inflammation [15-17] by the recruitment of macrophages and also by secreting proinflammatory cytokines such as TNF-alpha, IL-6 and chemokines such as MCP-1 collectively referred as adipokines [17]. For many years the principal cells of this tissue, mature adipocytes, were underestimated in their role in inflammation and the work of our team has reinforced the idea that these cells are largely responsible for low grade inflammation [18]. Indeed adipocytes are able to secrete various kinds of cytokines long before the infiltration of macrophages and some of these cytokines are secreted at higher levels compared to macrophages. In addition it has been proved that this inflammation is mediated in part by the presence of TLR2 and TLR4 on adipocytes [18].

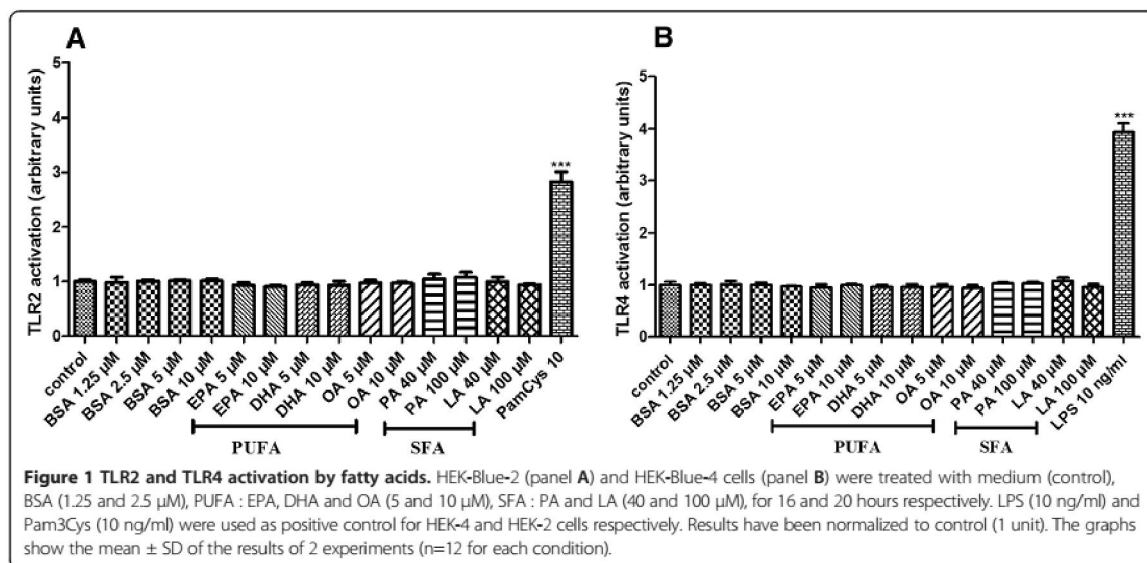
Confrontation of several studies leads to think that SFA could induce inflammation through TLR2/4 by leading to secretion of proinflammatory cytokines in a dose-dependent manner in different cells such as RAW264.7 [5,19], THP-1 [6,20], dendritic cells [21] and 3T3-L1 adipocytes [12]. Although these studies do not show a direct link between SFA and TLR2/4, it is quite possible since the extracellular domain of TLRs includes a leucine rich repeat (LRR) region which allows the association with hydrophobic ligands such as fatty acids, for example [22]. However, these data are controversial since Erridge and Sammani [23] have proved that this fatty acids mediated inflammation is due to endotoxin contaminants present in the BSA used to complex fatty acids. This raises a question on all data concerning the

signalling pathway of dietary fatty acids. Thus the purpose of this study is to determine the inflammatory role of dietary and endotoxin free PUFA and SFA on human adipose tissue in primary culture. We determined whether this effect is specifically mediated by mature adipocytes isolated from this tissue and through this study we aim to confirm the involvement of TLR2 and TLR4 in this process.

## Results

### TLR2 and TLR4 activation by fatty acids

In order to determine if fatty acids can activate TLR and also to test the possible presence of endotoxin in the BSA or media used for the experiments, we tested medium 199 with BSA on transfected HEK-293 cells (Figure 1). We could not detect activation of TLR2 and TLR4 on the HEK-2 (Figure 1A) and HEK-4 (Figure 1B) cells which cleared any doubt about possible presence of any endotoxin in the experimental set up. Based on this, we checked if SFA such as palmitic acid (PA) and lauric acid (LA) have the ability to activate TLR2 and TLR4. Our results show that both PA and LA did not lead to sAP activation which means that they do not activate TLR4 nor TLR2. Next we tested PUFA such as DHA, EPA and OA on both HEK-2 and HEK-4 to determine whether they could activate TLR2 and TLR4. Interestingly none of the fatty acids that we have used in our experiments was able to activate neither TLR2 nor TLR4. Treatment of HEK-2 and HEK-4 cells by Pam3Cys and LPS, natural ligands for TLR2 and TLR4 respectively, led to sAP activation which further validates the model.

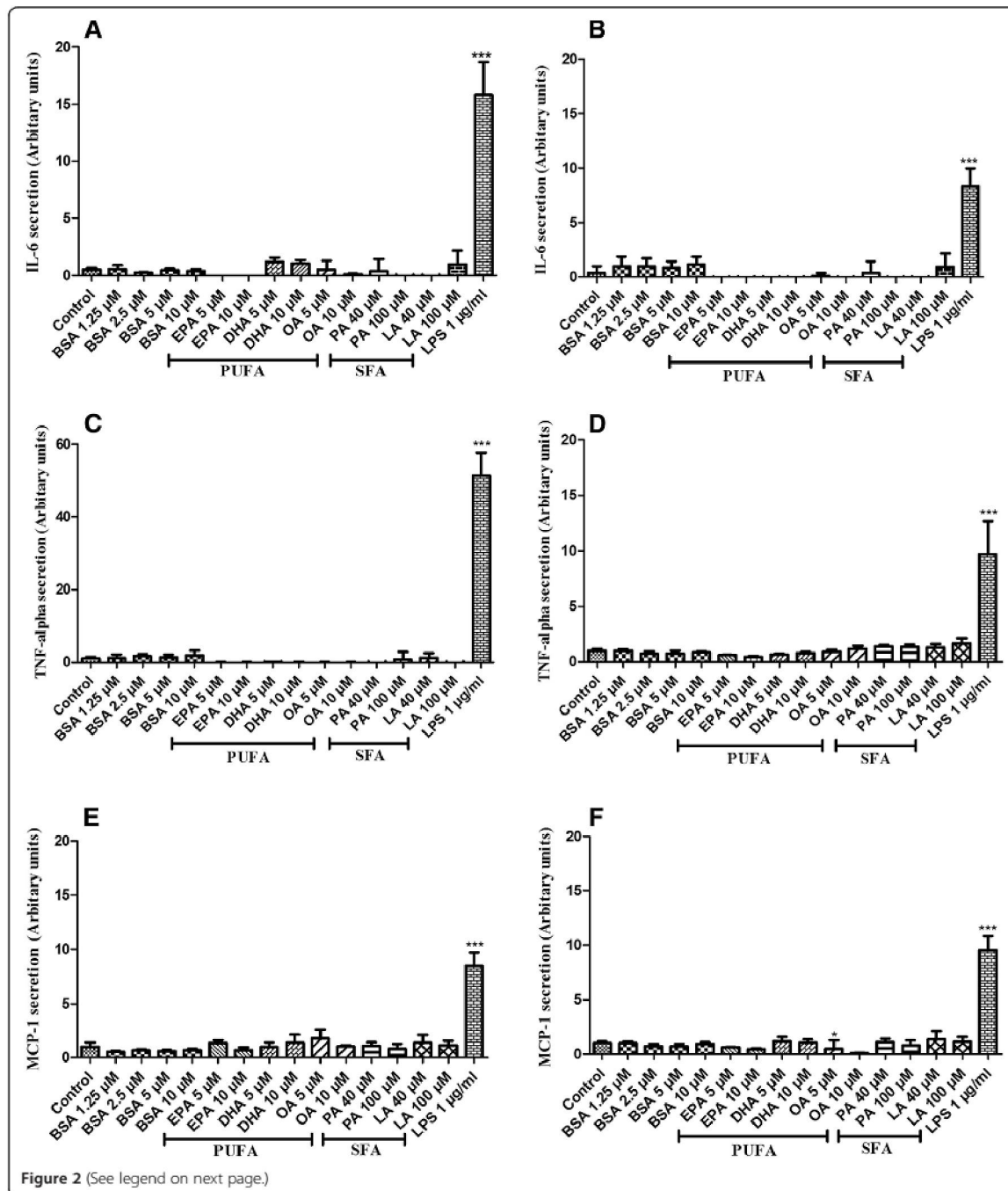




**Proinflammatory effects of SFA and PUFA on human adipose tissue and adipocytes**

In order to check whether dietary free fatty acids (SFA and PUFA) can promote inflammation on human adipose tissue and mature adipocytes, cytokines such as IL-

6, TNF-alpha and MCP-1 were measured from the media after incubation with BSA conjugated fatty acids. In Figure 2, we can see that levels of IL-6, TNF-alpha and MCP-1 in media after incubation with PUFA and SFA remained same as the negative controls (incubation



(See figure on previous page.)

**Figure 2 Proinflammatory effects of SFA and PUFA on human adipose tissue and adipocytes. A and B:** IL-6 secretion in the medium of adipose tissue (A) and adipocytes (B) culture after treatment with PUFA and SFA for 12 hours with LPS (1 µg/ml) as positive control. Results were expressed in arbitrary units normalised to control (1 represents from 15 to 20 ng/ml IL6, depending on the patient). The graph shows the mean ± SD of the results from 4 patients (n=6 for each condition, for each patient). **C and D:** TNF-alpha secretion in the medium of adipose tissue (C) and adipocytes (D) after treatment with PUFA and SFA for 6 hours, with LPS (1 µg/ml) as positive control. Results were expressed in arbitrary units, normalised to control (1 represents from 0.02 to 0.05 ng/ml TNF-alpha, depending on the patient). The graph shows the mean ± SD of the results from 4 patients (n=6 for each condition, for each patient). **E and F:** MCP-1 secretion in the medium of adipose tissue (E) and adipocytes (F) after treatment with PUFA and SFA for 12 hours, with LPS (1 µg/ml) as positive control. Results were expressed in arbitrary units, normalised to control (1 represents from 10 to 15 ng/ml MCP-1, depending on the patient). The graph shows the mean ± SD of the results from 3 patients (n=6 for each condition, for each patient).

only with media or BSA). This shows that both PUFA and SFA were not able to induce inflammation in adipose tissue nor specifically in adipocytes. These results are in accordance with the previous results from HEK-Blue cells which further confirms no activation of TLR2 and TLR4.

#### Anti-inflammatory effects of PUFA on human adipose tissue and adipocytes

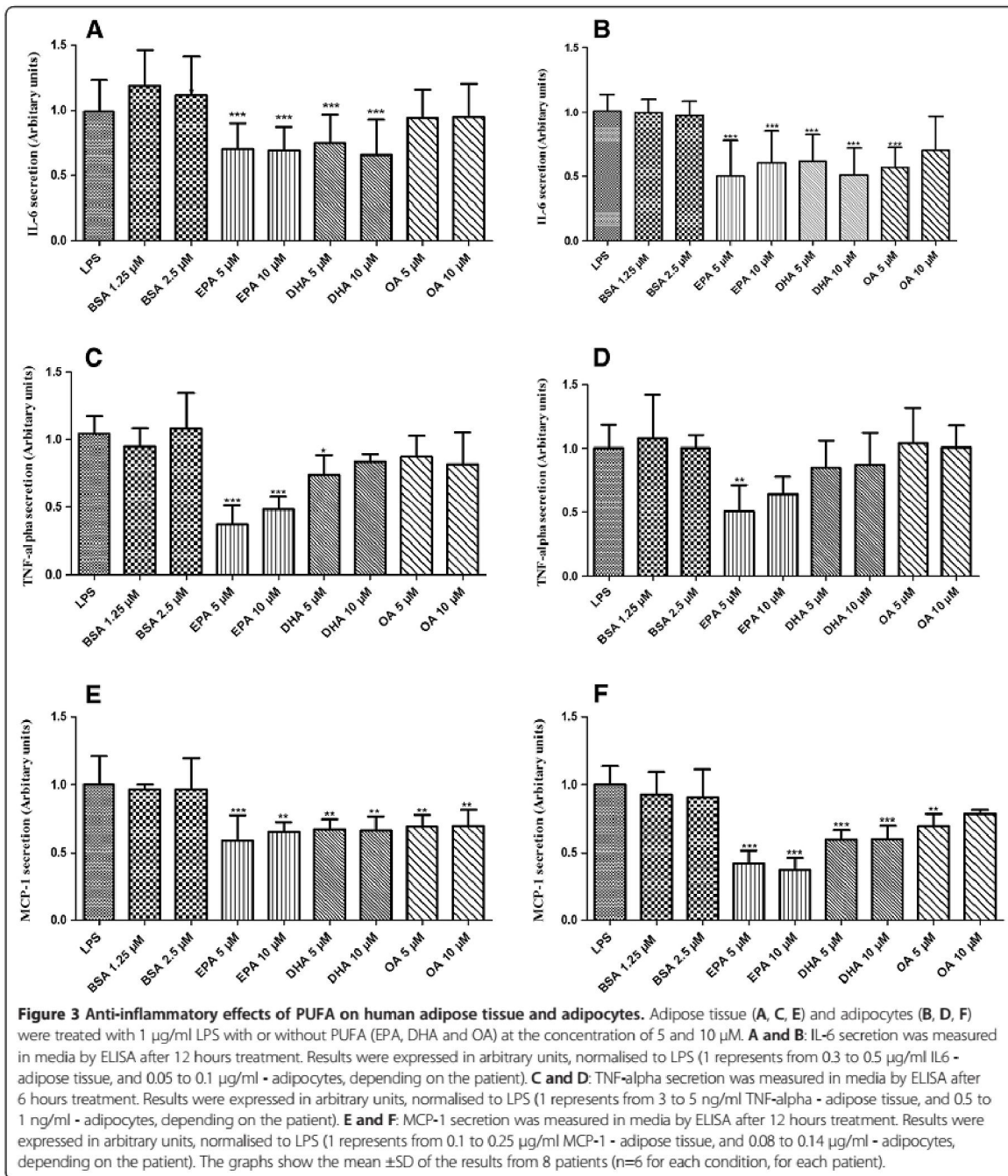
It has been reported that PUFA such as DHA and EPA showed anti-inflammatory effects on LPS induced 3T3-L1 adipocytes [24] and macrophages [8,11]. Then in order to check the anti-inflammatory effect of PUFA, adipose tissue and adipocytes were co-incubated with LPS and either DHA, EPA or OA (Figure 3). Interestingly the co-incubation with DHA or EPA led to down regulation of LPS induced cytokine secretion in both adipose tissue and mature adipocytes models. This down regulation was not due to cell death (data not shown). EPA showed significant anti-inflammatory effects by reducing 30% IL-6 (Figure 3A), 50% TNF-alpha (Figure 3C) and 45% MCP-1 (Figure 3E) secretion compared to LPS treatment in adipose tissue while, in mature adipocytes, it reduced 40% IL-6 (Figure 3B), 45% TNF-alpha (Figure 3D) and 55% MCP-1 (Figure 3F) secretion. DHA treatment reduced 40% IL-6 (Figure 3A), 20% TNF-alpha (Figure 3C) and 30% MCP-1 (Figure 3E) secretion in adipose tissue and 45% IL-6 (Figure 3B), 15% TNF-alpha (Figure 3D) and 45% MCP-1 (Figure 3F) secretion in mature adipocytes. OA reduced IL-6 and MCP-1 secretion in a non-significant way, in both adipose tissue and adipocytes (Figure 3A/B/E/F) and no significant change was noticed in case of TNF-alpha (Figure 3C/D).

#### Discussion

Controversial data are available regarding the proinflammatory and anti-inflammatory role of dietary free fatty acids. It has been shown that mice fed with SFA rich diet are prone to inflammation and promotes insulin resistance and atherosclerosis. Also this SFA mediated inflammation was attenuated in TLR4 and TLR2 *knock out* studies [25-29] and this leads to the suggestion that SFA

promote inflammation *via* direct activation of TLR2 and TLR4. Many research groups have claimed that PA and LA directly stimulate TLR2/4 [30,31] and induce secretion of proinflammatory cytokines in different cell models such as macrophages, endothelial cells and adipocytes. Recently Erridge and Samani [23] have shown that SFA mediated TLR2/4 activation is mainly due to the LPS and other lipopeptide contamination present in the BSA used for experimental setup. This creates doubt in the whole understanding of fatty acid mediated inflammation in different cell lines and animal models. In our previous work, we have shown the presence of functional TLR2/4 in adipocytes which were able to respond to TLR2 and TLR4 ligands (LTA and LPS respectively) by secreting TNF-alpha in a dose dependent manner [18]. Therefore in this present study we aimed to analyze the effect of fatty acids directly on the primary culture of human adipose tissue and mature adipocytes.

From our work, we showed that none of the fatty acids including saturated FA: PA and LA at the concentration of 40 and 100 µM were able to induce inflammation on primary culture of human adipose tissue and mature adipocytes. This has been confirmed by analyzing the three most important cytokines such as TNF-alpha, IL-6 and MCP-1 which are secreted by adipose tissue during inflammation. Our results are in contradiction with those from many studies regarding the inflammation induced by PA and LA. The first reason could be the concentrations of FFA used for the experiment. Indeed in those previous studies, PA and LA concentrations used were higher: in the range of 0.25-0.5 mM for 3T3-L1 adipocytes [12,32,33], myotubes [34], or CAEC's [35] treatment. We used PA and LA at 100 µM, which is in accordance with many recent studies [36]. However in our study, even at high concentrations (more than 100 µM, data not shown) no inflammatory effect could be observed on primary culture of mature adipocytes and adipose tissue. The second reason and probably the main reason for the inflammation induced by PA and LA in the earlier studies is the LPS and other lipopeptide contaminants present in the BSA used for fatty acid conjugation. Indeed Erridge and Samani [23] have previously shown that lipopeptide contaminants are present



in different BSA used for fatty acid conjugation. Also they have demonstrated that SFA conjugated with BSA (LPS and lipopeptide free) do not induce inflammation. For this reason, we used fatty acid poor and endotoxin free BSA all along the work. We confirmed the absence of endotoxin in our media or BSA used by testing with

HEK-Blue cells on which TLR4 and TLR2 are activated even with a low amount of LPS and Pam3Cys respectively. Also we tested the BSA conjugated SFA and PUFA on HEK-2 and HEK-4 and we found that none of the fatty acids are able to activate HEK-4 or HEK-2 cells. This proves that even if SFA are able to induce



proinflammatory effects in some cell models, this is not through TLR2 nor TLR4 activation. Our results are similar to Erridge [23], who demonstrates that PA and LA alone are not able to induce inflammation. Recently Chang [13] has shown in RAW264.7 cells that pre-treatment with PA but not LA increased the LPS induced proinflammatory mediators such as TNF-alpha and IL-6, while he could not detect any inflammatory effect of PA alone. This means that PA could act as an adjuvant to potentiate TLR4 activation by LPS. Personal data from Schwartz on the same model contradict this idea, whereas they confirm Chang's conclusion on human monocytes (THP-1), which proves that species and cell types have to be considered when we compare data [36]. Recently a new circulating hepatic glycoprotein: fetuin-A, was correlated with obesity and associated pathologies [37-39]. This protein was found to be a required endogenous TLR4 ligand and also crucial for the interaction of FFA with TLR4, permitting FFA proinflammatory effect through TLR4-pathway [40] which further supports our results. Thus on the basis of our results, we confirmed that no proinflammatory effect was induced by free fatty acids alone especially SFA on adipose tissue and mature adipocytes.

Moreover, we confirmed here that PUFA, like EPA, DHA and OA have anti-inflammatory properties. Furthermore EPA was found to have greater anti-inflammatory effect compared to DHA in both adipose tissue and mature adipocytes. OA was also found to have some anti-inflammatory effect to some extent but not comparable to EPA and DHA. These anti-inflammatory effects are in accordance with the previous studies on different cells such as RAW264.7 [13,41], THP-1 [11], HIMEC [10] and animal models [42,43]. In murine 3T3-L1 adipocytes it has been also shown that EPA and DHA are able to reduce MCP-1 expression and NFkB translocation [33]. Recently Oliver et al., [24] have shown in 3T3-L1 adipocytes and in macrophages that DHA has greater anti-inflammatory effect compared to EPA by attenuating the LPS induced NFkB activation and TNF-alpha secretion. This anti-inflammatory effect could be due to the activation of an alternate pathway through TLR4 [44] which is not NFkappaB-dependant or by a modulation of the LPS/TLR4 binding [45], through changes in lipid rafts for instance [46]. In our study, we showed that down regulation of LPS-induced cytokines secretion with DHA and EPA occurs in both models: adipose tissue and mature adipocytes. From this, we can hypothesize that mature adipocytes are more responsive to PUFA compared to other cells in the adipose tissue. This can be explained by the fact that in adipose tissue from normal BMI individuals, mature adipocytes are highly involved in inflammation process, in terms of high cytokine levels they are able to secrete and in terms

of the number of these cells within the tissue [47]. However under inflammation (LPS-treatment) we can't omit that stromal vascular cells can also be involved in this secretion by acting in a synergistic way with adipocytes. Indeed stromal vascular cells from obese patients show an up-regulation in inflammation-related genes [48].

Evidences suggest that these anti-inflammatory effects by PUFA are due to the conversion of these fatty acids into N-acyl ethanolamines (NAE), resolvins [22] and eicosanoids [49]. Balvers et al., [9] have shown in 3T3-L1 adipocytes that N-Acyl ethanolamine (NAE) such as DHEA and EPEA can be synthesized directly from their fatty acids precursors DHA and EPA respectively, through the transfer of fatty acid from membrane bound phospholipids to phosphatidylethanolamine (PE) to form N-acyl phosphatidylethanolamine (NAPE). Then NAPE specific phospholipase D cleaves NAPE to their respective NAE [50]. Balvers et al., [9] have further shown that these DHEA and EPEA act *via* PPAR- $\gamma$  and CB2 receptors for their anti-inflammatory properties. However, our team has previously shown that the anti-inflammatory effect of palmitoylethanolamide, the NAE synthesized from palmitic acid, is independent of CB1 and CB2 receptors on human mature adipocytes [49]. It is therefore difficult with current data, to accurately determine the mechanisms involved in the anti-inflammatory effects of these molecules.

## Conclusions

The inflammatory role and pathway of fatty acids are controversial for many years. Here we show that neither SFA nor PUFA have a pro-inflammatory effect on human adipose tissue and adipocytes as there are not able to activate TLR2 and TLR4. Moreover we show that PUFA have an anti-inflammatory effect, probably independent of TLRs on adipose tissue and especially on mature adipocytes. Finally, these results confirm the marked involvement of adipocytes in the relationship between dietary fatty acids and inflammation linked to obesity.

## Methods

### Reagents

Lipopolysaccharides (LPS from *E. coli* 0111:B4 strain, batch #LPE-32-02) was purchased from Sigma (Saint Quentin Fallavier, France), Pam3Cys (Catalog # tlr1-pmc) from InvivoGen, BSA (Fraction V, fatty acid poor and endotoxin free) from Calbiochem, Cat # 126579. Docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), palmitic acid (PA), oleic acid (OA) and lauric acid (LA) were purchased from Cayman chemicals and resuspended in DMSO and stored at -20°C for experiments.



#### TLR2 and TLR4 activation experiments

HEK-Blue™ LPS Detection Kit and Plasmotest™ were purchased from Invivogen, France. HEK-Blue-2 and HEK-Blue-4 cells are stably transfected with multiple genes from the TLR2 and TLR4 pathways respectively, and with a reporter gene (secreted alkaline phosphatase, sAP) which monitors the TLR binding through NFκB activation. Cells were maintained and plated according to manufacturers instructions. HEK-Blue-4 cells and HEK-Blue-2 cells were then treated with various fatty acids at different concentrations with or without 1X Positive Control (stock 1000X, provided along with the kit). HEK-Blue-2 and HEK-Blue-4 cells were incubated for 16 and 20 hours respectively, followed by collection of OD values at 640 nm.

#### Origin of human adipose tissue samples

Subcutaneous (abdominal, buttocks, hips and thighs) tissue samples of human white fat were obtained from normal weight or slightly overweight human subjects (exclusively females, mean body mass index = 23.3) undergoing liposuction, performed under general anaesthesia, for cosmetic reasons (aged between 25 and 60 years, mean 39 years). Apart from oral contraception, the subjects were not receiving treatment with prescribed medication at the time of liposuction. A total of 8 samples were obtained and the study was approved by the Ile de la Réunion ethics committee for the protection of persons undergoing biomedical research.

#### Primary culture of human adipose tissue

The adipose tissue was rinsed thrice with Ringer-Lactate buffer. 200 µl of tissue was distributed in 24-well tissue culture plates with medium 199 supplemented with 1% fatty acid free fetal bovine serum (FBS) (PAN Biotech, France), amphotericin B, (5 mg/ml), streptomycin (0.2 mg/ml) & penicillin (200 U/ml) (PAN Biotech, France), 66 nM insulin (Umuline Rapide, Lilly, France), 2 g/L glucose, 8 µg/ml biotin and 4 µg/ml pantothenate. Tissue was then maintained at 37°C in 5% CO<sub>2</sub> for a period of 24 hours prior to the experiments.

#### Primary culture of human adipocytes

Cultures were carried out as previously described [50]. Briefly after washing, tissue samples obtained by liposuction were digested for 30 min at 37°C in Ringer-Lactate buffer containing 1.5 mg/ml collagenase (NB5, SERVA, Germany, PZ activity 0.175 U/mg). The floating cells (mature adipocytes) were rinsed thrice in Ringer-Lactate. Cells were plated in 24-well (30 000 cells) or 6-well (120 000 cells) tissue culture plates with 300 µl or 1 ml medium 199 respectively. Cells were then maintained at 37°C in 5% CO<sub>2</sub> for a period of 24 hours prior to the experiments.

#### FA/BSA complex preparation and treatment

Cells and tissues were treated with complex of fatty acid and BSA. To do this, PUFA were reconstituted in DMSO directly and SFA were reconstituted in 0.1 N NaOH/DMSO, heated to 70°C until it gets dissolved. Reconstituted PUFA and SFA were then freshly conjugated to fatty acid free and endotoxin free BSA by incubating 2 mM of each FA with 0.5 mM of BSA (4:1 molar ratio) at 37 °C before experiments. Then they were dissolved directly into warmed culture medium with or without LPS (1 µg/ml). SFA were used at 40 and 100 µM with 10 and 25 µM BSA respectively and PUFA were used at 5 and 10 µM with 1.25 and 2.5 µM BSA respectively. After 6 and 12 hours of treatment the medium was collected and stored at -20 C for cytokine analysis. Further to verify the above prepared SFA are functional, adiponectin secretion and cholesterol assay were performed (data not shown). PA was able to decrease adiponectin secretion while LA up-regulated this secretion. Moreover both PA and LA induced cholesterol uptake from media (data not shown), as it is known for LPS treatment [51,52].

#### IL-6, TNF-alpha and MCP-1 analysis by ELISA

Following fatty acid treatment with and without LPS stimulation for 6 and 12 hours, media were assayed for IL-6, TNF-alpha and MCP-1 with Ready-SET-Go human ELISA kits (eBioscience, Cliniscience, Montrouge, France), according to manufacturer's instructions. ELISA sensitivity: 4 pg/ml for TNF-alpha, 2 pg/ml for IL-6 and 7 pg/ml for MCP-1.

#### Statistics

All values were measured as mean ± S.D. Statistical analysis was performed using Graph pad PRISM 5 software. Differences were tested for significance, treatments *versus* control cells, by the one-way ANOVA and Dunnett post-test. P < 0.05 (\*) or P < 0.01 (\*\*) or P < 0.001 (\*\*\*)

#### Abbreviations

TLR: Toll-like receptor; BSA: Bovin serum albumin; SFA: Saturated fatty acid; LA: Lauric acid; PA: Palmitic acid; PUFA: Poly-unsaturated fatty acid; EPA: Eicosapentaenoic acid; DHA: Docosahexaenoic acid; OA: Oleic acid; LPS: Lipopolysaccharides; NAPE: N-acyl phosphatidylethanolamine; NAE: N-acyl ethanolamine.

#### Competing interests

The authors declare that they have no competing interests.

#### Authors' contributions

RKM and MKG carried out the adipose tissue, mature adipocyte cultures and HEK-cells experiments. JKP participated in the fatty acid preparation. LG and KB participated in the ELISA assays. MKG and LH drafted the manuscript. FF, MC and RR conceived the study and LH participated in its design and coordination. MKG performed the statistical analysis. All authors read and approved the final manuscript.

#### Acknowledgements

We are grateful to the plastic surgeons: Hularud O., Delarue P. and Gonçalves J. who took part in this study and allowed the collection of subcutaneous adipose tissue samples, and to the Regional Council for the financial support. Finally, we would like to thank all patients who consented to the collection of tissue samples, and thus made this study possible.

Received: 31 May 2012 Accepted: 18 December 2012

Published: 21 December 2012

#### References

- Guilherme A, Virbasius JV, Puri V, Czech MP: Adipocyte dysfunctions linking obesity to insulin resistance and type 2 diabetes. *Nat Rev Mol Cell Biol* 2008, **9**(5):367–377.
- Aslan H, Altunkaynak BZ, Altunkaynak ME, Vuraler O, Kaplan S, Unal B: Effect of a high fat diet on quantitative features of adipocytes in the omentum: an experimental, stereological and ultrastructural study. *Obes Surg* 2006, **16**(11):1526–1534.
- Bullo M, Casas-Agustench P, Amigo-Correig P, Aranceta J, Salas-Salvado J: Inflammation, obesity and comorbidities: the role of diet. *Public Health Nutr* 2007, **10**(10A):1164–1172.
- Bays H, Mandarin L, DeFronzo RA: Role of the adipocyte, free fatty acids, and ectopic fat in pathogenesis of type 2 diabetes mellitus: peroxisomal proliferator-activated receptor agonists provide a rational therapeutic approach. *J Clin Endocrinol Metab* 2004, **89**(2):463–478.
- Suganami T, Tanimoto-Koyama K, Nishida J, Itoh M, Yuan X, Mizuarai S, Kotani H, Yamaoka S, Miyake K, Aoe S, et al: Role of the Toll-like receptor 4/NF-kappaB pathway in saturated fatty acid-induced inflammatory changes in the interaction between adipocytes and macrophages. *Arterioscler Thromb Vasc Biol* 2007, **27**(1):84–91.
- Bunn RC, Cockrell GE, Ou Y, Thrallkill KM, Lumpkin CK Jr, Fowlkes JL: Palmitate and insulin synergistically induce IL-6 expression in human monocytes. *Cardiovasc Diabetol* 2010, **9**:73.
- Tsimikas S, Reaven PD: The role of dietary fatty acids in lipoprotein oxidation and atherosclerosis. *Curr Opin Lipidol* 1998, **9**(4):301–307.
- Hao W, Wong OY, Liu X, Lee P, Chen Y, Wong KKY:  $\omega$ -3 fatty acids suppress inflammatory cytokine production by macrophages and hepatocytes. *J Pediatr Surg* 2010, **45**(12):2412–2418.
- Balvers MGJ, Verhoeckx KCM, Plastina P, Wortelboer HM, Meijerink J, Witkamp RF: Docosahexaenoic acid and eicosapentaenoic acid are converted by 3 T3-L1 adipocytes to N-acyl ethanolamines with anti-inflammatory properties. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* 2010, **1801**(10):1107–1114.
- Ibrahim A, Mboodji K, Hassan A, Aziz M, Boukhattala N, Coëffier M, Savoye G, Déchelotte P, Marion-Letellier R: Anti-inflammatory and anti-angiogenic effect of long chain n-3 polyunsaturated fatty acids in intestinal microvascular endothelium. *Clin Nutr* 2011, **30**(5):678–687.
- Mullen A, Loscher CE, Roche HM: Anti-inflammatory effects of EPA and DHA are dependent upon time and dose-response elements associated with LPS stimulation in THP-1-derived macrophages. *J Nutr Biochem* 2010, **21**(5):444–450.
- Ajuwon KM, Spurlock ME: Palmitate activates the NF-kappaB transcription factor and induces IL-6 and TNFalpha expression in 3 T3-L1 adipocytes. *J Nutr* 2005, **135**(8):1841–1846.
- Chang CF, Chau YP, Kung HN, Lu KS: The lipopolysaccharide-induced pro-inflammatory response in RAW264.7 cells is attenuated by an unsaturated fatty acid-bovine serum albumin complex and enhanced by a saturated fatty acid-bovine serum albumin complex. *Inflamm Res* 2011.
- Harvey KA, Walker CL, Pavlina TM, Xu Z, Zaloga GP, Siddiqui RA: Long-chain saturated fatty acids induce pro-inflammatory responses and impact endothelial cell growth. *Clin Nutr* 2010, **29**(4):492–500.
- Hotamisligil GS: Inflammation and metabolic disorders. *Nature* 2006, **444**(7121):860–867.
- Shoelson SE, Lee J, Goldfine AB: Inflammation and insulin resistance. *J Clin Invest* 2006, **116**(7):1793–1801.
- Trayhurn P, Wood IS: Adipokines: inflammation and the pleiotropic role of white adipose tissue. *Br J Nutr* 2004, **92**(3):347–355.
- Bès-Houtmann S, Roche R, Hoareau L, Gonthier M-P, Festy F, Caillens H, Gasque P, Lefebvre d'Hellencourt C, Cesari M: Presence of functional TLR2 and TLR4 on human adipocytes. *Histochem Cell Biol* 2006, **127**(2):131–137.
- Samokhvalov V, Bilan PJ, Schertzer JD, Antonescu CN, Klip A: Palmitate- and lipopolysaccharide-activated macrophages evoke contrasting insulin responses in muscle cells. *Am J Physiol Endocrinol Metab* 2009, **296**(1):E37–E46.
- Havens L, Danielsson KN, Fogelstrand L, Wiklund O: Induction of proinflammatory cytokines by long-chain saturated fatty acids in human macrophages. *Atherosclerosis* 2009, **202**(2):382–393.
- Weatherill AR, Lee JY, Zhao L, Lemay DG, Youn HS, Hwang DH: Saturated and polyunsaturated fatty acids reciprocally modulate dendritic cell functions mediated through TLR4. *J Immunol* 2005, **174**(9):5390–5397.
- Jin MS, Lee JO: Structures of the toll-like receptor family and its ligand complexes. *Immunity* 2008, **29**(2):182–191.
- Erridge C, Samani NJ: Saturated fatty acids Do Not directly stimulate toll-like receptor signaling. *Arterioscler Thromb Vasc Biol* 2009, **29**(11):1944–1949.
- Park JS, Lee EJ, Lee JC, Kim WK, Kim HS: Anti-inflammatory effects of short chain fatty acids in IFN-gamma-stimulated RAW 264.7 murine macrophage cells: involvement of NF-kappaB and ERK signaling pathways. *Int Immunopharmacol* 2007, **7**(1):70–77.
- Davis JE, Gabler NK, Walker-Daniels J, Spurlock ME: Tlr-4 deficiency selectively protects against obesity induced by diets high in saturated fat. *Obesity (Silver Spring)* 2008, **16**(6):1248–1255.
- Coenen KR, Gruen ML, Lee-Young RS, Puglisi MJ, Wasserman DH, Hasty AH: Impact of macrophage toll-like receptor 4 deficiency on macrophage infiltration into adipose tissue and the artery wall in mice. *Diabetologia* 2009, **52**(2):318–328.
- Mullick AE: Modulation of atherosclerosis in mice by toll-like receptor 2. *J Clin Invest* 2005, **115**(11):3149–3156.
- Poggi M, Bastelica D, Gual P, Iglesias MA, Gremeaux T, Knauf C, Peiretti F, Verdier M, Juhan-Vague I, Tanti JF, et al: C3H/HeJ mice carrying a toll-like receptor 4 mutation are protected against the development of insulin resistance in white adipose tissue in response to a high-fat diet. *Diabetologia* 2007, **50**(6):1267–1276.
- Radin MS, Sinha S, Bhatt BA, Dedousis N, O'Doherty RM: Inhibition or deletion of the lipopolysaccharide receptor toll-like receptor-4 confers partial protection against lipid-induced insulin resistance in rodent skeletal muscle. *Diabetologia* 2007, **51**(2):336–346.
- Lee JY: Saturated fatty acid activates but polyunsaturated fatty acid inhibits toll-like receptor 2 dimerized with toll-like receptor 6 or 1. *J Biol Chem* 2004, **279**(17):16971–16979.
- Lee JY, Sohn KH, Rhee SH, Hwang D: Saturated fatty acids, but not unsaturated fatty acids, induce the expression of cyclooxygenase-2 mediated through toll-like receptor 4. *J Biol Chem* 2001, **276**(20):16683–16689.
- Feng D, Tang Y, Kwon H, Hawkins M, Kitis RN, Pessin JE: High-fat diet-induced adipocyte cell death occurs through a cyclophilin D intrinsic signaling pathway independent of adipose tissue inflammation. *Diabetes* 2011, **60**(8):2134–2143.
- Han CY, Kargi AY, Omer M, Chan CK, Wabitsch M, O'Brien KD, Wight TN, Chait A: Differential effect of saturated and unsaturated free fatty acids on the generation of monocyte adhesion and chemotactic factors by adipocytes: dissociation of adipocyte hypertrophy from inflammation. *Diabetes* 2009, **59**(2):386–396.
- Weigert C, Brodbeck K, Staiger H, Kausch C, Machicao F, Haring HU, Schleicher ED: Palmitate, but not unsaturated fatty acids, induces the expression of interleukin-6 in human myotubes through proteasome-dependent activation of nuclear factor-kappaB. *J Biol Chem* 2004, **279**(23):23942–23952.
- Li W, Sama AE, Wang H: Role of HMGB1 in cardiovascular diseases. *Curr Opin Pharmacol* 2006, **6**(2):130–135.
- Schwartz EA, Zhang WY, Karnik SK, Borwege S, Anand VR, Laine PS, Su Y, Reaven PD: Nutrient modification of the innate immune response: a novel mechanism by which saturated fatty acids greatly amplify monocyte inflammation. *Arterioscler Thromb Vasc Biol* 2010, **30**(4):802–808.
- Ismail NA, Ragab S, El Dayem SM, Elbaky AA, Salah N, Hamed M, Assal H, Koura H: Fetuin-A levels in obesity: differences in relation to metabolic syndrome and correlation with clinical and laboratory variables. *Arch Med Sci* 2012, **8**(5):826–833.
- Erdmann J, Salmhofer H, Knauss A, Mayr M, Wagenpfeil S, Sympchenko O, Lupp P, Schudsziarra V: Relationship of fetuin-A levels to weight-dependent insulin resistance and type 2 diabetes mellitus. *Regul Pept* 2012, **178**(1–3):6–10.



39. Singh M, Sharma PK, Garg VK, Mondal SC, Singh AK, Kumar N: **Role of fetuin-A in atherosclerosis associated with diabetic patients.** *J Pharm Pharmacol* 2012, **64**(12):1703–1708.
40. Pal D, Dasgupta S, Kundu R, Maitra S, Das G, Mukhopadhyay S, Ray S, Majumdar SS, Bhattacharya S: **Fetuin-A acts as an endogenous ligand of TLR4 to promote lipid-induced insulin resistance.** *Nat Med* 2012, .
41. Skuladottir IH, Petursdottir DH, Hardardottir I: **The effects of omega-3 polyunsaturated fatty acids on TNF-alpha and IL-10 secretion by murine peritoneal cells in vitro.** *Lipids* 2007, **42**(8):699–706.
42. Gotoh N, Nagao K, Onoda S, Shirouchi B, Furuya K, Nagai T, Mizobe H, Ichioka K, Watanabe H, Yanagita T, et al: **Effects of three different highly purified n-3 series highly unsaturated fatty acids on lipid metabolism in C57BL/KsJ-db/dbMice.** *J Agric Food Chem* 2009, **57**(22):11047–11054.
43. Lu J, Jilling T, Li D, Caplan MS: **Polyunsaturated fatty acid supplementation alters proinflammatory gene expression and reduces the incidence of necrotizing enterocolitis in a neonatal rat model.** *Pediatr Res* 2007, **61**(4):427–432.
44. Panda SK, Kumar S, Tupperwar NC, Vaidya T, George A, Rath S, Bal V, Ravindran B: **Chitohexaose activates macrophages by alternate pathway through TLR4 and blocks endotoxemia.** *PLoS Pathog* 2012, **8**(5):e1002717.
45. Wang M, Chen Y, Zhang Y, Zhang L, Lu X, Chen Z: **Mannan-binding lectin directly interacts with toll-like receptor 4 and suppresses lipopolysaccharide-induced inflammatory cytokine secretion from THP-1 cells.** *Cell Mol Immunol* 2011, **8**(3):265–275.
46. Wong SW, Kwon MJ, Choi AM, Kim HP, Nakahira K, Hwang DH: **Fatty acids modulate toll-like receptor 4 activation through regulation of receptor dimerization and recruitment into lipid rafts in a reactive oxygen species-dependent manner.** *J Biol Chem* 2009, **284**(40):27384–27392.
47. Hoareau L, Bencharif K, Rondeau P, Murumalla R, Ravanan P, Tallet F, Delarue P, Cesari M, Roche R, Festy F: **Signaling pathways involved in LPS induced TNFalpha production in human adipocytes.** *J Inflamm (Lond)* 2010, **7**:1.
48. Nair S, Lee YH, Rousseau E, Cam M, Tataranni PA, Baier LJ, Bogardus C, Permana PA: **Increased expression of inflammation-related genes in cultured preadipocytes/stromal vascular cells from obese compared with non-obese Pima Indians.** *Diabetologia* 2005, **48**(9):1784–1788.
49. Hoareau L, Buyse M, Festy F, Ravanan P, Gonthier MP, Matias I, Petrosino S, Tallet F, d'Hellecourt CL, Cesari M, et al: **Anti-inflammatory effect of palmitoylethanolamide on human adipocytes.** *Obesity (Silver Spring)* 2009, **17**(3):431–438.
50. Fang S, Suh JM, Atkins AR, Hong SH, Leblanc M, Nofsinger RR, Yu RT, Downes M, Evans RM: **Corepressor SMRT promotes oxidative phosphorylation in adipose tissue and protects against diet-induced obesity and insulin resistance.** *Proc Natl Acad Sci U S A* 2011, **108**(8):3412–3417.
51. Ye Q, Chen Y, Lei H, Liu Q, Moorhead JF, Varghese Z, Ruan XZ: **Inflammatory stress increases unmodified LDL uptake via LDL receptor: an alternative pathway for macrophage foam-cell formation.** *Inflamm Res* 2009, **58**(11):809–818.
52. Eder K, Guan H, Sung HY, Francis SE, Crossman DC, Kiss-Toth E: **LDL uptake by monocytes in response to inflammation is MAPK dependent but independent of tribbles protein expression.** *Immunol Lett* 2008, **116**(2):178–183.

doi:10.1186/1476-511X-11-175

**Cite this article as:** Murumalla et al.: Fatty acids do not pay the toll: effect of SFA and PUFA on human adipose tissue and mature adipocytes inflammation. *Lipids in Health and Disease* 2012 **11**:175.

**Submit your next manuscript to BioMed Central and take full advantage of:**

- Convenient online submission
- Thorough peer review
- No space constraints or color figure charges
- Immediate publication on acceptance
- Inclusion in PubMed, CAS, Scopus and Google Scholar
- Research which is freely available for redistribution

Submit your manuscript at  
www.biomedcentral.com/submit



From this work, we have experimentally described and concluded that FFA especially SFA such as PA and LA are not able to induce inflammation in human mature adipocyte and adipose tissue in an endotoxin free conditions. Physiological stimuli alternative of LPS and Hsp60 that could induce inflammation in adipose tissue through TLR4 remains largely unknown. Recently HMGB1, a nuclear protein is identified to be secreted as a cytokine and activates TLR4 mediated inflammation. HMGB1 is found to be involved in various inflammatory diseases such as rheumatoid arthritis, cardiovascular disease, diabetes and atherosclerosis. Since adipose tissue plays a major role in this onset of these diseases, we hypothesized whether adipose tissue and derived cells under inflammation are able to express and secrete HMGB1 as a cytokine. Moreover we doubted whether HMGB1 plays a possible active role in adipose tissue inflammation and could function as a possible adipokine.

## 3. HMGB1

---

### 3.1 Introduction

High Mobility Group Box Protein 1 (HMGB1) is initially identified as non-histone chromosomal protein and then categorized as high mobility group protein (HMG-1) by Johns et.al, in 1975 [183]. Later in 1991, HMGB1 was rediscovered as amphoterin, a membrane bound protein from the advancing plasma membrane of neurites during embryonic brain development [184]. For decades, researchers characterized HMGB1 functionally as chromatin binding protein involved in DNA replication and repair, transcriptional regulation, protecting DNA integrity by DNA bending. In 1999, Tracey and his colleagues discovered that HMGB1 is secreted as a cytokine from macrophages when stimulated with LPS [185]. This discovery made researchers to study more on HMGB1 in the field of immunology and lead to an identification of many family members, later HMG-1 characterized as HMGB1 [186]. HMGB1 involvement in transcriptional regulation is reflected by the fact that HMGB1 knock-out mice are not able to survive for a couple of hours after birth due to hypoglycaemia [187].

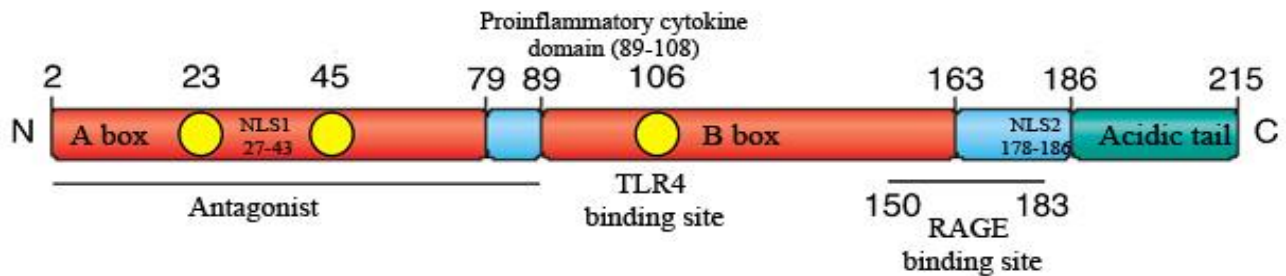


Figure 13: **Protein structure of HMGB1.** HMGB1 contains two DNA binding domains, A-box and B-box and an acidic C-terminal tail. Nuclear localization signals (NLS1 and NLS2), cysteine residues 23, 45 and 106 (yellow circles), other functionally important regions are noted in the figure. *Modified from* [188].

### **3.2 Structure of HMGB1**

HMGB1 is a highly conserved 25-kD protein with 99% homology in mammals. It is comprised of two positively charged DNA binding homologous regions termed A and B box, approximately 80 amino acids in length. It also has negatively charged C-terminal acidic tail composed of 30 aspartic and glutamic acids. The A-box (1-88 amino acids) is anti-inflammatory and acts as self-antagonist, while the B- box (89-185) is pro-inflammatory, containing TLR-4 and RAGE binding domain. There are also two nuclear-localization signals (NLS1 and NLS2) present in both the A and B-box respectively, which signals the nuclear and cytoplasmic translocations. There are also two nuclear emigration signals in the proximal part of A and B boxes respectively, which can bind to the nuclear exportin, CRM1 and mediates cytoplasmic translocation [189, 190].

### **3.3 Post translational modifications:**

Based on the inducing stimulus, the physical and chemical form of HMGB1 varies and undergoes extensive post-translational modifications such as glycosylation, acetylation, phosphorylation, methylation, redox changes of cysteine residues, which influence the receptor binding and downstream signaling events. Acetylation or phosphorylation of NLS regions signals HMGB1 to shift from nucleus and accumulate in the cytoplasm before subsequent extracellular secretion. Recently more evidences are accumulating on redox modifications of HMGB1, which determines the extracellular functionality. HMGB1 contains three cysteine residues, A box (C23 and C45) and B box (C106). A disulfide bridge is also been identified between C23 and C45 which plays a tremendous role in the cytokine function of the protein [191, 192]. In addition, reduced cysteine residue at C106 position is necessary for HMGB1 binding to TLR4 receptor which leads to subsequent inflammatory cytokine induction [193]. Studies on oxidation status of HMGB1 between viable and dead cells identifies that oxidized form is found only in apoptotic cells but not in live cells [194]. Further studies evidenced that oxidized form of HMGB1 from apoptotic cells lose their immune-stimulatory functions [195]. This process involves the caspase dependent production of reactive oxygen species (ROS) which in turn oxidize the HMGB1. A mutation experiment reveals that cysteine at C106 position is the critical residue when oxidized, lose its immune-stimulatory cytokine and chemokine properties of the protein. Induction of apoptosis in mice leads to major release of C106 oxidized form of

HMGB1. Further inhibition of caspase dependent oxidation of HMGB1 leads to release of reduced form of HMGB1 and immune cells recruitment in mouse model of liver injury [196].

### 3.4 HMGB1 location and functions:

HMGB1 plays varied functions based on its location, context and different post-translational modifications in individual cell types and during cell activation.

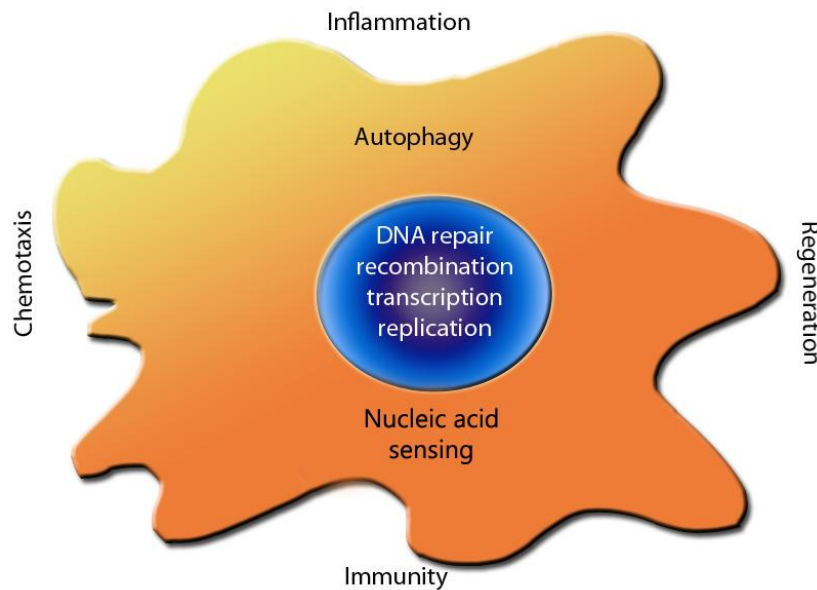


Figure 14: **HMGB1 nuclear, intracellular and extracellular functions.**

#### 3.4.1 Nuclear HMGB1

HMGB1 is an abundant non-histone chromosomal protein found in all nucleated cells binding to minor groove of DNA without sequence specificity. Both Box A and Box B domains contain three alpha-helices, named helix I, helix II and helix III and two loops, loop I and loop II [197, 198]. Both the domains adopt a unique “L” shape structure binding to the minor groove of the DNA. It also bends DNA duplex towards the major groove, which further confirms that HMGB1 can induce a significant change in DNA structures. This DNA bending capacity of HMGB1 allows it to participate in many biological processes [186, 199, 200] such as regulation of chromatin structure [201], transcription [187], DNA damage, repair and recombination [199]. Recent studies identified that HMGB1 is actively involved in modulating the efficiency of all four major DNA repair pathways, i.e., nucleotide excision repair (NER) [202, 203], base



excision repair (BER) [204], mismatch repair [205] and double strand break repair and nonhomologous end-joining [206, 207]. Additionally, it also facilitates proteins such as p53, homeobox proteins, NFkB and proteins involved in DNA recombination, repair and transcription to interact with DNA. HMGB1 is extremely mobile nuclear protein, which shuttles between nucleus and cytoplasm in most cells. The import to the nucleus is an active process while the cytoplasmic shift is via passive diffusion or by CRM-1 mediated transport. Moreover evidences illustrate that HMGB1 proteins “roam” the nucleus, do not show marked residential status by visiting each individual nucleosome every 2s on an average and they are described as “chromatin chaperone” or lubricant of chromatin [208, 209].

### **3.4.2 Intra cellular HMGB1**

Intra-cellular HMGB1 participates in autophagy, a lysosome-mediated catabolic process contributing to the maintenance of intracellular homeostasis and cell survival. It is evident that cytosolic reduced HMGB1 activates autophagy by disrupting the Beclin-1 and Beclin-2 complex formation, which is the crucial regulator between apoptosis and autophagy. The oxidation sensitive C106 of HMGB1 regulates nuclear-cytoplasmic translocation and intra-molecular disulfide between C23 and C45 assists HMGB1 binding to Beclin-1 and leads to induction of autophagy [210]. Under inflammation or autophagy, HMGB1 accumulates in the cytoplasmic exosome vesicles before secretion from the cells [189]. In the cytoplasm, HMGB1 plays a major role in nucleic acid sensing which is crucial for anti-viral immunity of the cell. HMGB1 binding to the immunogenic RNA is necessary for the recognition and binding to the specific PRR's and induction of immune response [211].

### **3.4.3 Extra cellular HMGB1**

Extracellular HMGB1 performs different functions based up on the nature of inducer and time of secretion. It acts as a danger signal and inflammatory mediator by binding to different cell surface receptors alone or by complex formation. At extracellular level, HMGB1-receptor interactions promotes inflammation and angiogenesis via activation of NFkB [212, 213] and tumor proliferation via activation of p44/p42, p38 and SAPK/JNK MAPKs [214, 215]. Three different redox states of HMGB1 such as all-thiol, disulfide and oxidized forms are found in extracellular space [216]. HMGB1 initially secreted in the reduced state as “all-thiol” form and

transforms in to other forms based up on the oxidized environment [217-219]. The “all- thiol” form acts as a chemoattractant, disulfide form acts pro-inflammatory and the oxidized form involves in the resolution of inflammation [216]. Accumulation evidences indicates that all-thiol HMGB1 is capable of stimulating migration of smooth muscle cells [220], neutrite cells [221], tumor cells [222], mesoangioblast stem cells [213], monocytes [223], dendritic cells [224] and neutrophils [212, 225]. Interestingly, HMGB1 is also capable of attracting stem cells [213] and may be important for skeletal muscle, heart tissue repair, and regeneration [226-228].

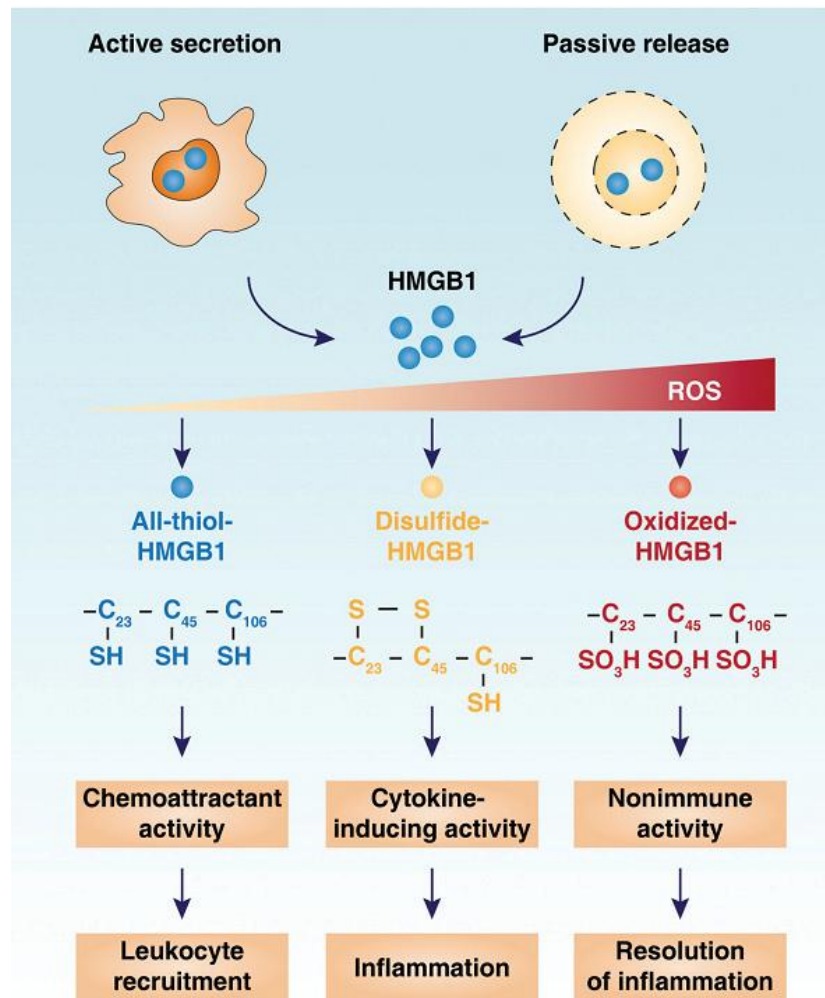


Figure 15: Extracellular HMGB1 - types and functions, [216]

Under certain cellular signals, cells secrete or release out HMGB1 by active or passive way in to the extracellular environment. These two different pathways differ in many aspects such as kinetics and post-translational modifications of the released protein.

#### **3.4.3.1 Active secretion:**

Many inflammatory cells such as monocytes, macrophages [185, 229], natural killer cells [230] and dendritic cells [231] actively secrete HMGB1. Also non-immune cells like pituitary cells [232], hepatocytes, platelets [233], astrocytes [234], neurons [235], endothelial cells [236] and tumor cells [237, 238] also has been reported to secrete HMGB1. Stimulated cells lead to both acetylation and phosphorylation of HMGB1 leading to translocation and accumulation in the cytoplasm [189]. HMGB1 secretion occurs via non-classical pathway, vesicle mediated [189] rather than classical Endoplasmic reticulum (ER) – Golgi signal peptide pathway. The exocytosis of HMGB-1 stored exosome vesicles is induced by lysophosphatidyl choline, which is produced after long hours in activated monocytes. This delay reflects in the extracellular secretion of HMGB1 unlike other pro-inflammatory cytokines such as TNF-alpha and hence HMGB1 is characterized as late mediator of inflammation [185, 189]. Most HMGB1 released during the first 12 hours is found to be preformed and only thereafter newly synthesized HMGB1 is released [185]. This active HMGB1 release can be induced in response to PAMPS or endogenous pro-inflammatory cytokines such as LPS, lipoteichoic acid, TNF, IL-1 $\beta$ , IL-2, IFN $\alpha$  and IFN $\gamma$  [229-232, 239, 240].

In other events like in cytotoxic stress, HMGB1 mobilized to the cytoplasm participates in Beclin dependent autophagy and in turn lead the cells secrete out HMGB1 [210, 241, 242]. These extracellular HMGB1 acts in an autocrine manner to protect the cells by autophagy-mediated inhibition of apoptosis and helps prolonged cell survival [243-246]. HMGB1 with all reduced cysteines is able to induce Beclin-1 dependent autophagy by interacting through RAGE but not through TLR4 or TLR2 receptor [244]. HMGB1 is also found to release actively during hypoxia, which requires TLR4 dependent production of ROS and calcium mediated signaling [247, 248].

### 3.4.3.2 Passive release

In necrotic cells, the loosely bound HMGB1 to DNA empties from the nucleus when the membrane integrity is lost and ends up released outside the cell passively as a cell death marker [220, 249]. HMGB1 released due to necrosis induces pro-inflammatory and also chemokine response [249]. During apoptosis, cells that undergo programmed cell death also release HMGB1. However, HMGB1 released via apoptosis is not pro-inflammatory due to irreversible oxidation of cysteine at position 106 [195]. Unlike apoptosis, inflammasomes activation induces pyroptosis, a pro-inflammatory form of programmed cell death results in robust release of HMGB1 [250]. Passive release of HMGB1 is rapid in comparison with active secretion of HMGB1, which requires more time [250]. Another difference in passive release is that HMGB1 is not acetylated, unlike in pyroptosis and active secretion.

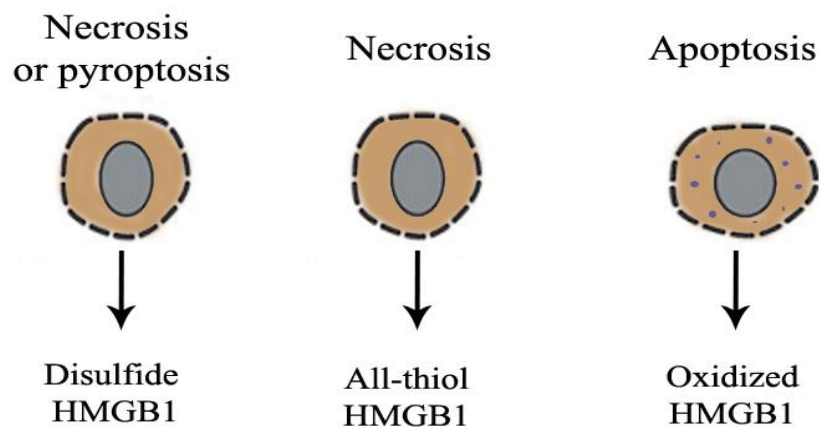


Figure 16: **Passive release of HMGB1 and its redox status**

### 3.5 HMGB1 – recognizing receptors and pathways

HMGB1 is identified to interact with wide range of molecules such as proteoglycans, lipids, carbohydrates, transcriptional factors and viral proteins. It binds with more than 12 different peptide sequences due to A-box, B-box or both. HMGB1 interacts with mediators like CXCL-12, IL-1, endotoxin (LPS and Pam<sub>3</sub>CSK<sub>4</sub>), DNA and other partner molecules forming immune-stimulatory complexes to promote inflammation [251-254]. RAGE was the first receptor described to recognize HMGB1. Later several other receptors such as TLR4, TLR2,

TLR9, CD24/Siglec, MAC-1 and TREM1 were identified to recognize HMGB1 alone or as complex.

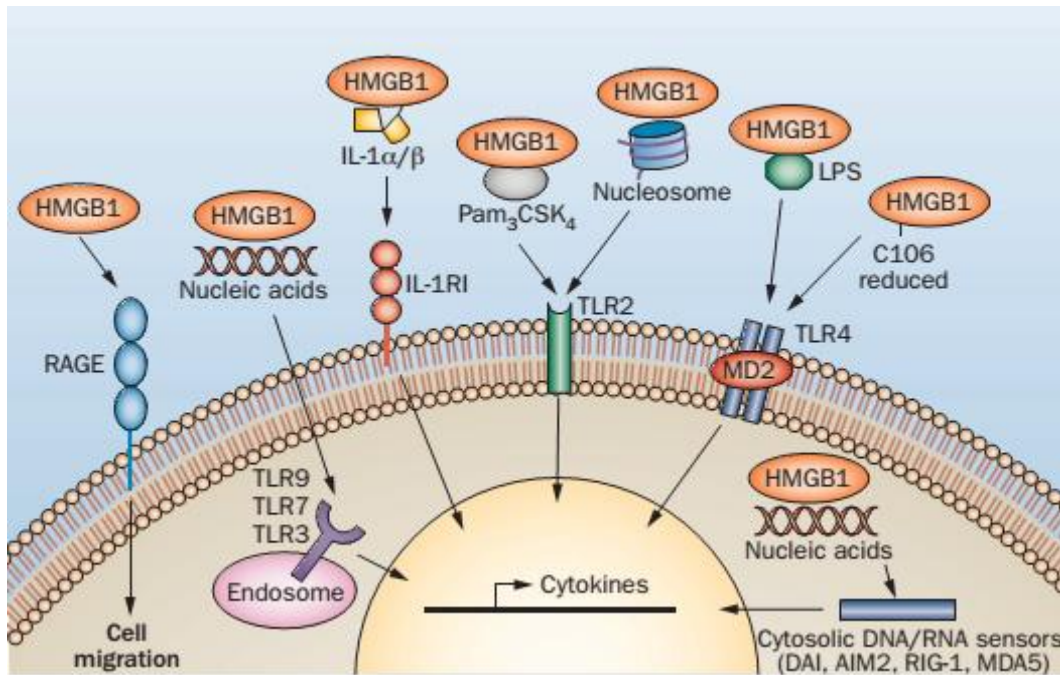


Figure 17: **HMGB1 recognizing receptors**, [255]

### 3.5.1 RAGE:

RAGE was the first receptor identified for HMGB1 and the binding site was mapped down to amino acids 150-183 in the B-box for cytokine stimulation [222, 256]. HMGB1 regulates cell migration through RAGE receptor in immune cells [212, 223, 224], stem cells [257, 258] and smooth muscle cells [220]. In rat smooth muscle cells HMGB1 has been to regulate cell migration independent of either A or B box of HMGB1 binding to RAGE.

- Interaction with RAGE signaling is involved in tumor growth and development [215].
- Binding to RAGE also promotes autophagy and limits apoptosis [215, 259].

- Activate inflammatory pathways [260, 261] and induces production of CXCL12, a chemokine that synergistically promotes chemotaxis in complex with HMGB1 [254, 262].
- Induction of RAGE activates MAP kinases p38 and JNK leading to NF- $\kappa$ B activation and translocation [215, 263].
- Signaling through RAGE mediates the up regulation of cell surface receptors including TLR4 and RAGE [264].

### **3.5.2 TLR4:**

HMGB1 recognition by TLR4 is demonstrated by FRET, immunoprecipitation and functionally by NF- $\kappa$ B activation [265]. HMGB1 binding to TLR4-MD2 complex activates cytokine production and reduced C106 with disulfide bond between C23 and C45 is demonstrated to be the crucial factor for the TLR4 activation [193]. HMGB1-TLR4 signaling mediates liver damage following hepatic ischemia-reperfusion injury [266]. HMGB1 and LPS initiate similar intra-cellular events such as activation of ERK1/2, JNK and p38 pathways leading to NF $\kappa$ B translocation [265, 267]. HMGB1 stimulation results in activation of both IKK- $\alpha$  and IKK- $\beta$ , while LPS activates only IKK- $\beta$  and gene array studies reveals the gene expression profile varies from LPS induced activation [265, 268].

### **3.5.3 TLR2:**

Apart from binding to bacterial derived ligands such as lipoteichoic acid [269], HMGB1 is also been reported to bind TLR2 and initiate NF $\kappa$ B activation and translocation [265, 268]. The cytokine effect of HMGB1 mediating through TLR2 is cell dependent, inducing HEK-293 cells while not in primary macrophage cells [270]. However nucleosome – HMGB1 complexes stimulate cytokine production in primary macrophages in a TLR-2 and MyD88-dependent manner [252]. The HMGB1/TLR2 axis performs a non-inflammatory role by mediating self renewal, tumorigenesis and metastatic stability in cancer stem cells (CSC) [271].

### **3.5.4 TLR9:**

TLR9 recognizes synthetic CpG oligonucleotides and unmethylated CpG motifs in bacterial and viral DNA [272] but also host DNA and RNA [273]. Normally TLR9 is localized in the endoplasmic reticulum but up on stimulation with CpG-DNA redistributes to early endosomes [274]. TLR9 is identified as a receptor for HMGB1 in complex with DNA while no direct interaction could be identified alone by immunoprecipitation. HMGB1 interacts with TLR9 in the endoplasmic reticulum Golgi intermediate compartment (ERGIC) in quiescent macrophages and accelerates the TLR9 redistribution to early endosomes in response to CpG-ODN stimulation. HMGB1-CpG-ODN complexes enhance cytokine production via TLR9 and MyD88 dependent manner but independent of TLR4 and TLR2 [275, 276].

### **3.6 HMGB1 role in sterile inflammation and autoimmune diseases:**

HMGB1 functions as a pro-inflammatory cytokine alone or as complex with other inflammatory agents in many infectious and inflammatory diseases. Elevated levels of HMGB1 has been reported in many sterile inflammatory diseases and successful targeting of HMGB1 through neutralizing antibodies or inhibitors shows promising therapeutic target in preclinical models. Some of the obesity-associated diseases where HMGB1 is involved are discussed below.

#### *3.6.1 Atherosclerosis*

Smooth muscle cells isolated from atherosclerotic plaques are found to secrete HMGB1 while not cells from normal arteries. Several cell types present in atherosclerotic plaques contribute to the increase in HMGB1 release including endothelial cells, foam cells, macrophages and platelets [233, 277-280]. They proliferate, migrate and actively secrete more HMGB1 when they are exposed to HMGB1. Endothelial cells treated with rHMGB1 induced inflammatory response including enhanced expression of ICAM-1, RAGE, TNF, CXCL8 and CCL2, plasminogen activator inhibitor (PAI-1) and tissue plasminogen activator (tPA) [267].

#### *3.6.2 Diabetes*

Higher levels of serum HMGB1 is observed in type II diabetes mellitus patients with all-cause mortality in association with coronary artery disease [280-282]. HMGB1 is passively released from damaged pancreatic  $\beta$  cells or secreted by islet infiltrated with immune cells such

as dendritic cells [283, 284]. Cardiac fibroblasts, cardiomyocytes and macrophages exposed to hyperglycemic condition led to time dependent upregulation of HMGB1 in RNA and protein level [285]. In the same way, NFkB activation due to hyperglycemia is found to be completely inhibited by knockdown of HMGB1 in fibroblasts and macrophages cells. Treatment with HMGB1 box A, a functional antagonist of extracellular HMGB1 cytokine activity and an inhibitor of HMGB1 interaction with RAGE, significantly prevents myocardial injury in diabetes mellitus and post-myocardial infarction tissue injury [285]. In-vivo studies by blocking HMGB1 in NOD mice reduce inflammation and delay the onset of diabetes [283].

### 3.6.3 Rheumatoid arthritis

HMGB1 levels are higher in serum, synovial tissue and fluid from rheumatoid arthritis patients [286-289] and joints from RA rodent models [290, 291] in comparison with normal. HMGB1 expression is particularly higher in areas where synovial tissue invades intra-articular cartilage and bone, which causes joint destruction. Traditional therapy such as gold salt administration reduces the HMGB1 release from macrophages [292]. Also neutralizing the expression or activation of HMGB1 by either antibodies [293] or other inhibitors [294-296] totally confers significant protection against the bone and cartilage destruction, and protection from inflammation.

## 3.7 HMGB1 expression in AT

Absolute absence of fat mass in HMGB1 knockout mice suggests the importance of HMGB1 expression and need for adipose tissue functions [187]. A strong positive correlation found between expression level of HMGB1 and FTO – fat mass and obesity associated gene, further enhances the link between HMGB1 and obese adipose tissue metabolism [297]. Proteomic profiling of adipose tissue from *Zmpste 24(-/-)* mice, a lipid dystrophy model surprisingly reveals altered expression of HMGB1 with cytoskeletal vimentin protein [298], which suggests the possible involvement of HMGB1 in adipocytes differentiation and plasticity. In HFD fed mice [299] and in peritumoral mice [300] adipose tissue, the nuclear HMGB1 level is significantly lower in comparison with the control mice due to necrosis. The *in vitro* differentiated 3T3-L1 adipocytes when exposed to necrotic inducers display increased level of



HMGB1 in the medium and decreased the nuclear HMGB1 while not by apoptosis inducers [299].

Adipocytes treatment with rHMGB1 or microvesicles carrying HMGB1 (isolated from tobacco smoke exposed macrophages, TSE-MV) induce the secretion of MCP-1 and impairs insulin signaling [301]. Inhibition of HMGB1 by glycyrrhizin or MCP-1 blocking, totally attenuates the monocyte transmigration and adherence to TSE-MV-exposed adipocytes [302]. NLRP3-ASC activated inflammasomes induced the release of HMGB1 by pyroptosis process in BMDM cells, which could also be induced by endotoxin [303] or PA [304]. Since both endotoxin and PA are known for adipose tissue inflammation and insulin resistance, we suspect whether HMGB1 could be secreted during adipose tissue inflammation. Very recently, increased serum level of HMGB1 in correspondence with other proinflammatory cytokines are reported from obese children and proposing HMGB1 as a biomarker for metabolic syndrome in obese children [305]. All these studies give possible insights of how HMGB1 could mediate sterile inflammation in adipocytes and infiltrated cells in adipose tissue.

HMGB1 in relation with adipose tissue inflammation remained unanswered and this interests us to investigate whether there is differential expression of HMGB1 between obese and lean person adipose tissue. Moreover we want to determine whether HMGB1 is secreted during adipose tissue inflammation and further we want to identify which cells are responsible for the increased secretion.



Contents lists available at ScienceDirect

Cytokine

journal homepage: [www.journals.elsevier.com/cytokine](http://www.journals.elsevier.com/cytokine)

## Inflammation triggers high mobility group box 1 (HMGB1) secretion in adipose tissue, a potential link to obesity

Manoj Kumar Gunasekaran<sup>a</sup>, Wildriss Viranaicken<sup>b</sup>, Anne-Claire Girard<sup>a,c</sup>, Franck Festy<sup>a,c</sup>, Maya Cesari<sup>a</sup>, Regis Roche<sup>a,c</sup>, Laurence Hoareau<sup>a,\*</sup>

<sup>a</sup> Groupe d'Etude sur l'Inflammation Chronique et l'Obésité (GEICO), University of Reunion Island, CYROI, 2 rue Maxime Rivière, 97 490 Sainte-Clotilde, Reunion

<sup>b</sup> Groupe de Recherche en Immunopathologies et maladies infectieuses (GRI), University of Reunion Island, CYROI, 2 rue Maxime Rivière, 97 490 Sainte-Clotilde, Reunion

<sup>c</sup> STEMGIS, CYROI, 2 rue Maxime Rivière, 97 490 Sainte-Clotilde, Reunion

### ARTICLE INFO

#### Article history:

Received 18 April 2013

Received in revised form 19 July 2013

Accepted 21 July 2013

Available online xxx

#### Keywords:

HMGB1

Human adipose tissue

Inflammation

Obesity

Primary culture

### ABSTRACT

**Background:** Low grade inflammation is one of the major metabolic disorders in case of obesity due to variable secretion of adipose derived cytokines called adipokines. Recently the nuclear protein HMGB1 was identified as an inflammatory alarmin in obesity associated diseases. However HMGB1 role in adipose tissue inflammation is not yet studied.

**Objectives:** The aim of this study was to prove the expression of HMGB1 in human adipose tissue and to assess the levels of expression between normo-weight and obese individuals. Furthermore we determined which type of cells within adipose tissue is involved in HMGB1 production under inflammatory signal.

**Methods:** Western-blot was performed on protein lysates from human normo-weight and obese adipose tissue to study the differential HMGB1 expression. Human normo-weight adipose tissue, adipose-derived stromal cells (ASCs) and adipocytes were cultured and stimulated with LPS to induce inflammation. HMGB1, IL-6 and MCP-1 secretion and gene expression were quantified by ELISA and Q-PCR respectively, as well as cell death by LDH assay. HMGB1 translocation during inflammation was tracked down by immunofluorescence in ASCs.

**Results:** HMGB1 was expressed 2-fold more in adipose tissue from obese compared to normo-weight individuals. LPS led to an up-regulation in HMGB1 secretion and gene expression in ASCs, while no change was noticed in adipocytes. Moreover, this HMGB1 release was not attributable to any cell death. In LPS-stimulated ASCs, HMGB1 translocation from nucleus to cytoplasm was detectable at 12 h and the nuclear HMGB1 was completely drained out after 24 h of treatment.

**Conclusion:** The expression level studies between adipose tissue from normo-weight and obese individuals together with *in vitro* results strongly suggest that adipose tissue secretes HMGB1 in response to inflammatory signals which characterized obesity.

© 2013 Elsevier Ltd. All rights reserved.

### 1. Introduction

Obesity has been well studied and everyone agrees on the fact that excess fat mass is critical for health. All the metabolic disorders encountered during obesity lead to deleterious metabolic and inflammatory diseases such as cardio-vascular diseases, arthritis, diabetes and even cancer [1]. Numerous studies have shown that excess fat mass leads first to low grade inflammation which is evidenced by high level of circulating pro-inflammatory cytokines and chemokines such as IL-6, TNF $\alpha$ , MCP-1, IL-1 $\beta$  and by a lower level of circulating anti-inflammatory cytokines like adiponectin [2–4]. More particularly, it has been proved that

adipose tissue is largely involved in this phenomenon by secreting huge amount of IL-6 [5,6]. This cytokines dysregulation is one of the starting point for obesity-related diseases [7].

Recently, a new protein is found to be involved in many infectious and non-infectious diseases: the high mobility group box 1 (HMGB1) protein [8–10]. In all cell types, the main and first role of this non-histone DNA-binding protein is to stabilize the nucleosomes, to help DNA bending and then to take part in DNA replication, transcription and repair [11]. Nevertheless under signals like stress, cell death, infection or inflammation, HMGB1 is found to be released from cell to act as a damage associated molecular pattern (DAMP) [12,13]. This phenomenon occurs in many tissues and cell types from different species such as hepatocytes [14,15], smooth muscle cells [16], testis [17] or periodontal ligament [18]. Once released, this protein triggers the secretion of many

\* Corresponding author. Tel.: +262 262 938 201.

E-mail address: [laurence.hoareau@wanadoo.fr](mailto:laurence.hoareau@wanadoo.fr) (L. Hoareau).

pro-inflammatory cytokines and is characterized as an 'alarmin'. Further, HMGB1 promotes the recruitment of inflammatory cells to damaged tissues [19,20], as in the case for atherosclerotic plaques [21,22].

In mouse model, *Hmgb1*<sup>-/-</sup> pups die within 24 h due to hypoglycemia and have no fat [23]. Moreover atherosclerotic hamsters fed with high fat diet exhibit higher level of circulating HMGB1 [24]. Mouse 3T3 fibroblasts and macrophages have been shown to express and release HMGB1 under signals such as oxidative stress (UVB) and inflammation (LPS) [25–27], and they are activated by HMGB1 [15,27]. As 3T3 fibroblasts are capable of differentiating into adipocytes and as macrophages infiltrate adipose tissue in case of obesity, these studies suggest a potential link between HMGB1 and obesity, although this link is not yet well understood. Moreover, a positive correlation has also been done between HMGB1 gene expression in human adipose tissue and one of the common polymorphism of the fat mass and obesity-associated (FTO) gene strongly linked to obesity [28]. Based on these data, we aimed to determine if HMGB1 is secreted by adipose tissue under low grade inflammation and which cell types within adipose tissue could be implicated in HMGB1 production.

The first aim of this work was to prove the expression of HMGB1 protein in human adipose tissue and to compare the expression level between normo-weight and obese individuals. Then from primary culture of human adipose tissue, adipocytes and adipose-derived stromal cells (ASCs) from normo-weight individuals, we compared the expression and secretion level of HMGB1 in normal and inflammatory conditions. Finally, immunostaining experiments were conducted on ASCs to study the cytoplasmic translocation of HMGB1 under inflammation.

## 2. Materials and methods

### 2.1. Reagents

Lipopolysaccharides (LPS from *E. coli* 0111:B4 strain, batch #LPE-32-02) and TRPreagent were purchased from Sigma, France. Collagenase (NB4, 0.12 PZU/mg) was from SERVA, Germany. Steri-Flip 100 µm was purchased from Millipore, France. All reagents for cell culture were purchased from PAN Biotech (France) except insulin (Umuline Rapide, Lilly, France) and reagents for adipocyte differentiation (Sigma, France). Ringer Lactate was obtained from B. Braun, France.

### 2.2. Origin of human adipose tissue samples and harvesting protocol

Subcutaneous (abdominal, buttocks, hips and thighs) adipose tissue samples were obtained from normal weight (BMI ≤ 25, mean body mass index = 22.65 ± 1.42 kg/m<sup>2</sup>) and obese women (BMI ≥ 30, mean body mass index = 35.6 ± 3.2 kg/m<sup>2</sup>) undergoing liposuction, performed under general anaesthesia for aesthetic reasons (aged from 25 to 60 years, mean 39 years). Apart from oral contraception, the subjects were not receiving any treatment with prescribed medication at the time of liposuction. Fat tissue was harvested mechanically with a vacuum pump after infiltration of a tumescence solution (40 mL lidocaine 2% + adrenalin 1 mg/L for 1 L Ringer Lactate) for local anaesthesia. A total of 22 samples were obtained and the study was approved by the Reunion Island ethics committee for the protection of persons undergoing biomedical research. Samples were handled within an hour after the end of the surgery.

### 2.3. Supplemented media

For all experiments, medium 199 was supplemented with 2 g/L glucose, 5 mg/mL amphotericin B, 0.2 mg/mL streptomycin and 200 U/mL penicillin. For adipose tissue and adipocytes, 1% fetal bovine serum (FBS) was added with 8 µg/mL biotin, 4 µg/mL pantothenate and 66 nM insulin. For ASCs, 20% FBS was added on the day of culture, then 10% the day after and for the rest of the culture period (media was changed each 2 days).

### 2.4. Adipose tissue preparation and primary culture

After liposuction, adipose tissue was rinsed thrice with Ringer Lactate. For primary culture, 200 µL of tissue was distributed in 24-well tissue culture plates with 300 µL of supplemented medium. Tissue was then maintained at 37 °C in 5% CO<sub>2</sub> for a period of 24 h prior to the experiments.

### 2.5. Adipocytes isolation and primary culture

After washing as described above, tissue was digested for 30 min at 37 °C in Ringer Lactate containing 0.5 mg/mL collagenase. The floating cells (adipocytes) were rinsed in Ringer-Lactate and plated (200 µL, ≈30,000 cells) in 24-well tissue culture plates with 300 µL of supplemented medium. Cells were then maintained at 37 °C in 5% CO<sub>2</sub> for a period of 24 h prior to the experiments.

### 2.6. Stromal cells extraction and ASCs primary culture

Tissue samples obtained by liposuction were digested for 1 h at 37 °C in Ringer Lactate containing 1 mg/mL collagenase. Digested tissue was centrifuged and the pellet was resuspended in Ringer Lactate, washed and filtered through Steri-Flip 100 µm. The isolated cells have already been analyzed by flow cytometry to assess the phenotypic characteristics of adipose stromal cells [29]. Cell counting and viability were assessed by Trypan blue dye exclusion. Around 200,000 cells/well were plated in 24-well culture plates with 500 µL of supplemented media. Cells were then maintained at 37 °C in 5% CO<sub>2</sub> for a period of 24 h prior to the experiments. By convention, the adherent and proliferated stromal vascular cells are called adipose-derived stromal cells (ASCs).

### 2.7. ASCs differentiation into adipocytes

The day after 80–90% confluence, a differentiation cocktail was added to the supplemented media [30]: 3% SVF, 200 nM insulin, 1 µM dexamethasone, 0.1 mM IBMX, 5 µM rosiglitazone, 16 µg/mL biotin and 7.4 µg/mL pantothenate. After 4 days, the differentiated adipocytes were maintained with 3% SVF, 200 nM insulin, 16 µg/mL biotin, 7.4 µg/mL pantothenate and medium was changed for every 3 days.

### 2.8. Adipose tissue protein extraction and western blot

Adipose tissue was lysed with protein lysis buffer (50 mM Tris-HCl, pH 7.6, 500 mM NaCl, 0.2 mM EDTA) using Dounce homogenizer (10 strokes) under ice-cold condition. The lysates were centrifuged at 15,000g for 30 min at 4 °C, the fat cake was removed and the aqueous protein phase was transferred to a new tube. Protein concentration was determined by Bradford method.

20 µg of proteins were separated by 12.5% SDS-PAGE and transferred to nitrocellulose membrane (Hybond C-EXTRA, Amersham bioscience, France). Membranes were blocked with 5% non-fat dried milk for 1 h at room temperature in Tris-buffered saline with 0.1% Tween-20 and then sequentially probed with mouse anti-human HMGB1 antibody (1:1000 dilution, clone 2F6, Sigma, France)



or control mouse anti-human  $\beta$ -actin antibody (1:1000 dilution, clone AC15, Sigma, France) and then with peroxidase labeled anti-mouse IgG (1:2000 dilution, Vector Laboratories Inc., USA).

Band detection using homemade chemiluminescent substrate (Luminol A8511 and p-Coumaric acid C9008, Sigma, France) was developed on a high performance autoradiography film (Amersham Hyperfilm™, France) and digitized using EPSON perfection 2480 photo. Band intensities were analyzed using image J software (NIH, USA). HMGB1 signal intensity from samples was averaged, normalized to signal from  $\beta$ -actin and used to calculate the relative signal intensity and the statistical analysis.

### 2.9. HMGB1, IL-6 and MCP-1 ELISA

Following treatment, media samples were collected and assayed for HMGB1 (IBL International, Germany), IL-6 and MCP-1 (eBioscience, France), according to the manufacturer's instructions. ELISA sensitivity: 0.2 ng/mL for HMGB1, 2 pg/mL for IL-6, 6 pg/mL for MCP-1.

### 2.10. Cytotoxicity assay

The release of lactate dehydrogenase (LDH) induced by plasma membrane disruption in culture medium was measured using LDH cytotoxicity assay kit from Science cell (Cliniscience, France) according to manufacturer's instructions. LDH release was measured through an enzymatic reaction and read at 490 nm. As a positive control for cell death, tissue and cells were treated with 1% Triton X-100 (Sigma, France).

### 2.11. RNA extraction, reverse transcription and real-time quantitative PCR

Following treatment, total RNA was isolated using TRIreagent according to the manufacturer's instructions. 2  $\mu$ g of total RNA was reverse-transcribed using random hexamers and Reverse Transcriptase™ (Invitrogen, France). Quantitative PCR was performed on 1  $\mu$ L of cDNA using ABI PRISM 7500 thermal cycler (Applied Biosystems, France) with qPCR supermix for ABI PRISM (Invitrogen, France). The 18S ribosomal RNA gene was used as a reference. Quantification of target mRNA was calculated using the comparative Ct method ( $\Delta\Delta$ CT) and was normalized to the reference gene expression. Analysis was performed on 6 samples per condition. Primers sequences are listed below:

IL-6	Forward: TCA CCT CTT CAG AAC GAA TTG ACA Reverse: AGT GCC TCT TTG CTG CTT TCA C
MCP-1	Forward: ATC ACC AGC AGC AAG TGT C Reverse: AGG TGG TCC ATG GAA TCC TG
HMGB1	Forward: ACC CAG ATG CTT CAG TCA AC Reverse: GGC GAT ACT CAG AGC AGA AG
18S	Forward: CGC CGC TAG AGG TGA AAT TCT Reverse: CAT TCT TGG CAA ATG CTT TC

### 2.12. Immunofluorescence

ASCs were cultured in monolayer on glass coverslips. Following treatment, the culture medium was removed completely and washed thrice with ice-cold PBS to be fixed with ice-cold absolute ethanol for 5 min. Cells were immediately stained for immunofluorescence or air dried and stored at  $-20^{\circ}\text{C}$  until staining was performed. Immunofluorescence staining was carried out according to a standard protocol.

The fixed cells were incubated primarily with mouse anti-human HMGB1 antibody (1:1000 dilution, clone 2F6, Sigma, France) overnight at  $4^{\circ}\text{C}$ . After PBS-Tween (0.05%) washing, cells were incubated with secondary antibody (1:2000 dilution, goat anti-mouse Alexa flour 488 IgG, Molecular Probes, Invitrogen, France) for 2 h in dark. Nuclei were stained with DAPI (D9542, Sigma, France) at a final concentration of 0.1 ng/mL. Coverslips were mounted in glass slides using Vectashield (Vector Labs, Cliniscience) and fluorescence was observed using a Nikon Eclips 80i microscope (Nikon, France). Images were obtained using the Nikon Digital camera system (Nikon, DXM1200C) and the imaging software NIS-Element BR version 3.1 (Nikon).

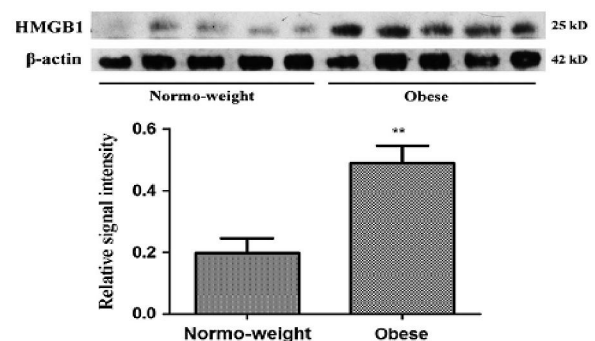
### 2.13. Statistics

All values were measured as mean  $\pm$  S.D. Statistical analysis was performed using Graph pad PRISM 5 software (Windows). Differences between normal and obese adipose tissue were tested using non-parametric T-test (Mann-Whitney test). Differences between control and treated samples were tested for significance by one-way ANOVA and Dunnett post-test.  $P < 0.05$  (\*);  $P < 0.01$  (\*\*);  $P < 0.001$  (\*\*\*) ;  $P < 0.0001$  (\*\*\*\*).

## 3. Results

### 3.1. High HMGB1 expression in adipose tissue from obese individuals

To determine the basal expression of HMGB1 protein in adipose tissue from normo-weight and obese individuals, western blot was performed. Regardless of the BMI, a specific band corresponding to HMGB1 protein was revealed from adipose tissue protein lysates of all the samples tested with the expected molecular weight (Fig. 1A). No oligomer or complex was revealed from the blot (data not shown). This ensures that HMGB1 protein was expressed in adipose tissue from both normal weight and obese persons. However, the HMGB1 bands from obese samples showed higher intensity in comparison with normal samples. This was confirmed after  $\beta$ -actin normalization of 20 samples analyzed: the average intensity for obese samples was significantly 2-fold higher than the average intensity for lean samples (Fig. 1B). Thus, HMGB1 protein was expressed more in adipose tissue from obese in comparison with normal weight individuals.



**Fig. 1.** HMGB1 protein expression in adipose tissue from lean and obese individuals. A. HMGB1 protein detected by western blot on adipose tissue lysates obtained from 5 lean individuals (BMI =  $22.9 \pm 1.6 \text{ kg/m}^2$ ) and 5 obese individuals (BMI =  $34.9 \pm 2.8 \text{ kg/m}^2$ ).  $\beta$ -actin was used as control. Results are representative of 10 lean and 10 obese tissue samples. B. HMGB1 densitometry calculated on 10 lean individuals (BMI =  $23.1 \pm 1.4 \text{ kg/m}^2$ ) and 10 obese individuals (BMI =  $35.6 \pm 3.2 \text{ kg/m}^2$ ). Data were normalized to  $\beta$ -actin and presented as mean  $\pm$  S.D (arbitrary units).  $P < 0.01$  (\*\*).

### 3.2. HMGB1 secretion is up-regulated without gene up-regulation in adipose tissue under inflammation

After LPS treatment, inflammation was confirmed by the huge increase in IL-6 and MCP-1 secretion from adipose tissue: around 200 fold for IL-6 and 100 fold for MCP-1 since 12 h, until 400 and 200 fold respectively for 48 h (Fig. 2). LDH measurement from media remained the same between control and LPS-treated tissue, regardless of the time point (Fig. 2).

Regarding HMGB1 secretion, we observe that human adipose tissue was able to release HMGB1 at a low basal level (less than 5 ng/mL, Fig. 2) and that there was no accumulation in culture media. More interestingly, we show that under LPS treatment this secretion was up-regulated from 24 h of incubation (3-fold increase) and kept increasing until 48 h (5-fold more) reaching 17 ng/mL.

At gene expression level, although IL-6 and MCP-1 mRNA were up-regulated, we cannot detect any significant change in HMGB1 mRNA level between control and LPS-treated adipose tissue (Fig. 3).

### 3.3. Adipocytes inflammation doesn't regulate HMGB1 secretion and gene expression

Despite the up-regulation of IL-6 and MCP-1 at both secretion (Fig. 4) and mRNA (Fig. 5) level, there was no change in HMGB1 secretion and mRNA expression in adipocytes after LPS treatment, regardless of the incubation time. Nevertheless, a basal level of released HMGB1 was detected (less than 5 ng/mL), without any accumulation in the culture media. Moreover, adipocytes viability was not affected by LPS treatment since there is no increase in LDH release (Fig. 4).

### 3.4. HMGB1 secretion and gene expression up-regulation in ASCs under inflammation

LPS-treated ASCs showed inflammation, as there was a significant up-regulation in IL-6 and MCP-1 secretion since 12–48 h

(Fig. 6). HMGB1 was also released in higher levels after LPS treatment (Fig. 6). This increase was significant since 12 h although the level was low (less than 5 ng/mL) and at 48 h the increase was about 10-fold to reach more than 10 ng/mL. Moreover, LPS treatment did not induce any cell death in ASCs, as confirmed by the LDH assay (Fig. 6).

At gene expression level, inflammation was characterized by IL-6 and MCP-1 up-regulation (Fig. 7). Regarding HMGB1 mRNA, a 20% increase was found at 12 h treatment and at 24 h expression levels were doubled compared to control (Fig. 7). After 48 h treatment, HMGB1 gene expression was no more up-regulated and returned to normal.

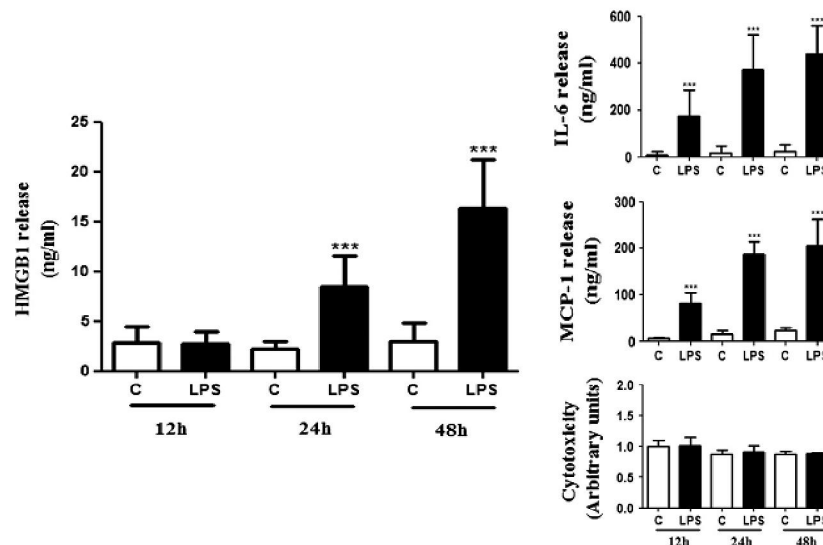
### 3.5. HMGB1 secretion is not modulated by LPS in in vitro-differentiated adipocytes

At day 16, adipose differentiation was confirmed by multilocular droplets accumulation inside cells, and identified as triglycerides with Oil Red O staining (Fig. 8). These fully differentiated and hypertrophied cells secreted more than 3-fold IL-6 after 24 h LPS treatment, whereas no significant change was noticed in HMGB1 secretion. No cell death was detected insofar as LDH dosage was not significantly higher after LPS treatment (and compared to the first day of culture, data not shown).

### 3.6. HMGB1 translocation in ASCs under inflammation

In control cells, nuclei were stained in blue with DAPI, surrounded and punctuated by green points corresponding to HMGB1 (Fig. 9). The nuclear DAPI staining completely matched with HMGB1 staining, in accordance with the function of HMGB1 as a nuclear protein. In control cells, there was no change in HMGB1 localization until 24 h of culture.

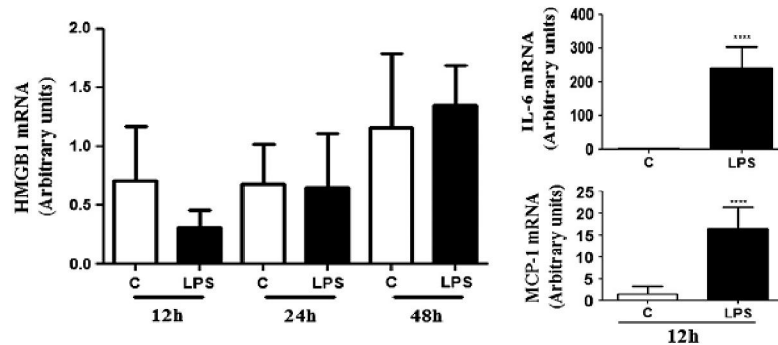
Under LPS treatment at 6 h, the HMGB1 intra-nuclear localization was slightly modified to be higher in the nuclear crown. At 12 h treatment, HMGB1 translocation was effective and clearly visible, resulting in a cytoplasmic localization, although a slight signal



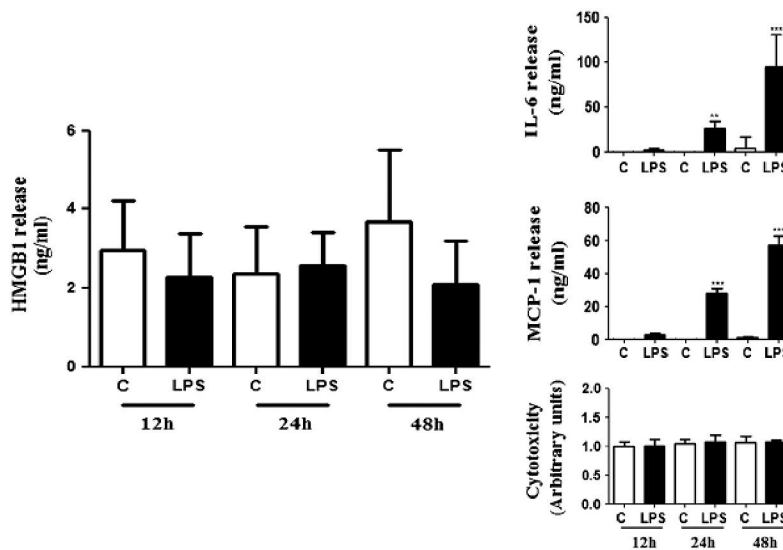
**Fig. 2.** HMGB1 secretion from adipose tissue in culture, normal versus inflammatory conditions. HMGB1, IL-6 and MCP-1 concentration measured by ELISA in media from adipose tissue after 12 h, 24 h and 48 h incubation with or without 1  $\mu$ g/mL LPS. Results are expressed in ng/mL. LDH release from adipose tissue after 12 h, 24 h and 48 h incubation with or without 1  $\mu$ g/mL LPS. Results are expressed in arbitrary units normalized to 12 h control. The graphs show the mean  $\pm$  S.D. of the results from 6 different tissue samples ( $n = 6$  for each condition, for each tissue sample).  $P < 0.001$  (\*\*\*).

Please cite this article in press as: Gunasekaran MK et al. Inflammation triggers high mobility group box 1 (HMGB1) secretion in adipose tissue, a potential link to obesity. Cytokine (2013), <http://dx.doi.org/10.1016/j.cyto.2013.07.017>





**Fig. 3.** HMGB1 gene expression from adipose tissue in culture, normal versus inflammatory conditions. HMGB1 gene expression determined by Q-PCR after 12 h, 24 and 48 h incubation with or without 1  $\mu\text{g}/\text{mL}$  LPS. IL-6 and MCP-1 gene expression determined by Q-PCR on the 12 h samples. Results are expressed in arbitrary units reported to respective time control. The graphs show the mean  $\pm$  S.D of the results from 6 different tissue samples ( $n = 6$  for each condition, for each tissue sample).  $P < 0.0001$  (\*\*\*\*).



**Fig. 4.** HMGB1 secretion from adipocytes in culture, normal versus inflammatory conditions. HMGB1, IL-6 and MCP-1 concentration measured by ELISA in media from adipocytes after 12 h, 24 h and 48 h incubation with or without 1  $\mu\text{g}/\text{mL}$  LPS. Results are expressed in ng/mL. LDH release from adipose tissue after 12 h, 24 h and 48 h incubation with or without 1  $\mu\text{g}/\text{mL}$  LPS. Results are expressed in arbitrary units normalized to 12 h control. The graphs show the mean  $\pm$  S.D of the results from 6 different tissue samples ( $n = 6$  for each condition, for each tissue sample).  $P < 0.01$  (\*\*);  $P < 0.001$  (\*\*\*).

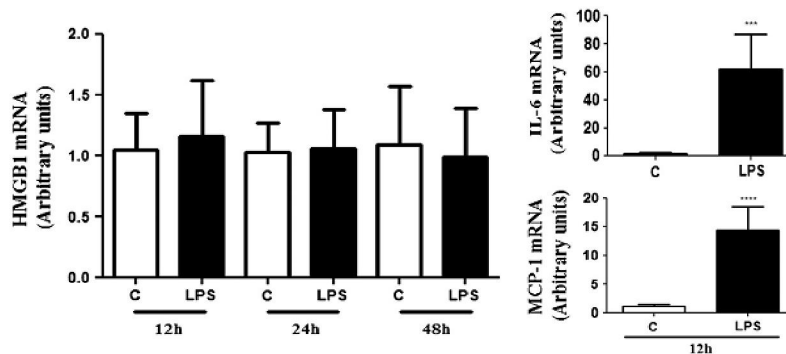
was detected in the nucleus. After 24 h treatment, HMGB1 was completely drained out from nucleus to the cytoplasm, resulting in green signal only in the cytoplasmic compartment.

#### 4. Discussion

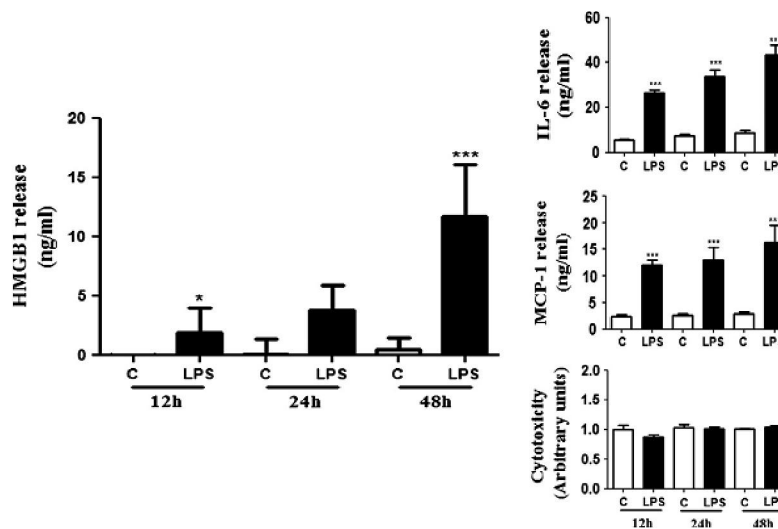
The role of HMGB1 in the initiation of certain diseases has been reported as well as its expression [31]. For instance, this is the case for the non-alcoholic fatty liver disease [32] or for atherosclerosis [16,24,33]. The common feature is low-grade inflammation, which participates from the onset to the maintenance and aggravation of these diseases. In the context of obesity, a recent work from Arrigo et al., has identified blood HMGB1 as a new biomarker for metabolic syndrome [34] in obese children, but nothing is known about HMGB1 expression and its role in human adipose tissue. Here we

have investigated if HMGB1 could be possibly linked to adipose tissue inflammation and obesity.

On all patients analyzed in this study, we observed that there was a strong positive correlation between BMI and HMGB1 protein expression within adipose tissue: the expression was higher in adipose tissue from obese persons than normal weight individuals. Our results further supports the study of Lappalainen et al., in which they showed a positive correlation between HMGB1 gene expression in adipose tissue and one of the common polymorphism of the FTO gene strongly linked to obesity [28]. Our study further confirms that adipose tissue HMGB1 is dysregulated and strongly expressed in case of obesity. This increase in HMGB1 protein level could probably partly due to leukocytes infiltration, which happens in case of obesity [35,36], as these cells are known to be a source of HMGB1 [25,37]. We wanted next to determine if adipose cells themselves (stromal cells or adipocytes) have a role in



**Fig. 5.** HMGB1 gene expression from adipocytes in culture, normal versus inflammatory conditions. HMGB1 gene expression determined by Q-PCR after 12 h, 24 and 48 h incubation with or without 1  $\mu$ g/mL LPS. IL-6 and MCP-1 gene expression determined by Q-PCR on the 12 h samples. Results are expressed in arbitrary units reported to respective time control. The graphs show the mean  $\pm$  S.D. of the results from 6 different tissue samples ( $n = 6$  for each condition, for each tissue sample).  $P < 0.001$  (\*\*);  $P < 0.0001$  (\*\*\*\*).



**Fig. 6.** HMGB1 secretion in ASCs, normal versus inflammatory conditions. HMGB1, IL-6 and MCP-1 concentration measured by ELISA in media from ASCs after 12 h, 24 h and 48 h incubation with or without 1  $\mu$ g/mL LPS. Results are expressed in ng/mL. LDH release from adipose tissue after 12 h, 24 h and 48 h incubation with or without 1  $\mu$ g/mL LPS. Results are expressed in arbitrary units normalized to 12 h control. The graphs show the mean  $\pm$  S.D. of the results from 6 different tissue samples ( $n = 6$  for each condition, for each tissue sample).  $P < 0.05$  (\*);  $P < 0.001$  (\*\*).

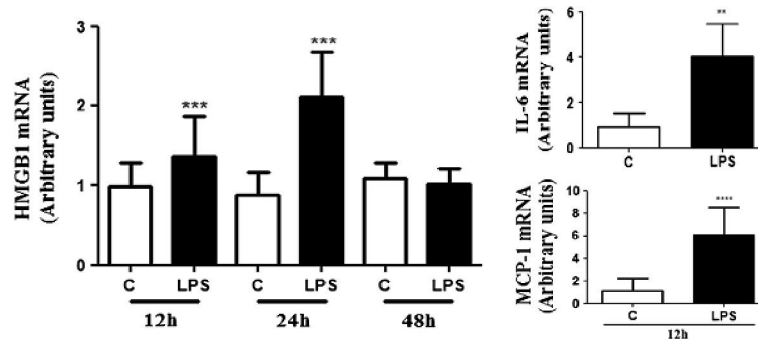
this expression. Then, for all *in vitro* experiments we have conducted, we used samples from normo-weight individuals, which contain no or really few infiltrated leukocytes (unpublished data).

It's now well established that overweight and obesity exhibit a chronic and low-grade inflammation [38,39]. Thus, in order to know if the high level of HMGB1 expression in case of obesity is linked to low grade inflammation, adipose tissue from normal weight patients was cultured and treated with LPS to induce inflammation. We proved for the first time that human adipose tissue was able to release HMGB1 at a low basal level. More interestingly, we showed that under inflammation, HMGB1 secretion was upregulated. This increase in HMGB1 secretion following LPS treatment has never been published concerning adipose tissue, and this new result is totally in accordance with *in vivo* and *in vitro* studies in other models [25,40].

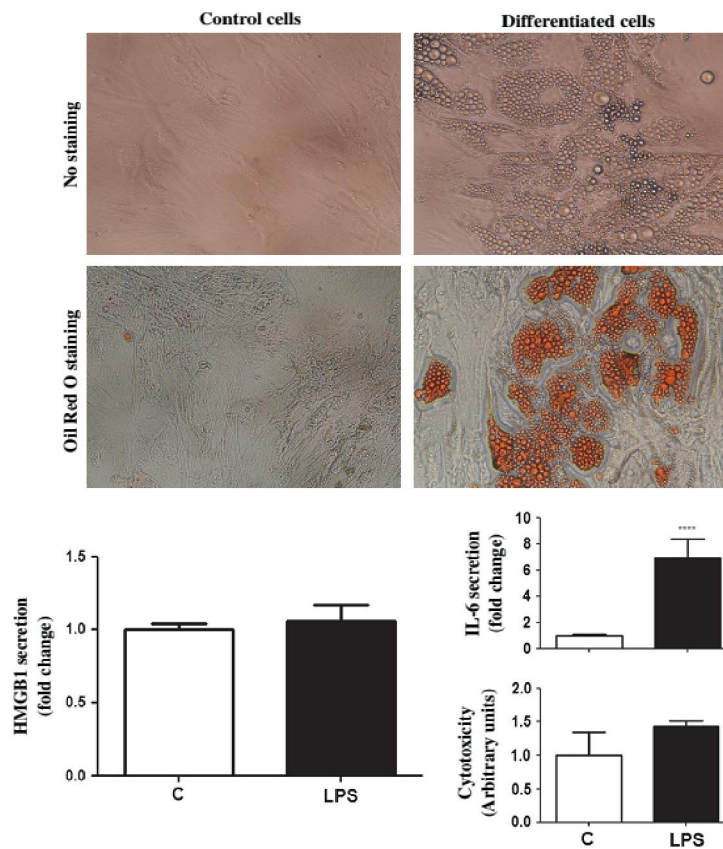
Since HMGB1 has been considered for a long time only as a cell death marker released passively by necrotic cells [41], we have also

performed cytotoxicity assay by measuring lactate dehydrogenase (LDH) release. In our experiments, the level of LDH was low and the same in all supernatants (control versus LPS), ruling out the possibility that LPS-induced HMGB1 secretion was due to cell lysis. This secretion is thus specifically attributable to inflammation and could be associated to an active and regulated secretion, different from the passive mechanism that occurs during cell death. Nevertheless, the HMGB1 basal detectable level noticed in control adipose tissue could probably be attributable to basal cell death in culture, at least partly.

Our data were totally new and led us to investigate which type of cells within adipose tissue was responsible for this HMGB1 up-regulation. Indeed, adipose tissue is composed by different kind of cells: after collagenase digestion, we can separate the floating adipocytes and the pelleted stromal vascular fraction. These two cell populations were then plated separately and inflammation was induced through LPS incubation.



**Fig. 7.** HMGB1 gene expression in ASCs, normal versus inflammatory conditions. HMGB1 gene expression determined by Q-PCR after 12 h, 24 and 48 h incubation with or without 1  $\mu$ g/mL LPS. IL-6 and MCP-1 gene expression determined by Q-PCR on the 12 h samples. Results are expressed in arbitrary units reported to respective time control. The graphs show the mean  $\pm$  S.D of the results from 6 different tissue samples ( $n = 6$  for each condition, for each tissue sample).  $P < 0.01$  (\*\*);  $P < 0.001$  (\*\*\*);  $P < 0.0001$  (\*\*\*\*).

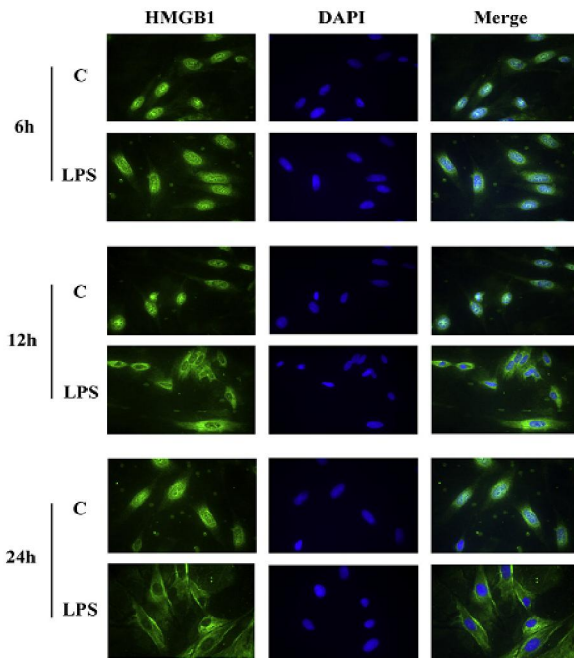


**Fig. 8.** HMGB1 secretion from *in vitro* differentiated adipocytes. ASCs were induced to differentiate into adipocytes with appropriate media. To confirm differentiation, Oil Red O staining was performed after 16 days (D16) and pictures were taken with a phase contrast light microscope and are representative of 2 different experiments (400 $\times$  magnification). At D16, the medium was changed and the fully differentiated cells were treated with or without LPS (1  $\mu$ g/mL) for 24 h and HMGB1 secretion was quantified by ELISA together with IL-6 and LDH release. Results are expressed in arbitrary units (normalized to control cells, 0.575 ng/mL for HMGB1 and 0.641 ng/mL for IL-6). The graphs show the mean  $\pm$  S.D of the results from 2 different tissue samples ( $n = 6$  for each condition, for each tissue sample).  $P < 0.0001$  (\*\*\*\*).

From our results, we can conclude that the general HMGB1 production observed in adipose tissue comes exclusively from cells from the stromal vascular fraction, insofar as ASCs were able to

respond to inflammation by up-regulating HMGB1 gene expression and secretion in a time-dependant manner. Neither freshly cultured adipocytes, nor *in vitro*-differentiated adipocytes showed a





**Fig. 9.** HMGB1 localization in ASCs, normal versus inflammatory conditions. HMGB1 localization detected by immunofluorescence. After 6 h, 12 h and 24 h incubation with or without 1  $\mu$ g/ml LPS, ASCs were stained with anti-HMGB1 antibody (green) and DAPI (blue). 400 $\times$  magnification. Photographs were taken with an inverted fluorescent microscope and are representative of 3 independent experiments on 3 different tissue samples.

modification in their HMGB1 profile. This difference could explain why in adipose tissue samples there was no increase in HMGB1 mRNA: the number of stromal cells within adipose tissue is less compared to that of ASCs in primary culture and the resulting mRNA expression reflected the adipocyte mRNA expression.

In case of obesity, the leukocytes infiltration could be partly induced by the high level of MCP-1 released from adipose tissue during inflammation but also due to HMGB1 release itself, which is known to promote the recruitment of inflammatory cells [19,42].

In the present study, HMGB1 secretion was noticed until 48 h, which further confirms its characterization as a late inflammatory mediator, as it has been related in other models [25,43–45]. This late secretion from adipose tissue could also occur secondarily through TNF $\alpha$  effect, as it has been shown in epithelial cells [46] or in hepatocyte cell line [47]. Indeed, after LPS treatment, TNF $\alpha$  is secreted earlier from adipocytes (peak secretion at 6 h, *in vitro* [48]) and could then stimulate stromal cells through a paracrine effect, to release HMGB1.

Furthermore, the immunofluorescence pictures on undifferentiated ASCs showed a translocation of HMGB1 from nucleus to cytoplasm after LPS treatment. These results are in accordance with literature (HepG2, mouse cardiac fibroblasts and RAW264.7 [25,47,49]). Other events as oxidative stress could lead to HMGB1 translocation (in human epithelial cells [50]). As inflammation and oxidative stress are underlying phenomena in obesity, we can hypothesize that HMGB1 translocation could occur in obesity-linked status.

The translocation study further strengthens the hypothesis that under inflammation signal HMGB1 secretion could mainly be attributable to an active and regulated phenomenon, contrary to the passive release happened during cell necrosis [51,52].

Moreover, no one has elucidated how this nuclear HMGB1 is secreted because HMGB1 does not have a leader sequence to follow the classical pathway of endoplasmic reticulum and Golgi apparatus [52]. For instance, in LPS-activated monocytic cells, it has been shown that once released from nucleus, HMGB1 is accumulated in lysosomal vesicles [53,54], whereas it is spread in all cytoplasm in mouse fibroblasts [53]. This lysosomes exocytosis is triggered by lysophosphatidyl choline, which is generated later under inflammation [54]. Nevertheless, it has been suggested that most of the early release (until 12 h) is from preformed HMGB1 (probably in nucleus) in activated mouse RAW264.7 cells [43]. From our results, although no cytoplasmic vesicle was seen, we need additional experiments to well understand this mechanism in this new model.

## 5. Conclusions

In summary, we propose through our study that HMGB1 is more expressed in adipose tissue from obese persons and that this dysregulation is attributable to inflammation. We have also identified ASCs as the major source of HMGB1 in adipose tissue during inflammation. In the context of using HMGB1 as a therapeutic target to prevent and treat obesity associated diseases [55], further studies have to be investigated on human adipose cells to study what are the signal transduction pathways and the paracrine or autocrine effects due to this damage signal.

## Acknowledgements

We are grateful to José Gonçalves M.D, Olivier Hulard M.D and Pierre Delarue M.D for the collection of adipose tissue samples and their cooperation on the study. We thank the Clinifutur group, and the personnel from the Orchidées and St-Vincent clinics. This work was supported by the Ministère de l'Enseignement Supérieur et de la Recherche, the Ministère d'Outre-Mer and the European Union. MKG received a graduate studentship award from the Regional Council of La Réunion. We would like to thank all patients who consented to the collection of adipose tissue.

## References

- [1] Grundy SM. Metabolic complications of obesity. *Endocrine* 2000;13:155–65.
- [2] Tataranni PA, Ortega E. A burning question: does an adipokine-induced activation of the immune system mediate the effect of overnutrition on type 2 diabetes. *Diabetes* 2005;54:917–27.
- [3] Ferroni P, Basili S, Falco A, Davi G. Inflammation, insulin resistance, and obesity. *Curr Atheroscler Rep* 2004;6:424–31.
- [4] Stienstra R, Tack CJ, Kanneganti TD, Joosten LA, Netea MG. The inflammasome puts obesity in the danger zone. *Cell Metab* 2012;15:10–8.
- [5] Harwood Jr HJ. The adipocyte as an endocrine organ in the regulation of metabolic homeostasis. *Neuropharmacology* 2012;63:57–75.
- [6] Kim JH, Bachmann RA, Chen J. Interleukin-6 and insulin resistance. *Vitam Horm* 2009;80:613–33.
- [7] Gnancinska M, Malgorzewicz S, Stojek M, Lysiak-Szydłowska W, Sworczak K. Role of adipokines in complications related to obesity: a review. *Adv Med Sci* 2009;54:150–7.
- [8] Gougeon ML, Melki MT, Saidi H. HMGB1, an alarmin promoting HIV dissemination and latency in dendritic cells. *Cell Death Differ* 2012;19:96–106.
- [9] Harris HE, Andersson U, Pisetsky DS. HMGB1: a multifunctional alarmin driving autoimmune and inflammatory disease. *Nat Rev Rheumatol* 2012;8:195–202.
- [10] Ohmori H, Luo Y, Kuniyasu H. Non-histone nuclear factor HMGB1 as a therapeutic target in colorectal cancer. *Expert Opin Ther Targets* 2011;15:183–93.
- [11] Liu Y, Prasad R, Wilson SH. HMGB1: roles in base excision repair and related function. *Biochim Biophys Acta* 2010;180:119–30.
- [12] Naglova H, Bucova M. HMGB1 and its physiological and pathological roles. *Bratisl Lek Listy* 2012;113:163–71.
- [13] Czura CJ, Wang H, Tracey KJ. Dual roles for HMGB1: DNA binding and cytokine. *J Endotoxin Res* 2001;7:315–21.
- [14] Evankovich J, Cho SW, Zhang R, Cardinal J, Dhupar R, Zhang L, et al. High mobility group box 1 release from hepatocytes during ischemia and



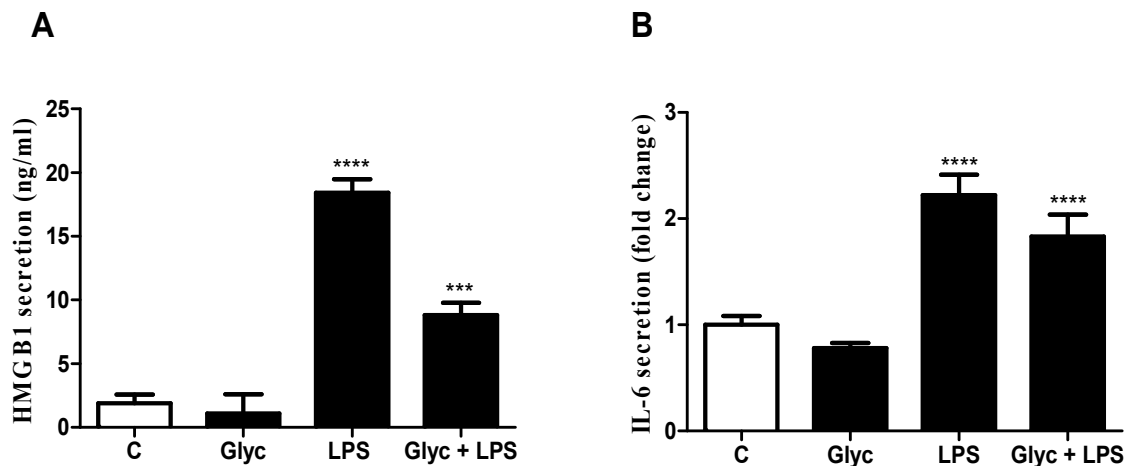
- reperfusion injury is mediated by decreased histone deacetylase activity. *J Biol Chem* 2010;285:39888–97.
- [15] Dragomir AC, Laskin JD, Laskin DL. Macrophage activation by factors released from acetaminophen-injured hepatocytes: potential role of HMGB1. *Toxicol Appl Pharmacol* 2011;253:170–7.
- [16] Porto A, Palumbo R, Pieroni M, Aprigliano G, Chiesa R, Sanvito F, et al. Smooth muscle cells in human atherosclerotic plaques secrete and proliferate in response to high mobility group box 1 protein. *Faseb J* 2006;20:2565–6.
- [17] Zetterstrom CK, Strand ML, Soder O. The high mobility group box chromosomal protein 1 is expressed in the human and rat testis where it may function as an antibacterial factor. *Hum Reprod* 2006;21:2801–9.
- [18] Wolf M, Lossdorfer S, Abuduwali N, Jager A. Potential role of high mobility group box protein 1 and intermittent PTH (1–34) in periodontal tissue repair following orthodontic tooth movement in rats. *Clin Oral Investig*; 2013.
- [19] Schiraldi M, Raucci A, Munoz LM, Livoti E, Celona B, Venereau E, et al. HMGB1 promotes recruitment of inflammatory cells to damaged tissues by forming a complex with CXCL12 and signaling via CXCR4. *J Exp Med* 2012;209:551–63.
- [20] Palumbo R, De Marchis F, Pusterla T, Conti A, Alessio M, Bianchi ME. Src family kinases are necessary for cell migration induced by extracellular HMGB1. *J Leukoc Biol* 2009;86:617–23.
- [21] Kanellakis P, Agrotis A, Kyaw TS, Koulis C, Ahrens I, Mori S, et al. High-mobility group box protein 1 neutralization reduces development of diet-induced atherosclerosis in apolipoprotein e-deficient mice. *Arterioscler Thromb Vasc Biol* 2011;31:313–9.
- [22] de Souza AW, Westra J, Limburg PC, Bijl M, Kallenberg CG. HMGB1 in vascular diseases: Its role in vascular inflammation and atherosclerosis. *Autoimmun Rev* 2012;11:909–17.
- [23] Calogero S, Grassi F, Aguzzi A, Voigtlander T, Ferrier P, Ferrari S, et al. The lack of chromosomal protein Hmg1 does not disrupt cell growth but causes lethal hypoglycaemia in newborn mice. *Nat Genet* 1999;22:276–80.
- [24] Haraba R, Suica VI, Uyy E, Ivan L, Antohe F. Hyperlipidemia stimulates the extracellular release of the nuclear high mobility group box 1 protein. *Cell Tissue Res* 2011;346:361–8.
- [25] Wu CX, Sun H, Liu Q, Guo H, Gong JP. LPS induces HMGB1 relocation and release by activating the NF-kappaB-CBP signal transduction pathway in the murine macrophage-like cell line RAW264.7. *J Surg Res* 2012;175:88–100.
- [26] Chakraborty R, Bhatt KH, Sodhi A. Ultraviolet B induces high mobility group box 1 release from mouse peritoneal macrophages in vitro via caspase-1 mediated secretion pathway. *Immunobiology*; 2013.
- [27] Ranzato E, Patrone M, Pedrazzi M, Burlando B. Hmg1 promotes wound healing of 3T3 mouse fibroblasts via RAGE-dependent ERK1/2 activation. *Cell Biochem Biophys* 2010;57:9–17.
- [28] Lappalainen T, Kolehmainen M, Schwab U, Pulkkinen L, de Mello VD, Vaitinen M, et al. Gene expression of FTO in human subcutaneous adipose tissue, peripheral blood mononuclear cells and adipocyte cell line. *J Nutrigenet Nutrigenomics* 2010;3:37–45.
- [29] Festy F, Hoareau L, Bes-Houtmann S, Pequin AM, Gonthier MP, Munstun A, et al. Surface protein expression between human adipose tissue-derived stromal cells and mature adipocytes. *Histochem Cell Biol* 2005;124:113–21.
- [30] Yu G, Floyd ZE, Wu X, Hebert T, Halvorsen YD, Buehrer BM, et al. Adipogenic differentiation of adipose-derived stem cells. *Methods Mol Biol* 2011;702:193–200.
- [31] Ulloa L, Messmer D. High-mobility group box 1 (HMGB1) protein: friend and foe. *Cytokine Growth Factor Rev* 2006;17:189–201.
- [32] Li L, Chen L, Hu L, Liu Y, Sun HY, Tang J, et al. Nuclear factor high-mobility group box1 mediating the activation of Toll-like receptor 4 signaling in hepatocytes in the early stage of nonalcoholic fatty liver disease in mice. *Hepatology* 2011;54:1620–30.
- [33] Inoue K, Kawahara K, Biswas KK, Ando K, Mitsudo K, Nobuyoshi M, et al. HMGB1 expression by activated vascular smooth muscle cells in advanced human atherosclerosis plaques. *Cardiovasc Pathol* 2007;16:136–43.
- [34] Arrigo T, Chirico V, Salpietro V, Munafo C, Ferrau V, Gitto E, et al. High-mobility group protein B1: a new biomarker of metabolic syndrome in obese children. *Eur J Endocrinol* 2013;168:631–8.
- [35] Harford KA, Reynolds CM, McGillicuddy FC, Roche HM. Fats, inflammation and insulin resistance: insights to the role of macrophage and T-cell accumulation in adipose tissue. *Proc Nutr Soc* 2011;70:408–17.
- [36] Sell H, Eckel J. Adipose tissue inflammation: novel insight into the role of macrophages and lymphocytes. *Curr Opin Clin Nutr Metab Care* 2010;13:366–70.
- [37] Jiang W, Pisetsky DS. The role of IFN-alpha and nitric oxide in the release of HMGB1 by RAW 264.7 cells stimulated with polyinosinic-polycytidylic acid or lipopolysaccharide. *J Immunol* 2006;177:3337–43.
- [38] Scarpellini E, Tack J. Obesity and metabolic syndrome: an inflammatory condition. *Dig Dis* 2012;30:148–53.
- [39] Clement K, Vignes S. Inflammation, adipokines and obesity. *Rev Med Interne* 2009;30:824–32.
- [40] Sass G, Heinlein S, Agli A, Bang R, Schumann J, Tiegs G. Cytokine expression in three mouse models of experimental hepatitis. *Cytokine* 2002;19:115–20.
- [41] Scaffidi P, Misteli T, Bianchi ME. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. *Nature* 2002;418:191–5.
- [42] Venereau E, Schiraldi M, Ugucioni M, Bianchi ME. HMGB1 and leukocyte migration during trauma and sterile inflammation. *Mol Immunol*; 2013.
- [43] Wang H, Bloom O, Zhang M, Vishnubhakat JM, Ombrellino M, Che J, et al. HMGB-1 as a late mediator of endotoxin lethality in mice. *Science* 1999;285:248–51.
- [44] Czura CJ, Tracey KJ. Targeting high mobility group box 1 as a late-acting mediator of inflammation. *Crit Care Med* 2003;31:S46–50.
- [45] Lutz W, Sterkiewicz J. High mobility group box 1 protein as a late-acting mediator of acute lung inflammation. *Int J Occup Med Environ Health* 2004;17:245–54.
- [46] Willenbrock S, Braun O, Baumgart J, Lange S, Junghans C, Heisterkamp A, et al. TNF-alpha induced secretion of HMGB1 from non-immune canine mammary epithelial cells (MTH53A). *Cytokine* 2012;57:210–20.
- [47] Zhou RR, Zhao SS, Zou MX, Zhang P, Zhang BX, Dai XH, et al. HMGB1 cytoplasmic translocation in patients with acute liver failure. *BMC Gastroenterol* 2011;11:21.
- [48] Bes-Houtmann S, Roche R, Hoareau L, Gonthier MP, Festy F, Caillens H, et al. Presence of functional TLR2 and TLR4 on human adipocytes. *Histochem Cell Biol* 2007;127:131–7.
- [49] Yin JP, Su ZL, Wang YM, Wang T, Tian SS, Xu XX, et al. Release of HMGB1 by LPS-treated cardiac fibroblasts and its contribution to the production of collagen type I and III. *Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi* 2012;28:785–8.
- [50] Hou C, Zhao H, Li W, Cai S. Hydrogen peroxide induces high mobility group box 1 release in human bronchial epithelial cells. *Nan Fang Yi Ke Da Xue Xue Bao* 2012;32:1131–4.
- [51] Gauley J, Pisetsky DS. The translocation of HMGB1 during cell activation and cell death. *Autoimmunity* 2009;42:299–301.
- [52] Muller S, Ronfani L, Bianchi ME. Regulated expression and subcellular localization of HMGB1, a chromatin protein with a cytokine function. *J Intern Med* 2004;255:332–43.
- [53] Bonaldi T, Talamo F, Scaffidi P, Ferrera D, Porto A, Bachi A, et al. Monocytic cells hyperacetylate chromatin protein HMGB1 to redirect it towards secretion. *Embo J* 2003;22:5551–60.
- [54] Gardella S, Andrei C, Ferrera D, Lotti LV, Torrisi MR, Bianchi ME, et al. The nuclear protein HMGB1 is secreted by monocytes via a non-classical, vesicle-mediated secretory pathway. *EMBO Rep* 2002;3:995–1001.
- [55] Nogueira-Machado JA, de Oliveira Volpe CM. HMGB-1 as a target for inflammation controlling. *Recent Pat Endocr Metab Immune Drug Discov* 2012;6:201–9.

We have shown from this work, higher level of HMGB1 protein expression in adipose tissue from obese persons. By *in vitro* studies we have proposed that TLR4 mediated inflammation induces the active secretion of HMGB1 from adipose derived stem cells (ASC's) from stromal vascular fractions (SVF) while not by differentiated adipocytes and mature adipocytes [306]. As adipose tissue is able to secrete HMGB1 in response to inflammation, however the role of HMGB1 on human adipose tissue inflammation has not been studied yet. In the following study we have investigated the proinflammatory secretion profiles of rHMGB1 treated human mature adipocytes. Further we aimed to explore which receptor and intracellular signaling pathways are responsible for the inflammation process.

## HMGB1 – an inflammatory adipokine

### HMGB1 autocrine effects on LPS stimulated ASC's

HMGB1 is found to be secreted as a late mediator of inflammation by immune cells such as monocytes, macrophages and involve in the progression of inflammation. Other than immune cells, we have shown in our earlier studies that human ASC's also actively secrete HMGB1 due to LPS mediated inflammation. As HMGB1 secreted during inflammation functions as a cytokine, we investigated whether LPS induced HMGB1 could act in an autocrine manner, by inhibiting the extracellular HMGB1 with glycyrrhizin, a natural inhibitor compound from licorice which is known to bind HMGB1 directly and inhibits its cytokine activities. In this study, we measured HMGB1 secretion from the supernatants conditioned by human ASC's exposed to LPS for 24 h with and without glycyrrhizin pre-treatment for 2 hours.



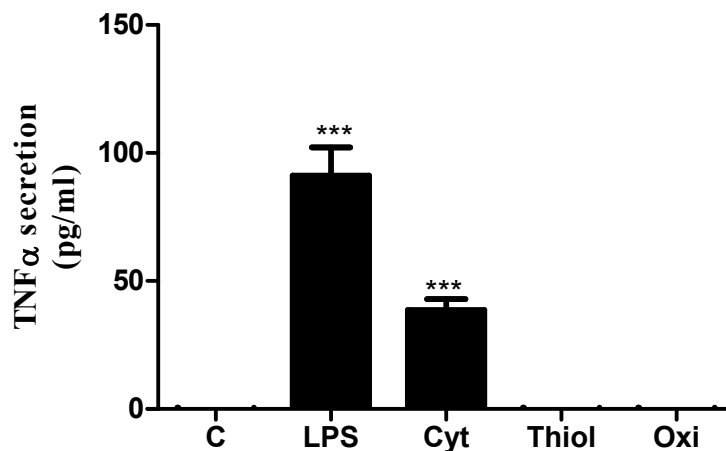
**Fig – 1:** Human ASC's are treated for 24h with LPS (1  $\mu\text{g/ml}$ ) with or without glycyrrhizin (1  $\mu\text{M}$ ) and the conditioned medium are analyzed for HMGB1 (A) and IL-6 (B) secretion in comparison with their respective control. Results are shown as mean  $\pm$  SD of three independent experiments (n=6, \*\*\*\*,  $P < 0.0001$ ; \*\*\*,  $P < 0.001$ ; using unpaired t-test).

We confirm that LPS stimulation increased HMGB1 secretion significantly from ASC's. More interestingly, the glycyrrhizin pre-treatment reduced significantly almost half of the LPS

induced HMGB1 secretion (around 52%). In addition, the glycyrrhizin pre-treatment also reduced (16 %) the IL-6 secretion from LPS treated ASC's. This suggests that LPS stimulated HMGB1 secretion, in turn the extracellular HMGB1 induce autocrine effects on ASC's which was inhibited by glycyrrhizin. This gives an insight on how HMGB1 secreted from ASC's might acts in an autocrine manner to promote inflammation in adipose tissue.

### HMGB1 proinflammatory effects on PBMC cells

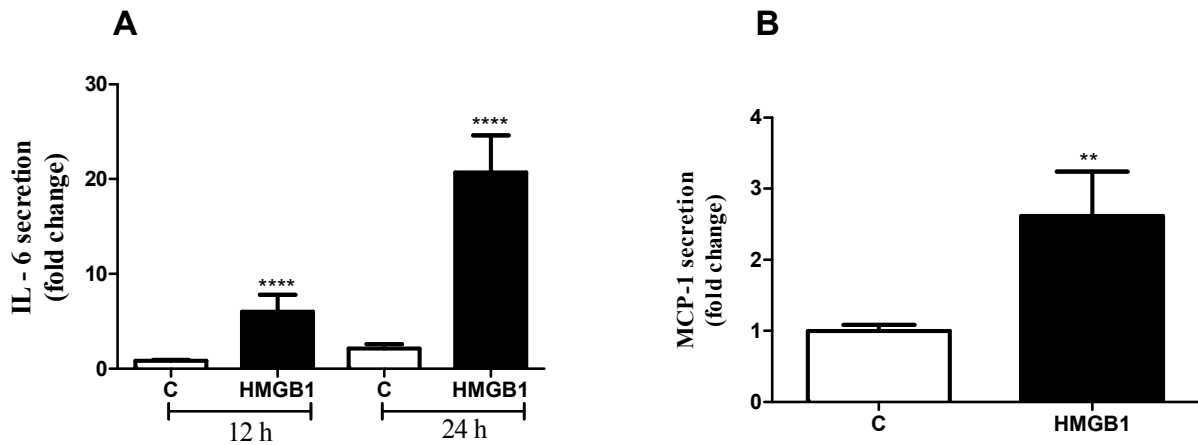
Extracellular HMGB1 due to the redox modifications results in different forms of HMGB1 such as reduced disulfide (C23-C45) – cytHMGB1, all-thiol and oxidized HMGB1. Only reduced disulfide HMGB1 is found to activate TLR4 receptor and lead to the secretion of pro-inflammatory cytokines. We tested all the three forms of rHMGB1 primarily on human PBMC cells to identify which form is able to induce TNF $\alpha$  secretion in comparison with LPS (50 ng/ml). Among all the three isoforms, only cyt-HMGB1 triggers inflammation and lead to the secretion of TNF $\alpha$ , even though less in comparison with LPS. From this we confirm that only cyt-HMGB1 is able to induce inflammation. Moreover from this result, we can conclude that there is very minimal or no LPS contamination in all the three forms of rHMGB1 since they are produced and purified in the same manner.



**Fig – 2:** Human PBMC are treated with either 50 ng/ml LPS or 2.5  $\mu$ g/ml of different form of rHMGB1 for 4 h and the conditioned medium are analyzed for TNF $\alpha$  secretion in comparison with respective control. Results are shown as mean  $\pm$  SD of three independent experiments (n=6, \*\*\*, P < 0.001 using unpaired t-test).

### HMGB1 proinflammatory effects on mature adipocytes

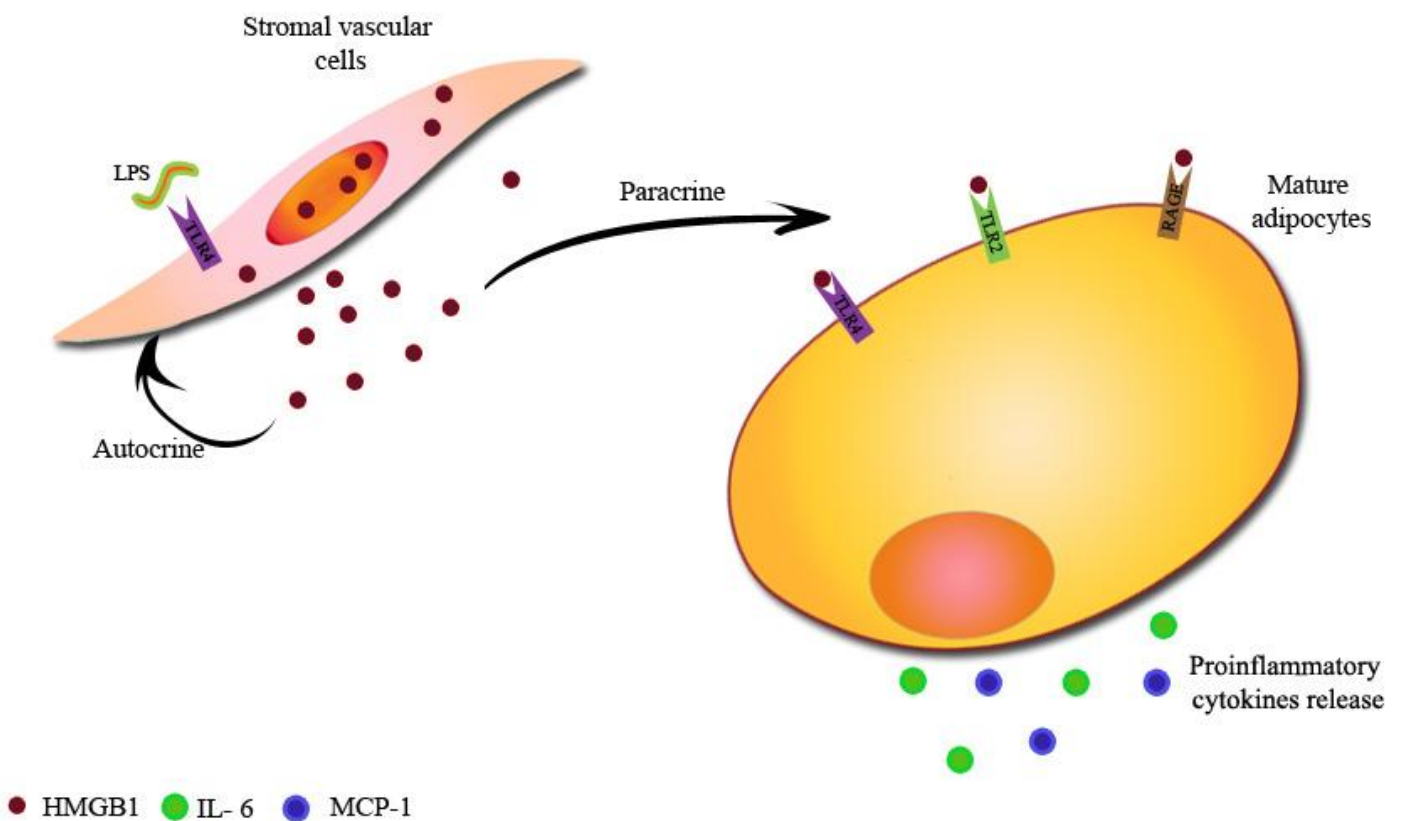
To address whether HMGB1 could induce inflammation in mature adipocytes, cytokine and all-thiol HMGB1 was treated with mature adipocytes. Only the cytokine form induces inflammation and activates the cells to secrete IL-6 and MCP-1 but not TNF $\alpha$  (data not shown).



**Fig – 3:** Human mature adipocytes treated with cytokine form of rHMGB1 (2.5  $\mu$ g/ml) for 12 and 24 h and the conditioned medium are analyzed for IL-6 (A) and MCP-1 at 24h (B) secretion in comparison with respective control. Results are shown as mean  $\pm$  SD of three independent experiments (n=6, \*\*\*\*, P<0.0001; \*\*, P<0.01 using unpaired t-test).

These results indicate that the proinflammatory effects of HMGB1 varies according to the cell types, since cyt-HMGB1 induces TNF secretion from PBMC but not on mature adipocytes regardless of the time and concentration (data not shown).

From these results we suggest that chronic low grade inflammation in adipose tissue is partially due to or regulated by HMGB1 secretion after TLR4 activation. Extracellular HMGB1 acts in a paracrine manner to induce inflammation on mature adipocytes as an inflammatory adipokine, resulting in the secretion of IL-6 and MCP-1. The increase in MCP-1 secretion gives an idea of how inflammatory cells could be recruited in to the adipose tissue during adipose tissue inflammation.



**Fig – 4:** Schematics of autocrine and paracrine effects of HMGB1 secreted from ASC's during adipose tissue inflammation with possible HMGB1 receptors.

## General summary

During my thesis work, I have studied how TLR4 activation regulates adipose tissue inflammation and contributes to the onset of metabolic diseases. Through *in vitro* and *ex vivo* samples from lean and obese adipose tissue, I have investigated the physiological trigger which activates TLR4 mediated inflammation. The main findings of my thesis work are summarized as follows:

- 1) FFA do not pay the toll and activates no inflammation
  - FFA - SFA and PUFA alone in endotoxin free conditions neither activate TLR4 and TLR2 nor induce inflammation in human mature adipocytes and adipose tissue.
  - PUFA in the contrary reduces LPS mediated inflammation and act as anti-inflammatory agent.
- 2) HMGB1 expression in human adipose tissue
  - HMGB1 is expressed 2- fold more at protein level in adipose tissue from obese persons.
  - TLR4 activation by LPS in human adipose tissue induced the secretion of HMGB1 as a late mediator of inflammation.
  - ASCs from SVF fractions are responsible for the increase in secretion of HMGB1 during adipose tissue inflammation.
  - Mature adipocytes and *in vitro* differentiated ASCs in to adipocytes do not secrete HMGB1 under LPS induced inflammation.
- 3) HMGB1 – a novel inflammatory adipokine
  - HMGB1 is partly involved in regulating LPS induced inflammation in ASCs in an autocrine manner.



- Cyt - rHMGB1 triggered inflammation in human mature adipocytes by the secretion of IL-6 and MCP-1 but not TNF $\alpha$ .

## **General discussion and conclusion**

The accumulating evidence on novel bio molecules that are secreted from adipose tissue which can regulate lipid and glucose metabolism, appetite and energy balance, insulin sensitivity, inflammation and immunity, strengthens adipose tissue as an endocrine organ. The fatal consequence of excess adipose deposition is mainly due to the increase in adipose tissue inflammation by the increase in endogenous and exogenous ligands leading to metabolic dysfunctions. Earlier work from our team has shown the presence of functional toll like receptors (TLR2 and TLR4) [154] and endocannabinoid receptors (CB1 and CB2) [307] expression in human mature adipocytes. These receptors when activated induced the secretion of proinflammatory cytokines which gives an insight of how these receptors participates in overall adipose tissue inflammation. As TLR receptors are abundantly expressed in adipocytes (similar to macrophages), this suggests that the paracrine effect between macrophages and adipocytes are due to TLR activation resulting in adipose tissue inflammation.

FFA released from dying adipocytes especially SFA is suggested to act as an endogenous ligand to activate TLR4 and induce adipose tissue inflammation. This was supported by many *in vitro* studies where BSA conjugated SFA induces inflammation in many cell models. Recently, Erridge and Sammani disproved this concept and shown that, in an endotoxin free condition the FFA's alone were not able to induce inflammation in different cells including macrophages and adipocytes [182]. Following their contrary statement, we have confirmed in primary culture of human adipose tissue and mature adipocytes that SFA alone is not able to neither engage TLR2 and TLR4 receptors nor induce inflammation under endotoxin free condition. However the PUFA reduces LPS induced inflammation and acts as an anti-inflammatory agent [308].

HMGB1 is recently identified as an endogenous TLR4 ligand, acting as proinflammatory cytokine secreted by immune cells and other cells during inflammation in many metabolic diseases which are associated with obesity. In HFD fed mice and in peritumoral adipose tissue, HMGB1 is reported to be released due to necrosis and in adipocyte cell line when treated with necrotic inducers [299, 300]. Recently, increase in circulatory levels of HMGB1 has been

observed in obese children correlating with other increased proinflammatory cytokines suggesting HMGB1 as a novel biomarker for metabolic syndrome [305]. In this context, increased HMGB1 expression between obese adipose tissue compared to lean indicates possible role of HMGB1 during obesity [306]. Similar to immune cells, the increase in HMGB1 secretion similar to other proinflammatory cytokines from ASC's gives an insight of how HMGB1 could acts as a late mediator for adipose tissue inflammation during obesity [306]. In addition, the increase in IL-6 and MCP-1 secretion from mature adipocytes after rHMGB1 treatment describes its inflammatory and chemoattractant role during the adipose tissue inflammation.

Other than inflammation, ER stress [309] and Hypoxia [310] is also found to release HMGB1 due to necrosis. Recently PKR mediated inflammasome activation initiates the onset of pyroptosis, a form of programmed inflammatory cell death is found to induce robust release of hyper acetylated HMGB1, by a highly regulated process in mouse peritoneal macrophages and in BMDM [311]. Surprisingly, the inflammasomes could also be activated by PA under endotoxin free condition [304], which could possibly trigger pyroptosis and release HMGB1. HMGB1 released via pyroptosis also releases all-thiol HMGB1 isoform with strong chemotactic behavior which can recruits CXCR4-expressing cell populations such as monocytes, lymphocytes and neutrophils to the inflammatory sites [312]. Altogether, these studies suggest a possible mechanism of how HMGB1 may participate in the recruitment of macrophages to investigate the dying adipocytes and triggers inflammation in adipose tissue. In contrary, PUFA reduced the vascular HMGB1 and RAGE expression in rat endothelial cells [313]. All these studies shows an strong relation between fatty acid and HMGB1, however direct effects of fatty acids on HMGB1 expression have not been studied yet and could be interesting to be investigated directly on adipose cells. Also as HMGB1 is known to interact with many exogenous and endogenous molecules to form complex, possible interaction with fatty acids which may facilitate the HMGB1 binding to receptors remain unanswered.

As HMGB1 released via inflammation or necrosis can promotes inflammation, this gives an idea of how HMGB1 could possibly play a role in the onset of inflammation in adipose tissue. Also as HMGB1 activates stem cell migration and plays a role in angiogenesis, it may also involve in the increase in adipose tissue plasticity. Altogether, the extracellular HMGB1 from adipose tissue and its contribution to inflammation indicates its importance in the pathogenesis

of obesity. Taken together, my results suggest HMGB1 as a novel adipokine taking part in adipose tissue inflammation.

Based on the results from my studies and others, I propose the following hypothesis on adipose tissue inflammation,

- SFA do not activate TLR, instead it may activate inflammasomes and pyroptosis inducing inflammation in adipose tissue
- During adipose tissue inflammation, ASC's and immune cells secrete various forms of HMGB1 which may play a big part in inflammation, recruitment and resolution of inflammation.
  - Cytokine form of HMGB1 and other forms could act in an autocrine and paracrine manner alone or as a complex with other endogenous ligands by activating TLR4 and other receptors, inducing inflammation and releasing proinflammatory cytokines.
  - The all-thiol HMGB1 which acts as a chemo attractant could contribute to the infiltration of monocyte, macrophages and other immune cells into the adipose tissue during inflammation.

### **Future perspective**

In many diseases such as arthritis, sepsis, endotoxemia, diabetes mellitus, post-myocardial infarction tissue injury and in hemorrhagic shock, blocking HMGB1 using neutralizing antibodies, A-box and natural compounds such as glycyrrhizin showed decrease in inflammation. In this regard, considering HMGB1 as an adipokine and to develop therapeutics to reduce adipose tissue inflammation mediated chronic diseases, more studies on HMGB1 role on adipose tissue has to be investigated such as

- Receptors and signaling pathways activated by HMGB1 on adipocytes
- FFA effects on HMGB1 secretion from adipocytes and possible complex formation.

- Real time secretion profile of various forms of HMGB1 during adipose tissue inflammation.
- Development of antibodies that specifically targets acetylated, phosphorylated and disulfide HMGB1 to reduce the inflammation.
- Blocking specific receptors through which HMGB1 mediate chronic inflammation.

Through my scientific data contribution to the research community, I hope that more research on HMGB1 role in adipose tissue will be carried out in future. By advanced promising therapeutics discovery, blocking selective deleterious role of HMGB1 in adipose tissue might reduce the risk of obesity associated diseases.

## **Acknowledgements**

**Regis**, thanks a lot for your interest in offering me a PhD position in your team when I request you via my first mail. You have given me the freedom to think, plan and work independently has let me to gain more experience and knowledge in the field of adipose tissue inflammation research.

**Laurence**, a simple thanks will not fulfill for all what you have done and still doing for me. You have taken care of me right from the first day of arrival at Reunion airport and still continuing on at this moment. I have learned all the professional and laboratory skills from you. Your presence in the lab creates positive atmosphere in the lab and you proved “everything is possible by team work”. You are a good role model for women in science and achievements. You have stand by my side and make me feel confident both at happiness and difficult situations. I am lucky and happy to meet and have you in my life.

**Maya**, thanks for letting me in GEICO lab and introducing me to the world of adipose tissue metabolism. You have welcomed me all the times with your smile and kept the doors always open when I am in need of your help.

**Kshitish Acharya**, my hearty thanks to you. Without you, I would have never made it in this place where I am now. I admire and salute you for your teaching skills, by setting up strong foundation on basic science in each one of your student in your class. You are my all time favorite teacher.

Thanks to Anne Claire, Karima, Cynthia, Lydie Gence, Sophie, Dorothy, Aurelie who advised and assisted me technically in the lab.

Wildriss and Thomas, thanks for all your scientific, technical advice and help throughout my PhD.

Thanks to CYROI work force that drives to maintain such a wonderful research facility, my special thanks to Christian, Christophe, Gregory and Joan.

Erwan, thanks for your tips and advices on photography.....

My dear Aurore, Yann, Lisa, Li peng, Janina, Max and Gaelle, it is not possible without you all...you were there for me all the time and we had wonderful time and good memories to cherish.

Thanks to all the coffee mates in CYROI with whom I spared my time discussing scientifically, socially.....and most of the time planning for the weekend. Our contribution has made scientific persons to be “heavy coffee drinkers in the world” in a recent survey.

My special thanks to Reunion Island and its people who treated me as a part of their family. I have spent my three years with loads of memories and adventures during my stay.

Finally, my hearty thanks to mom, dad and to my late sister, with all your support and wishes I am here because of you, thanks for believing in me. You are the reason for everything where I am up to now in my scientific world and where I will be in future.

My special thanks to everyone at CYROI and GEICO who comfort me with your smile and warm wishes each and every day.

## References:

- [1] Malik VS, Popkin BM, Bray GA, Despres JP, Hu FB. Sugar-sweetened beverages, obesity, type 2 diabetes mellitus, and cardiovascular disease risk. *Circulation*. 2010;121:1356-64.
- [2] James WP. WHO recognition of the global obesity epidemic. *International journal of obesity*. 2008;32 Suppl 7:S120-6.
- [3] Cannon B, Nedergaard J. Brown adipose tissue: function and physiological significance. *Physiological reviews*. 2004;84:277-359.
- [4] Ricquier D. Fundamental mechanisms of thermogenesis. *Comptes rendus biologiques*. 2006;329:578-86; discussion 653-5.
- [5] Nedergaard J, Bengtsson T, Cannon B. Unexpected evidence for active brown adipose tissue in adult humans. *American journal of physiology Endocrinology and metabolism*. 2007;293:E444-52.
- [6] Timmons JA, Wennmalm K, Larsson O, Walden TB, Lassmann T, Petrovic N, et al. Myogenic gene expression signature establishes that brown and white adipocytes originate from distinct cell lineages. *Proceedings of the National Academy of Sciences of the United States of America*. 2007;104:4401-6.
- [7] Frontini A, Cinti S. Distribution and development of brown adipocytes in the murine and human adipose organ. *Cell metabolism*. 2010;11:253-6.
- [8] Seale P, Bjork B, Yang W, Kajimura S, Chin S, Kuang S, et al. PRDM16 controls a brown fat/skeletal muscle switch. *Nature*. 2008;454:961-7.
- [9] Cederberg A, Gronning LM, Ahren B, Tasken K, Carlsson P, Enerback S. FOXC2 is a winged helix gene that counteracts obesity, hypertriglyceridemia, and diet-induced insulin resistance. *Cell*. 2001;106:563-73.
- [10] Leonardsson G, Steel JH, Christian M, Pocock V, Milligan S, Bell J, et al. Nuclear receptor corepressor RIP140 regulates fat accumulation. *Proceedings of the National Academy of Sciences of the United States of America*. 2004;101:8437-42.
- [11] Seale P, Conroe HM, Estall J, Kajimura S, Frontini A, Ishibashi J, et al. Prdm16 determines the thermogenic program of subcutaneous white adipose tissue in mice. *The Journal of clinical investigation*. 2011;121:96-105.
- [12] Cryer A, Van RL. Characterization of the collagen types synthesized by human and rat adipocyte precursors in vitro. *European journal of clinical investigation*. 1982;12:235-8.
- [13] Mariman EC, Wang P. Adipocyte extracellular matrix composition, dynamics and role in obesity. *Cellular and molecular life sciences : CMLS*. 2010;67:1277-92.
- [14] Divoux A, Clement K. Architecture and the extracellular matrix: the still unappreciated components of the adipose tissue. *Obesity reviews : an official journal of the International Association for the Study of Obesity*. 2011;12:e494-503.
- [15] Ouchi N, Parker JL, Lugus JJ, Walsh K. Adipokines in inflammation and metabolic disease. *Nature reviews Immunology*. 2011;11:85-97.
- [16] Bjorntorp P, Bengtsson C, Blohme G, Jonsson A, Sjostrom L, Tibblin E, et al. Adipose tissue fat cell size and number in relation to metabolism in randomly selected middle-aged men and women. *Metabolism: clinical and experimental*. 1971;20:927-35.
- [17] Bjorntorp P, Sjostrom L, SJOSTROM L. Number and size of adipose tissue fat cells in relation to metabolism in human obesity. *Metabolism: clinical and experimental*. 1971;20:703-13.
- [18] Skurk T, Alberti-Huber C, Herder C, Hauner H. Relationship between adipocyte size and adipokine expression and secretion. *The Journal of clinical endocrinology and metabolism*. 2007;92:1023-33.
- [19] Jernas M, Palming J, Sjoholm K, Jennische E, Svensson PA, Gabrielsson BG, et al. Separation of human adipocytes by size: hypertrophic fat cells display distinct gene expression. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. 2006;20:1540-2.

- [20] Ohsaki Y, Cheng J, Suzuki M, Shinohara Y, Fujita A, Fujimoto T. Biogenesis of cytoplasmic lipid droplets: from the lipid ester globule in the membrane to the visible structure. *Biochimica et biophysica acta*. 2009;1791:399-407.
- [21] Verstraeten VL, Renes J, Ramaekers FC, Kamps M, Kuijpers HJ, Verheyen F, et al. Reorganization of the nuclear lamina and cytoskeleton in adipogenesis. *Histochemistry and cell biology*. 2011;135:251-61.
- [22] Sato K, Umeno H, Nakashima T. Histological investigation of liposuctioned fat for injection laryngoplasty. *American journal of otolaryngology*. 2005;26:219-25.
- [23] Suganami T, Ogawa Y. Adipose tissue macrophages: their role in adipose tissue remodeling. *Journal of leukocyte biology*. 2010;88:33-9.
- [24] Wronska A, Kmiec Z. Structural and biochemical characteristics of various white adipose tissue depots. *Acta physiologica*. 2012;205:194-208.
- [25] Wajchenberg BL. Subcutaneous and visceral adipose tissue: their relation to the metabolic syndrome. *Endocrine reviews*. 2000;21:697-738.
- [26] Ahmadian M, Wang Y, Sul HS. Lipolysis in adipocytes. *The international journal of biochemistry & cell biology*. 2010;42:555-9.
- [27] Walker GE, Verti B, Marzullo P, Savia G, Mencarelli M, Zurleni F, et al. Deep subcutaneous adipose tissue: a distinct abdominal adipose depot. *Obesity*. 2007;15:1933-43.
- [28] Gealekman O, Guseva N, Hartigan C, Apotheke S, Gorgoglione M, Gurav K, et al. Depot-specific differences and insufficient subcutaneous adipose tissue angiogenesis in human obesity. *Circulation*. 2011;123:186-94.
- [29] Canello R, Tordjman J, Poitou C, Guilhem G, Bouillot JL, Hugol D, et al. Increased infiltration of macrophages in omental adipose tissue is associated with marked hepatic lesions in morbid human obesity. *Diabetes*. 2006;55:1554-61.
- [30] Coelho M, Oliveira T, Fernandes R. Biochemistry of adipose tissue: an endocrine organ. *Archives of medical science : AMS*. 2013;9:191-200.
- [31] Friedman JM, Halaas JL. Leptin and the regulation of body weight in mammals. *Nature*. 1998;395:763-70.
- [32] Hotamisligil GS, Shargill NS, Spiegelman BM. Adipose expression of tumor necrosis factor- $\alpha$ : direct role in obesity-linked insulin resistance. *Science*. 1993;259:87-91.
- [33] Siiteri PK. Adipose tissue as a source of hormones. *The American journal of clinical nutrition*. 1987;45:277-82.
- [34] Flier JS, Cook KS, Usher P, Spiegelman BM. Severely impaired adiponin expression in genetic and acquired obesity. *Science*. 1987;237:405-8.
- [35] Bluher M. Adipose tissue dysfunction contributes to obesity related metabolic diseases. *Best practice & research Clinical endocrinology & metabolism*. 2013;27:163-77.
- [36] Tan CY, Vidal-Puig A. Adipose tissue expandability: the metabolic problems of obesity may arise from the inability to become more obese. *Biochemical Society transactions*. 2008;36:935-40.
- [37] Stefan N, Kantartzis K, Machann J, Schick F, Thamer C, Rittig K, et al. Identification and characterization of metabolically benign obesity in humans. *Archives of internal medicine*. 2008;168:1609-16.
- [38] Spencer M, Unal R, Zhu B, Rasouli N, McGehee RE, Jr., Peterson CA, et al. Adipose tissue extracellular matrix and vascular abnormalities in obesity and insulin resistance. *The Journal of clinical endocrinology and metabolism*. 2011;96:E1990-8.
- [39] Halberg N, Khan T, Trujillo ME, Wernstedt-Asterholm I, Attie AD, Sherwani S, et al. Hypoxia-inducible factor 1 $\alpha$  induces fibrosis and insulin resistance in white adipose tissue. *Molecular and cellular biology*. 2009;29:4467-83.
- [40] Kovsan J, Bluher M, Tarnovscki T, Kloting N, Kirshtein B, Madar L, et al. Altered autophagy in human adipose tissues in obesity. *The Journal of clinical endocrinology and metabolism*. 2011;96:E268-77.



- [41] Keuper M, Bluher M, Schon MR, Moller P, Dzyakanchuk A, Amrein K, et al. An inflammatory micro-environment promotes human adipocyte apoptosis. *Molecular and cellular endocrinology*. 2011;339:105-13.
- [42] Canello R, Henegar C, Viguerie N, Taleb S, Poitou C, Rouault C, et al. Reduction of macrophage infiltration and chemoattractant gene expression changes in white adipose tissue of morbidly obese subjects after surgery-induced weight loss. *Diabetes*. 2005;54:2277-86.
- [43] Harman-Boehm I, Bluher M, Redel H, Sion-Vardy N, Ovadia S, Avinoach E, et al. Macrophage infiltration into omental versus subcutaneous fat across different populations: effect of regional adiposity and the comorbidities of obesity. *The Journal of clinical endocrinology and metabolism*. 2007;92:2240-7.
- [44] Kabon B, Nagele A, Reddy D, Eagon C, Fleshman JW, Sessler DI, et al. Obesity decreases perioperative tissue oxygenation. *Anesthesiology*. 2004;100:274-80.
- [45] Blaak EE, van Baak MA, Kemerink GJ, Pakbiers MT, Heidendal GA, Saris WH. Beta-adrenergic stimulation and abdominal subcutaneous fat blood flow in lean, obese, and reduced-obese subjects. *Metabolism: clinical and experimental*. 1995;44:183-7.
- [46] Andersson J, Sjostrom LG, Karlsson M, Wiklund U, Hultin M, Karpe F, et al. Dysregulation of subcutaneous adipose tissue blood flow in overweight postmenopausal women. *Menopause*. 2010;17:365-71.
- [47] Pasarica M, Sereda OR, Redman LM, Albarado DC, Hymel DT, Roan LE, et al. Reduced adipose tissue oxygenation in human obesity: evidence for rarefaction, macrophage chemotaxis, and inflammation without an angiogenic response. *Diabetes*. 2009;58:718-25.
- [48] West DB, Prinz WA, Francendese AA, Greenwood MR. Adipocyte blood flow is decreased in obese Zucker rats. *The American journal of physiology*. 1987;253:R228-33.
- [49] Pasarica M, Rood J, Ravussin E, Schwarz JM, Smith SR, Redman LM. Reduced oxygenation in human obese adipose tissue is associated with impaired insulin suppression of lipolysis. *The Journal of clinical endocrinology and metabolism*. 2010;95:4052-5.
- [50] Goossens GH, Blaak EE. Adipose tissue oxygen tension: implications for chronic metabolic and inflammatory diseases. *Current opinion in clinical nutrition and metabolic care*. 2012;15:539-46.
- [51] Hosogai N, Fukuhara A, Oshima K, Miyata Y, Tanaka S, Segawa K, et al. Adipose tissue hypoxia in obesity and its impact on adipocytokine dysregulation. *Diabetes*. 2007;56:901-11.
- [52] Rausch ME, Weisberg S, Vardhana P, Tortoriello DV. Obesity in C57BL/6J mice is characterized by adipose tissue hypoxia and cytotoxic T-cell infiltration. *International journal of obesity*. 2008;32:451-63.
- [53] Ye J, Gao Z, Yin J, He Q. Hypoxia is a potential risk factor for chronic inflammation and adiponectin reduction in adipose tissue of ob/ob and dietary obese mice. *American journal of physiology Endocrinology and metabolism*. 2007;293:E1118-28.
- [54] Trayhurn P. Hypoxia and adipose tissue function and dysfunction in obesity. *Physiological reviews*. 2013;93:1-21.
- [55] Semenza GL. Targeting HIF-1 for cancer therapy. *Nature reviews Cancer*. 2003;3:721-32.
- [56] He Q, Gao Z, Yin J, Zhang J, Yun Z, Ye J. Regulation of HIF-1{alpha} activity in adipose tissue by obesity-associated factors: adipogenesis, insulin, and hypoxia. *American journal of physiology Endocrinology and metabolism*. 2011;300:E877-85.
- [57] Zhang X, Lam KS, Ye H, Chung SK, Zhou M, Wang Y, et al. Adipose tissue-specific inhibition of hypoxia-inducible factor 1{alpha} induces obesity and glucose intolerance by impeding energy expenditure in mice. *The Journal of biological chemistry*. 2010;285:32869-77.
- [58] Koumenis C, Naczki C, Koritzinsky M, Rastani S, Diehl A, Sonenberg N, et al. Regulation of protein synthesis by hypoxia via activation of the endoplasmic reticulum kinase PERK and phosphorylation of the translation initiation factor eIF2alpha. *Molecular and cellular biology*. 2002;22:7405-16.

- [59] Zhang L, Ebenezer PJ, Dasuri K, Fernandez-Kim SO, Francis J, Mariappan N, et al. Aging is associated with hypoxia and oxidative stress in adipose tissue: implications for adipose function. *American journal of physiology Endocrinology and metabolism*. 2011;301:E599-607.
- [60] Yin J, Gao Z, He Q, Zhou D, Guo Z, Ye J. Role of hypoxia in obesity-induced disorders of glucose and lipid metabolism in adipose tissue. *American journal of physiology Endocrinology and metabolism*. 2009;296:E333-42.
- [61] Chen B, Lam KS, Wang Y, Wu D, Lam MC, Shen J, et al. Hypoxia dysregulates the production of adiponectin and plasminogen activator inhibitor-1 independent of reactive oxygen species in adipocytes. *Biochemical and biophysical research communications*. 2006;341:549-56.
- [62] Grosfeld A, Andre J, Hauguel-De Mouzon S, Berra E, Pouyssegur J, Guerre-Millo M. Hypoxia-inducible factor 1 transactivates the human leptin gene promoter. *The Journal of biological chemistry*. 2002;277:42953-7.
- [63] Bashan N, Dorfman K, Tarnovscki T, Harman-Boehm I, Liberty IF, Bluher M, et al. Mitogen-activated protein kinases, inhibitory-kappaB kinase, and insulin signaling in human omental versus subcutaneous adipose tissue in obesity. *Endocrinology*. 2007;148:2955-62.
- [64] Rudich A, Kanety H, Bashan N. Adipose stress-sensing kinases: linking obesity to malfunction. *Trends in endocrinology and metabolism: TEM*. 2007;18:291-9.
- [65] Gregor MF, Hotamisligil GS. Thematic review series: Adipocyte Biology. Adipocyte stress: the endoplasmic reticulum and metabolic disease. *Journal of lipid research*. 2007;48:1905-14.
- [66] Shen X, Zhang K, Kaufman RJ. The unfolded protein response--a stress signaling pathway of the endoplasmic reticulum. *Journal of chemical neuroanatomy*. 2004;28:79-92.
- [67] Nishitoh H, Matsuzawa A, Tobiume K, Saegusa K, Takeda K, Inoue K, et al. ASK1 is essential for endoplasmic reticulum stress-induced neuronal cell death triggered by expanded polyglutamine repeats. *Genes & development*. 2002;16:1345-55.
- [68] Hu P, Han Z, Couvillon AD, Kaufman RJ, Exton JH. Autocrine tumor necrosis factor alpha links endoplasmic reticulum stress to the membrane death receptor pathway through IRE1alpha-mediated NF-kappaB activation and down-regulation of TRAF2 expression. *Molecular and cellular biology*. 2006;26:3071-84.
- [69] Manna SK, Babajan B, Raghavendra PB, Raviprakash N, Sureshkumar C. Inhibiting TRAF2-mediated activation of NF-kappaB facilitates induction of AP-1. *The Journal of biological chemistry*. 2010;285:11617-27.
- [70] Tripathi YB, Pandey V. Obesity and endoplasmic reticulum (ER) stresses. *Frontiers in immunology*. 2012;3:240.
- [71] Gargalovic PS, Gharavi NM, Clark MJ, Pagnon J, Yang WP, He A, et al. The unfolded protein response is an important regulator of inflammatory genes in endothelial cells. *Arteriosclerosis, thrombosis, and vascular biology*. 2006;26:2490-6.
- [72] Eizirik DL, Cardozo AK, Cnop M. The role for endoplasmic reticulum stress in diabetes mellitus. *Endocrine reviews*. 2008;29:42-61.
- [73] Wojtczak L, Schonfeld P. Effect of fatty acids on energy coupling processes in mitochondria. *Biochimica et biophysica acta*. 1993;1183:41-57.
- [74] Brookheart RT, Michel CI, Schaffer JE. As a matter of fat. *Cell metabolism*. 2009;10:9-12.
- [75] Hotamisligil GS. Inflammation and metabolic disorders. *Nature*. 2006;444:860-7.
- [76] Roberts CK, Sindhu KK. Oxidative stress and metabolic syndrome. *Life sciences*. 2009;84:705-12.
- [77] Chen XH, Zhao YP, Xue M, Ji CB, Gao CL, Zhu JG, et al. TNF-alpha induces mitochondrial dysfunction in 3T3-L1 adipocytes. *Molecular and cellular endocrinology*. 2010;328:63-9.
- [78] Lin Y, Berg AH, Iyengar P, Lam TK, Giacca A, Combs TP, et al. The hyperglycemia-induced inflammatory response in adipocytes: the role of reactive oxygen species. *The Journal of biological chemistry*. 2005;280:4617-26.

- [79] Gao CL, Zhu C, Zhao YP, Chen XH, Ji CB, Zhang CM, et al. Mitochondrial dysfunction is induced by high levels of glucose and free fatty acids in 3T3-L1 adipocytes. *Molecular and cellular endocrinology*. 2010;320:25-33.
- [80] Houstis N, Rosen ED, Lander ES. Reactive oxygen species have a causal role in multiple forms of insulin resistance. *Nature*. 2006;440:944-8.
- [81] Wang T, Si Y, Shirihai OS, Si H, Schultz V, Corkey RF, et al. Respiration in adipocytes is inhibited by reactive oxygen species. *Obesity*. 2010;18:1493-502.
- [82] Furukawa S, Fujita T, Shimabukuro M, Iwaki M, Yamada Y, Nakajima Y, et al. Increased oxidative stress in obesity and its impact on metabolic syndrome. *The Journal of clinical investigation*. 2004;114:1752-61.
- [83] Meigs JB, Larson MG, Fox CS, Keaney JF, Jr., Vasan RS, Benjamin EJ. Association of oxidative stress, insulin resistance, and diabetes risk phenotypes: the Framingham Offspring Study. *Diabetes care*. 2007;30:2529-35.
- [84] Zhang Y, Goldman S, Baerga R, Zhao Y, Komatsu M, Jin S. Adipose-specific deletion of autophagy-related gene 7 (atg7) in mice reveals a role in adipogenesis. *Proceedings of the National Academy of Sciences of the United States of America*. 2009;106:19860-5.
- [85] Baerga R, Zhang Y, Chen PH, Goldman S, Jin S. Targeted deletion of autophagy-related 5 (atg5) impairs adipogenesis in a cellular model and in mice. *Autophagy*. 2009;5:1118-30.
- [86] Singh R, Xiang Y, Wang Y, Baikati K, Cuervo AM, Luu YK, et al. Autophagy regulates adipose mass and differentiation in mice. *The Journal of clinical investigation*. 2009;119:3329-39.
- [87] Maixner N, Kovsan J, Harman-Boehm I, Bluher M, Bashan N, Rudich A. Autophagy in adipose tissue. *Obesity facts*. 2012;5:710-21.
- [88] Zhou L, Liu F. Autophagy: roles in obesity-induced ER stress and adiponectin downregulation in adipocytes. *Autophagy*. 2010;6:1196-7.
- [89] Koh EH, Park JY, Park HS, Jeon MJ, Ryu JW, Kim M, et al. Essential role of mitochondrial function in adiponectin synthesis in adipocytes. *Diabetes*. 2007;56:2973-81.
- [90] Xu H, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ, et al. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *The Journal of clinical investigation*. 2003;112:1821-30.
- [91] Curat CA, Miranville A, Sengenès C, Diehl M, Tonus C, Busse R, et al. From blood monocytes to adipose tissue-resident macrophages: induction of diapedesis by human mature adipocytes. *Diabetes*. 2004;53:1285-92.
- [92] Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW, Jr. Obesity is associated with macrophage accumulation in adipose tissue. *The Journal of clinical investigation*. 2003;112:1796-808.
- [93] Christiansen T, Richelsen B, Bruun JM. Monocyte chemoattractant protein-1 is produced in isolated adipocytes, associated with adiposity and reduced after weight loss in morbid obese subjects. *International journal of obesity*. 2005;29:146-50.
- [94] Sartipy P, Loskutoff DJ. Monocyte chemoattractant protein 1 in obesity and insulin resistance. *Proceedings of the National Academy of Sciences of the United States of America*. 2003;100:7265-70.
- [95] Skurk T, Herder C, Kraft I, Müller-Scholze S, Hauner H, Kolb H. Production and release of macrophage migration inhibitory factor from human adipocytes. *Endocrinology*. 2005;146:1006-11.
- [96] Xie L, Ortega MT, Mora S, Chapes SK. Interactive changes between macrophages and adipocytes. *Clinical and vaccine immunology : CVI*. 2010;17:651-9.
- [97] Chazenbalk G, Bertolotto C, Heneidi S, Jumabay M, Trivax B, Aronowitz J, et al. Novel pathway of adipogenesis through cross-talk between adipose tissue macrophages, adipose stem cells and adipocytes: evidence of cell plasticity. *PloS one*. 2011;6:e17834.

- [98] Khazen W, M'Bika J P, Tomkiewicz C, Benelli C, Chany C, Achour A, et al. Expression of macrophage-selective markers in human and rodent adipocytes. *FEBS letters*. 2005;579:5631-4.
- [99] Itoh M, Suganami T, Hachiya R, Ogawa Y. Adipose tissue remodeling as homeostatic inflammation. *International journal of inflammation*. 2011;2011:720926.
- [100] Lehrke M, Lazar MA. Inflamed about obesity. *Nature medicine*. 2004;10:126-7.
- [101] Charriere G, Cousin B, Arnaud E, Andre M, Bacou F, Penicaud L, et al. Preadipocyte conversion to macrophage. Evidence of plasticity. *The Journal of biological chemistry*. 2003;278:9850-5.
- [102] Anderson EK, Gutierrez DA, Hasty AH. Adipose tissue recruitment of leukocytes. *Current opinion in lipidology*. 2010;21:172-7.
- [103] Schäffler A, Schölmerich J, Salzberger B. Adipose tissue as an immunological organ: Toll-like receptors, C1q/TNFs and CTRPs. *Trends in immunology*. 2007;28:393-9.
- [104] Xu H, Sethi JK, Hotamisligil GS. Transmembrane tumor necrosis factor (TNF)-alpha inhibits adipocyte differentiation by selectively activating TNF receptor 1. *The Journal of biological chemistry*. 1999;274:26287-95.
- [105] Huang S, Rutkowski JM, Snodgrass RG, Ono-Moore KD, Schneider DA, Newman JW, et al. Saturated fatty acids activate TLR-mediated proinflammatory signaling pathways. *Journal of lipid research*. 2012;53:2002-13.
- [106] Suganami T, Nishida J, Ogawa Y. A paracrine loop between adipocytes and macrophages aggravates inflammatory changes: role of free fatty acids and tumor necrosis factor alpha. *Arteriosclerosis, thrombosis, and vascular biology*. 2005;25:2062-8.
- [107] Suganami T, Tanimoto-Koyama K, Nishida J, Itoh M, Yuan X, Mizuarai S, et al. Role of the Toll-like receptor 4/NF-kappaB pathway in saturated fatty acid-induced inflammatory changes in the interaction between adipocytes and macrophages. *Arteriosclerosis, thrombosis, and vascular biology*. 2007;27:84-91.
- [108] Trayhurn P. Adipose tissue in obesity--an inflammatory issue. *Endocrinology*. 2005;146:1003-5.
- [109] Festa A, D'Agostino R, Jr., Williams K, Karter AJ, Mayer-Davis EJ, Tracy RP, et al. The relation of body fat mass and distribution to markers of chronic inflammation. *International journal of obesity and related metabolic disorders : journal of the International Association for the Study of Obesity*. 2001;25:1407-15.
- [110] Delarue J, Magnan C. Free fatty acids and insulin resistance. *Current opinion in clinical nutrition and metabolic care*. 2007;10:142-8.
- [111] Kruszynska YT, Worrall DS, Ofrecio J, Frias JP, Macaraeg G, Olefsky JM. Fatty acid-induced insulin resistance: decreased muscle PI3K activation but unchanged Akt phosphorylation. *The Journal of clinical endocrinology and metabolism*. 2002;87:226-34.
- [112] Yu C, Chen Y, Cline GW, Zhang D, Zong H, Wang Y, et al. Mechanism by which fatty acids inhibit insulin activation of insulin receptor substrate-1 (IRS-1)-associated phosphatidylinositol 3-kinase activity in muscle. *The Journal of biological chemistry*. 2002;277:50230-6.
- [113] Hotamisligil GS, Peraldi P, Budavari A, Ellis R, White MF, Spiegelman BM. IRS-1-mediated inhibition of insulin receptor tyrosine kinase activity in TNF-alpha- and obesity-induced insulin resistance. *Science*. 1996;271:665-8.
- [114] Ruan H, Hacoen N, Golub TR, Van Parijs L, Lodish HF. Tumor necrosis factor-alpha suppresses adipocyte-specific genes and activates expression of preadipocyte genes in 3T3-L1 adipocytes: nuclear factor-kappaB activation by TNF-alpha is obligatory. *Diabetes*. 2002;51:1319-36.
- [115] Campbell PJ, Carlson MG, Nurjhan N. Fat metabolism in human obesity. *The American journal of physiology*. 1994;266:E600-5.
- [116] Yu YH, Ginsberg HN. Adipocyte signaling and lipid homeostasis: sequelae of insulin-resistant adipose tissue. *Circulation research*. 2005;96:1042-52.

- [117] Nolan CJ, Madiraju MS, Delghingaro-Augusto V, Peyot ML, Prentki M. Fatty acid signaling in the beta-cell and insulin secretion. *Diabetes*. 2006;55 Suppl 2:S16-23.
- [118] Zhao YF, Feng DD, Chen C. Contribution of adipocyte-derived factors to beta-cell dysfunction in diabetes. *The international journal of biochemistry & cell biology*. 2006;38:804-19.
- [119] Patane G, Anello M, Piro S, Vigneri R, Purrello F, Rabuazzo AM. Role of ATP production and uncoupling protein-2 in the insulin secretory defect induced by chronic exposure to high glucose or free fatty acids and effects of peroxisome proliferator-activated receptor-gamma inhibition. *Diabetes*. 2002;51:2749-56.
- [120] Cnop M, Welsh N, Jonas JC, Jorns A, Lenzen S, Eizirik DL. Mechanisms of pancreatic beta-cell death in type 1 and type 2 diabetes: many differences, few similarities. *Diabetes*. 2005;54 Suppl 2:S97-107.
- [121] de Jongh RT, Serne EH, Ijzerman RG, de Vries G, Stehouwer CD. Free fatty acid levels modulate microvascular function: relevance for obesity-associated insulin resistance, hypertension, and microangiopathy. *Diabetes*. 2004;53:2873-82.
- [122] Bachmayer C, Kemmer A, Ehrmann N, Hasenberg T, Lammert A, Hammes HP. Adipokines and endothelial dysfunction in obesity WHO degrees III. *Microvascular research*. 2013.
- [123] Antoniadou C, Antonopoulos AS, Tousoulis D, Stefanadis C. Adiponectin: from obesity to cardiovascular disease. *Obesity reviews : an official journal of the International Association for the Study of Obesity*. 2009;10:269-79.
- [124] Pasceri V, Willerson JT, Yeh ET. Direct proinflammatory effect of C-reactive protein on human endothelial cells. *Circulation*. 2000;102:2165-8.
- [125] !!! INVALID CITATION !!!
- [126] Murphy TK, Calle EE, Rodriguez C, Kahn HS, Thun MJ. Body mass index and colon cancer mortality in a large prospective study. *American journal of epidemiology*. 2000;152:847-54.
- [127] Carmichael AR. Obesity as a risk factor for development and poor prognosis of breast cancer. *BJOG : an international journal of obstetrics and gynaecology*. 2006;113:1160-6.
- [128] Ursin G, Longnecker MP, Haile RW, Greenland S. A meta-analysis of body mass index and risk of premenopausal breast cancer. *Epidemiology*. 1995;6:137-41.
- [129] Haakinson DJ, Leeds SG, Dueck AC, Gray RJ, Wasif N, Stucky CC, et al. The impact of obesity on breast cancer: a retrospective review. *Annals of surgical oncology*. 2012;19:3012-8.
- [130] Fader AN, Arriba LN, Frasure HE, von Gruenigen VE. Endometrial cancer and obesity: epidemiology, biomarkers, prevention and survivorship. *Gynecologic oncology*. 2009;114:121-7.
- [131] Bergstrom A, Pisani P, Tenet V, Wolk A, Adami HO. Overweight as an avoidable cause of cancer in Europe. *International journal of cancer Journal international du cancer*. 2001;91:421-30.
- [132] Cirillo D, Rachiglio AM, la Montagna R, Giordano A, Normanno N. Leptin signaling in breast cancer: an overview. *Journal of cellular biochemistry*. 2008;105:956-64.
- [133] Chen J. Multiple signal pathways in obesity-associated cancer. *Obesity reviews : an official journal of the International Association for the Study of Obesity*. 2011;12:1063-70.
- [134] Jarde T, Perrier S, Vasson MP, Caldefie-Chezet F. Molecular mechanisms of leptin and adiponectin in breast cancer. *European journal of cancer*. 2011;47:33-43.
- [135] Bromberg J, Wang TC. Inflammation and cancer: IL-6 and STAT3 complete the link. *Cancer cell*. 2009;15:79-80.
- [136] Cabodi S, Taverna D. Interfering with inflammation: a new strategy to block breast cancer self-renewal and progression? *Breast cancer research : BCR*. 2010;12:305.
- [137] Subbaramaiah K, Howe LR, Bhardwaj P, Du B, Gravaghi C, Yantiss RK, et al. Obesity is associated with inflammation and elevated aromatase expression in the mouse mammary gland. *Cancer prevention research*. 2011;4:329-46.
- [138] Korkaya H, Liu S, Wicha MS. Breast cancer stem cells, cytokine networks, and the tumor microenvironment. *The Journal of clinical investigation*. 2011;121:3804-9.

- [139] Lemaitre B, Nicolas E, Michaut L, Reichhart JM, Hoffmann JA. The dorsoventral regulatory gene cassette *spatzle/Toll/cactus* controls the potent antifungal response in *Drosophila* adults. *Cell*. 1996;86:973-83.
- [140] Nomura N, Miyajima N, Sazuka T, Tanaka A, Kawarabayasi Y, Sato S, et al. Prediction of the coding sequences of unidentified human genes. I. The coding sequences of 40 new genes (KIAA0001-KIAA0040) deduced by analysis of randomly sampled cDNA clones from human immature myeloid cell line KG-1. *DNA research : an international journal for rapid publication of reports on genes and genomes*. 1994;1:27-35.
- [141] Rock FL, Hardiman G, Timans JC, Kastelein RA, Bazan JF. A family of human receptors structurally related to *Drosophila* Toll. *Proceedings of the National Academy of Sciences of the United States of America*. 1998;95:588-93.
- [142] Akira S, Takeda K. Toll-like receptor signalling. *Nature reviews Immunology*. 2004;4:499-511.
- [143] Davis JE, Gabler NK, Walker-Daniels J, Spurlock ME. Tlr-4 deficiency selectively protects against obesity induced by diets high in saturated fat. *Obesity*. 2008;16:1248-55.
- [144] Tsukumo DM, Carvalho-Filho MA, Carvalheira JB, Prada PO, Hirabara SM, Schenka AA, et al. Loss-of-function mutation in Toll-like receptor 4 prevents diet-induced obesity and insulin resistance. *Diabetes*. 2007;56:1986-98.
- [145] Kim F, Pham M, Luttrell I, Bannerman DD, Tupper J, Thaler J, et al. Toll-like receptor-4 mediates vascular inflammation and insulin resistance in diet-induced obesity. *Circulation research*. 2007;100:1589-96.
- [146] Hwa Cho H, Bae YC, Jung JS. Role of toll-like receptors on human adipose-derived stromal cells. *Stem Cells*. 2006;24:2744-52.
- [147] Batra A, Pietsch J, Fedke I, Glauben R, Okur B, Stroh T, et al. Leptin-Dependent Toll-Like Receptor Expression and Responsiveness in Preadipocytes and Adipocytes. *The American Journal of Pathology*. 2007;170:1931-41.
- [148] Pietsch J, Batra A, Stroh T, Fedke I, Glauben R, Okur B, et al. Toll-Like Receptor Expression and Response to Specific Stimulation in Adipocytes and Preadipocytes: On the Role of Fat in Inflammation. *Annals of the New York Academy of Sciences*. 2006;1072:407-9.
- [149] Chung S, Lapoint K, Martinez K, Kennedy A, Boysen Sandberg M, McIntosh MK. Preadipocytes mediate lipopolysaccharide-induced inflammation and insulin resistance in primary cultures of newly differentiated human adipocytes. *Endocrinology*. 2006;147:5340-51.
- [150] Poulain-Godefroy O, Froguel P. Preadipocyte response and impairment of differentiation in an inflammatory environment. *Biochemical and biophysical research communications*. 2007;356:662-7.
- [151] Khazen W, M'Bika JP, Collinet M, Tramonni M, Chany C, Achour A, et al. Differentiation-dependent expression of interferon gamma and toll-like receptor 9 in 3T3-F442A adipocytes. *Biochimie*. 2007;89:669-75.
- [152] Lin Y. The Lipopolysaccharide-activated Toll-like Receptor (TLR)-4 Induces Synthesis of the Closely Related Receptor TLR-2 in Adipocytes. *Journal of Biological Chemistry*. 2000;275:24255-63.
- [153] Murakami K, Bujo H, Unoki H, Saito Y. High fat intake induces a population of adipocytes to co-express TLR2 and TNFalpha in mice with insulin resistance. *Biochemical and biophysical research communications*. 2007;354:727-34.
- [154] Bès-Houtmann S, Roche R, Hoareau L, Gonthier M-P, Festy F, Caillens H, et al. Presence of functional TLR2 and TLR4 on human adipocytes. *Histochemistry and cell biology*. 2006;127:131-7.
- [155] Vitseva OI, Tanriverdi K, Tchkonja TT, Kirkland JL, McDonnell ME, Apovian CM, et al. Inducible Toll-like Receptor and NF-κB Regulatory Pathway Expression in Human Adipose Tissue. *Obesity*. 2008;16:932-7.

- [156] Anderson PD, Mehta NN, Wolfe ML, Hinkle CC, Pruscino L, Comiskey LL, et al. Innate immunity modulates adipokines in humans. *The Journal of clinical endocrinology and metabolism*. 2007;92:2272-9.
- [157] Kopp A, Buechler C, Neumeier M, Weigert J, Aslanidis C, Scholmerich J, et al. Innate immunity and adipocyte function: ligand-specific activation of multiple Toll-like receptors modulates cytokine, adipokine, and chemokine secretion in adipocytes. *Obesity*. 2009;17:648-56.
- [158] Hoareau L, Bencharif K, Rondeau P, Murumalla R, Ravanan P, Tallet F, et al. Signaling pathways involved in LPS induced TNF $\alpha$  production in human adipocytes. *Journal of Inflammation*. 2010;7:1.
- [159] Lin Y, Lee H, Berg AH, Lisanti MP, Shapiro L, Scherer PE. The lipopolysaccharide-activated toll-like receptor (TLR)-4 induces synthesis of the closely related receptor TLR-2 in adipocytes. *The Journal of biological chemistry*. 2000;275:24255-63.
- [160] Cani PD, Amar J, Iglesias MA, Poggi M, Knauf C, Bastelica D, et al. Metabolic endotoxemia initiates obesity and insulin resistance. *Diabetes*. 2007;56:1761-72.
- [161] Amar J, Burcelin R, Ruidavets JB, Cani PD, Fauvel J, Alessi MC, et al. Energy intake is associated with endotoxemia in apparently healthy men. *The American journal of clinical nutrition*. 2008;87:1219-23.
- [162] Rabot S, Membrez M, Bruneau A, Gerard P, Harach T, Moser M, et al. Germ-free C57BL/6J mice are resistant to high-fat-diet-induced insulin resistance and have altered cholesterol metabolism. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. 2010;24:4948-59.
- [163] Backhed F, Ding H, Wang T, Hooper LV, Koh GY, Nagy A, et al. The gut microbiota as an environmental factor that regulates fat storage. *Proceedings of the National Academy of Sciences of the United States of America*. 2004;101:15718-23.
- [164] Grunfeld C, Feingold KR. Endotoxin in the gut and chylomicrons: translocation or transportation? *Journal of lipid research*. 2009;50:1-2.
- [165] Ghoshal S, Witta J, Zhong J, de Villiers W, Eckhardt E. Chylomicrons promote intestinal absorption of lipopolysaccharides. *Journal of lipid research*. 2009;50:90-7.
- [166] Ilan Y. Leaky gut and the liver: a role for bacterial translocation in nonalcoholic steatohepatitis. *World journal of gastroenterology : WJG*. 2012;18:2609-18.
- [167] Ding S, Lund PK. Role of intestinal inflammation as an early event in obesity and insulin resistance. *Current opinion in clinical nutrition and metabolic care*. 2011;14:328-33.
- [168] Kim KA, Gu W, Lee IA, Joh EH, Kim DH. High fat diet-induced gut microbiota exacerbates inflammation and obesity in mice via the TLR4 signaling pathway. *PloS one*. 2012;7:e47713.
- [169] de La Serre CB, Ellis CL, Lee J, Hartman AL, Rutledge JC, Raybould HE. Propensity to high-fat diet-induced obesity in rats is associated with changes in the gut microbiota and gut inflammation. *American journal of physiology Gastrointestinal and liver physiology*. 2010;299:G440-8.
- [170] Ding S, Chi MM, Scull BP, Rigby R, Schwerbrock NM, Magness S, et al. High-fat diet: bacteria interactions promote intestinal inflammation which precedes and correlates with obesity and insulin resistance in mouse. *PloS one*. 2010;5:e12191.
- [171] Kol A, Lichtman AH, Finberg RW, Libby P, Kurt-Jones EA. Cutting edge: heat shock protein (HSP) 60 activates the innate immune response: CD14 is an essential receptor for HSP60 activation of mononuclear cells. *Journal of immunology*. 2000;164:13-7.
- [172] More SH, Breloer M, von Bonin A. Eukaryotic heat shock proteins as molecular links in innate and adaptive immune responses: Hsp60-mediated activation of cytotoxic T cells. *International immunology*. 2001;13:1121-7.
- [173] Frostegard J, Kjellman B, Gidlund M, Andersson B, Jindal S, Kiessling R. Induction of heat shock protein in monocytic cells by oxidized low density lipoprotein. *Atherosclerosis*. 1996;121:93-103.

- [174] Zanin-Zhorov A, Nussbaum G, Franitza S, Cohen IR, Lider O. T cells respond to heat shock protein 60 via TLR2: activation of adhesion and inhibition of chemokine receptors. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. 2003;17:1567-9.
- [175] Habich C, Kempe K, van der Zee R, Burkart V, Kolb H. Different heat shock protein 60 species share pro-inflammatory activity but not binding sites on macrophages. *FEBS letters*. 2003;533:105-9.
- [176] Osterloh A, Veit A, Gessner A, Fleischer B, Breloer M. Hsp60-mediated T cell stimulation is independent of TLR4 and IL-12. *International immunology*. 2008;20:433-43.
- [177] Cohen-Sfady M, Nussbaum G, Pevsner-Fischer M, Mor F, Carmi P, Zanin-Zhorov A, et al. Heat shock protein 60 activates B cells via the TLR4-MyD88 pathway. *Journal of immunology*. 2005;175:3594-602.
- [178] Habich C, Baumgart K, Kolb H, Burkart V. The receptor for heat shock protein 60 on macrophages is saturable, specific, and distinct from receptors for other heat shock proteins. *Journal of immunology*. 2002;168:569-76.
- [179] Marker T, Sell H, Zillessen P, Glode A, Kriebel J, Ouwens DM, et al. Heat shock protein 60 as a mediator of adipose tissue inflammation and insulin resistance. *Diabetes*. 2012;61:615-25.
- [180] Gulden E, Mollerus S, Bruggemann J, Burkart V, Habich C. Heat shock protein 60 induces inflammatory mediators in mouse adipocytes. *FEBS letters*. 2008;582:2731-6.
- [181] Ajuwon KM, Spurlock ME. Palmitate activates the NF-kappaB transcription factor and induces IL-6 and TNFalpha expression in 3T3-L1 adipocytes. *The Journal of nutrition*. 2005;135:1841-6.
- [182] Erridge C, Samani NJ. Saturated fatty acids do not directly stimulate Toll-like receptor signaling. *Arteriosclerosis, thrombosis, and vascular biology*. 2009;29:1944-9.
- [183] Johns EW GC, Walker JM, Sanders C. Chromosomal proteins related to histones. *Ciba Found Symp*. 1975;28:95-112.
- [184] Merenmies J, Pihlaskari R, Laitinen J, Wartiovaara J, Rauvala H. 30-kDa heparin-binding protein of brain (amphoterin) involved in neurite outgrowth. Amino acid sequence and localization in the filopodia of the advancing plasma membrane. *The Journal of biological chemistry*. 1991;266:16722-9.
- [185] Wang H, Bloom O, Zhang M, Vishnubhakat JM, Ombrellino M, Che J, et al. HMG-1 as a late mediator of endotoxin lethality in mice. *Science*. 1999;285:248-51.
- [186] Bustin M. Revised nomenclature for high mobility group (HMG) chromosomal proteins. *Trends Biochem Sci*. 2001;26:152-3.
- [187] Calogero S, Grassi F, Aguzzi A, Voigtlander T, Ferrier P, Ferrari S, et al. The lack of chromosomal protein Hmg1 does not disrupt cell growth but causes lethal hypoglycaemia in newborn mice. *Nat Genet*. 1999;22:276-80.
- [188] Venereau E, Schiraldi M, Ugucioni M, Bianchi ME. HMGB1 and leukocyte migration during trauma and sterile inflammation. *Molecular immunology*. 2013;55:76-82.
- [189] Gardella S, Andrei C, Ferrera D, Lotti LV, Torrisi MR, Bianchi ME, et al. The nuclear protein HMGB1 is secreted by monocytes via a non-classical, vesicle-mediated secretory pathway. *EMBO Rep*. 2002;3:995-1001.
- [190] Bonaldi T, Talamo F, Scaffidi P, Ferrera D, Porto A, Bachi A, et al. Monocytic cells hyperacetylate chromatin protein HMGB1 to redirect it towards secretion. *The EMBO journal*. 2003;22:5551-60.
- [191] Hoppe G, Talcott KE, Bhattacharya SK, Crabb JW, Sears JE. Molecular basis for the redox control of nuclear transport of the structural chromatin protein Hmgb1. *Exp Cell Res*. 2006;312:3526-38.
- [192] Rouhiainen A, Tumova S, Valmu L, Kalkkinen N, Rauvala H. Pivotal advance: analysis of proinflammatory activity of highly purified eukaryotic recombinant HMGB1 (amphoterin). *Journal of leukocyte biology*. 2007;81:49-58.
- [193] Yang H, Hreggvidsdottir HS, Palmblad K, Wang H, Ochani M, Li J, et al. A critical cysteine is required for HMGB1 binding to Toll-like receptor 4 and activation of macrophage cytokine release. *Proceedings of the National Academy of Sciences of the United States of America*. 2010;107:11942-7.



- [194] Urbonaviciute V, Meister S, Furnrohr BG, Frey B, Guckel E, Schett G, et al. Oxidation of the alarmin high-mobility group box 1 protein (HMGB1) during apoptosis. *Autoimmunity*. 2009;42:305-7.
- [195] Kazama H, Ricci JE, Herndon JM, Hoppe G, Green DR, Ferguson TA. Induction of immunological tolerance by apoptotic cells requires caspase-dependent oxidation of high-mobility group box-1 protein. *Immunity*. 2008;29:21-32.
- [196] Antoine DJ, Williams DP, Kipar A, Laverty H, Park BK. Diet restriction inhibits apoptosis and HMGB1 oxidation and promotes inflammatory cell recruitment during acetaminophen hepatotoxicity. *Molecular medicine*. 2010;16:479-90.
- [197] Ohndorf UM, Rould MA, He Q, Pabo CO, Lippard SJ. Basis for recognition of cisplatin-modified DNA by high-mobility-group proteins. *Nature*. 1999;399:708-12.
- [198] Weir HM, Kraulis PJ, Hill CS, Raine AR, Laue ED, Thomas JO. Structure of the HMG box motif in the B-domain of HMG1. *The EMBO journal*. 1993;12:1311-9.
- [199] Lange SS, Vasquez KM. HMGB1: the jack-of-all-trades protein is a master DNA repair mechanic. *Mol Carcinog*. 2009;48:571-80.
- [200] Bustin M, Reeves R. High-mobility-group chromosomal proteins: architectural components that facilitate chromatin function. *Prog Nucleic Acid Res Mol Biol*. 1996;54:35-100.
- [201] Catez F, Yang H, Tracey KJ, Reeves R, Misteli T, Bustin M. Network of dynamic interactions between histone H1 and high-mobility-group proteins in chromatin. *Molecular and cellular biology*. 2004;24:4321-8.
- [202] Krynetskaia N, Xie H, Vucetic S, Obradovic Z, Krynetskiy E. High mobility group protein B1 is an activator of apoptotic response to antimetabolite drugs. *Mol Pharmacol*. 2008;73:260-9.
- [203] Lange SS, Mitchell DL, Vasquez KM. High mobility group protein B1 enhances DNA repair and chromatin modification after DNA damage. *Proceedings of the National Academy of Sciences of the United States of America*. 2008;105:10320-5.
- [204] Prasad R, Liu Y, Deterding LJ, Poltoratsky VP, Kedar PS, Horton JK, et al. HMGB1 is a cofactor in mammalian base excision repair. *Mol Cell*. 2007;27:829-41.
- [205] Yuan F, Gu L, Guo S, Wang C, Li GM. Evidence for involvement of HMGB1 protein in human DNA mismatch repair. *The Journal of biological chemistry*. 2004;279:20935-40.
- [206] Stros M, Cherny D, Jovin TM. HMG1 protein stimulates DNA end joining by promoting association of DNA molecules via their ends. *Eur J Biochem*. 2000;267:4088-97.
- [207] van Gent DC, Hiom K, Paull TT, Gellert M. Stimulation of V(D)J cleavage by high mobility group proteins. *The EMBO journal*. 1997;16:2665-70.
- [208] Bianchi ME, Manfredi A. Chromatin and cell death. *Biochimica et biophysica acta*. 2004;1677:181-6.
- [209] Phair RD, Scaffidi P, Elbi C, Vecerova J, Dey A, Ozato K, et al. Global nature of dynamic protein-chromatin interactions in vivo: three-dimensional genome scanning and dynamic interaction networks of chromatin proteins. *Molecular and cellular biology*. 2004;24:6393-402.
- [210] Tang D, Kang R, Livesey KM, Cheh CW, Farkas A, Loughran P, et al. Endogenous HMGB1 regulates autophagy. *The Journal of cell biology*. 2010;190:881-92.
- [211] Yanai H, Ban T, Wang Z, Choi MK, Kawamura T, Negishi H, et al. HMGB proteins function as universal sentinels for nucleic-acid-mediated innate immune responses. *Nature*. 2009;462:99-103.
- [212] Orlova VV, Choi EY, Xie C, Chavakis E, Bierhaus A, Ihanus E, et al. A novel pathway of HMGB1-mediated inflammatory cell recruitment that requires Mac-1-integrin. *The EMBO journal*. 2007;26:1129-39.
- [213] Palumbo R, Galvez BG, Pusterla T, De Marchis F, Cossu G, Marcu KB, et al. Cells migrating to sites of tissue damage in response to the danger signal HMGB1 require NF-kappaB activation. *The Journal of cell biology*. 2007;179:33-40.

- [214] Riuzzi F, Sorci G, Donato R. The amphoterin (HMGB1)/receptor for advanced glycation end products (RAGE) pair modulates myoblast proliferation, apoptosis, adhesiveness, migration, and invasiveness. Functional inactivation of RAGE in L6 myoblasts results in tumor formation in vivo. *The Journal of biological chemistry*. 2006;281:8242-53.
- [215] Taguchi A, Blood DC, del Toro G, Canet A, Lee DC, Qu W, et al. Blockade of RAGE-amphoterin signalling suppresses tumour growth and metastases. *Nature*. 2000;405:354-60.
- [216] Tang D, Billiar TR, Lotze MT. A Janus tale of two active high mobility group box 1 (HMGB1) redox states. *Molecular medicine*. 2012;18:1360-2.
- [217] Zandarashvili L, Sahu D, Lee K, Lee YS, Singh P, Rajarathnam K, et al. Real-time kinetics of high-mobility group box 1 (HMGB1) oxidation in extracellular fluids studied by in situ protein NMR spectroscopy. *The Journal of biological chemistry*. 2013;288:11621-7.
- [218] Rubartelli A, Lotze MT. Inside, outside, upside down: damage-associated molecular-pattern molecules (DAMPs) and redox. *Trends in immunology*. 2007;28:429-36.
- [219] Tang D, Kang R, Zeh HJ, 3rd, Lotze MT. High-mobility group box 1, oxidative stress, and disease. *Antioxidants & redox signaling*. 2011;14:1315-35.
- [220] Degryse B, Bonaldi T, Scaffidi P, Muller S, Resnati M, Sanvito F, et al. The high mobility group (HMG) boxes of the nuclear protein HMG1 induce chemotaxis and cytoskeleton reorganization in rat smooth muscle cells. *The Journal of cell biology*. 2001;152:1197-206.
- [221] Fages C, Nolo R, Huttunen HJ, Eskelinen E, Rauvala H. Regulation of cell migration by amphoterin. *Journal of cell science*. 2000;113 ( Pt 4):611-20.
- [222] Huttunen HJ, Fages C, Kuja-Panula J, Ridley AJ, Rauvala H. Receptor for advanced glycation end products-binding COOH-terminal motif of amphoterin inhibits invasive migration and metastasis. *Cancer Res*. 2002;62:4805-11.
- [223] Rouhiainen A, Kuja-Panula J, Wilkman E, Pakkanen J, Stenfors J, Tuominen RK, et al. Regulation of monocyte migration by amphoterin (HMGB1). *Blood*. 2004;104:1174-82.
- [224] Yang D, Chen Q, Yang H, Tracey KJ, Bustin M, Oppenheim JJ. High mobility group box-1 protein induces the migration and activation of human dendritic cells and acts as an alarmin. *Journal of leukocyte biology*. 2007;81:59-66.
- [225] Wang H, Ward MF, Sama AE. Novel HMGB1-inhibiting therapeutic agents for experimental sepsis. *Shock*. 2009;32:348-57.
- [226] De Mori R, Straino S, Di Carlo A, Mangoni A, Pompilio G, Palumbo R, et al. Multiple effects of high mobility group box protein 1 in skeletal muscle regeneration. *Arteriosclerosis, thrombosis, and vascular biology*. 2007;27:2377-83.
- [227] Germani A, Limana F, Capogrossi MC. Pivotal advances: high-mobility group box 1 protein--a cytokine with a role in cardiac repair. *Journal of leukocyte biology*. 2007;81:41-5.
- [228] Li W, Sama AE, Wang H. Role of HMGB1 in cardiovascular diseases. *Current opinion in pharmacology*. 2006;6:130-5.
- [229] Chen G, Li J, Ochani M, Rendon-Mitchell B, Qiang X, Susarla S, et al. Bacterial endotoxin stimulates macrophages to release HMGB1 partly through CD14- and TNF-dependent mechanisms. *Journal of leukocyte biology*. 2004;76:994-1001.
- [230] Semino C, Angelini G, Poggi A, Rubartelli A. NK/iDC interaction results in IL-18 secretion by DCs at the synaptic cleft followed by NK cell activation and release of the DC maturation factor HMGB1. *Blood*. 2005;106:609-16.
- [231] Dumitriu IE, Baruah P, Valentinis B, Voll RE, Herrmann M, Nawroth PP, et al. Release of high mobility group box 1 by dendritic cells controls T cell activation via the receptor for advanced glycation end products. *Journal of immunology*. 2005;174:7506-15.

- [232] Wang H, Vishnubhakat JM, Bloom O, Zhang M, Ombrellino M, Sama A, et al. Proinflammatory cytokines (tumor necrosis factor and interleukin 1) stimulate release of high mobility group protein-1 by pituicytes. *Surgery*. 1999;126:389-92.
- [233] Rouhiainen A, Imai S, Rauvala H, Parkkinen J. Occurrence of amphoterin (HMG1) as an endogenous protein of human platelets that is exported to the cell surface upon platelet activation. *Thromb Haemost*. 2000;84:1087-94.
- [234] Passalacqua M, Patrone M, Picotti GB, Del Rio M, Sparatore B, Melloni E, et al. Stimulated astrocytes release high-mobility group 1 protein, an inducer of LAN-5 neuroblastoma cell differentiation. *Neuroscience*. 1998;82:1021-8.
- [235] Rauvala H, Pihlaskari R. Isolation and some characteristics of an adhesive factor of brain that enhances neurite outgrowth in central neurons. *The Journal of biological chemistry*. 1987;262:16625-35.
- [236] Mullins GE, Sundén-Cullberg J, Johansson AS, Rouhiainen A, Erlandsson-Harris H, Yang H, et al. Activation of human umbilical vein endothelial cells leads to relocation and release of high-mobility group box chromosomal protein 1. *Scand J Immunol*. 2004;60:566-73.
- [237] Melloni E, Sparatore B, Patrone M, Pessino A, Passalacqua M, Pontremoli S. Identity in molecular structure between "differentiation enhancing factor" of murine erythroleukemia cells and the 30 kD heparin-binding protein of developing rat brain. *Biochemical and biophysical research communications*. 1995;210:82-9.
- [238] Rauvala H, Merenmies J, Pihlaskari R, Korkolainen M, Huhtala ML, Panula P. The adhesive and neurite-promoting molecule p30: analysis of the amino-terminal sequence and production of antipeptide antibodies that detect p30 at the surface of neuroblastoma cells and of brain neurons. *The Journal of cell biology*. 1988;107:2293-305.
- [239] Jiang W, Li J, Gallowitsch-Puerta M, Tracey KJ, Pisetsky DS. The effects of CpG DNA on HMGB1 release by murine macrophage cell lines. *Journal of leukocyte biology*. 2005;78:930-6.
- [240] Jiang W, Pisetsky DS. The role of IFN-alpha and nitric oxide in the release of HMGB1 by RAW 264.7 cells stimulated with polyinosinic-polycytidylic acid or lipopolysaccharide. *Journal of immunology*. 2006;177:3337-43.
- [241] Livesey KM, Tang D, Zeh HJ, Lotze MT. Autophagy inhibition in combination cancer treatment. *Current opinion in investigational drugs*. 2009;10:1269-79.
- [242] Skinner M. Autophagy: in the hands of HMGB1. *Nature reviews Molecular cell biology*. 2010;11:756-7.
- [243] Zhan Z, Li Q, Wu P, Ye Y, Tseng HY, Zhang L, et al. Autophagy-mediated HMGB1 release antagonizes apoptosis of gastric cancer cells induced by vincristine via transcriptional regulation of Mcl-1. *Autophagy*. 2012;8:109-21.
- [244] Tang D, Kang R, Cheh CW, Livesey KM, Liang X, Schapiro NE, et al. HMGB1 release and redox regulates autophagy and apoptosis in cancer cells. *Oncogene*. 2010;29:5299-310.
- [245] Liu L, Yang M, Kang R, Wang Z, Zhao Y, Yu Y, et al. HMGB1-induced autophagy promotes chemotherapy resistance in leukemia cells. *Leukemia*. 2011;25:23-31.
- [246] Liu L, Yang M, Kang R, Wang Z, Zhao Y, Yu Y, et al. DAMP-mediated autophagy contributes to drug resistance. *Autophagy*. 2011;7:112-4.
- [247] Tsung A, Klune JR, Zhang X, Jeyabalan G, Cao Z, Peng X, et al. HMGB1 release induced by liver ischemia involves Toll-like receptor 4 dependent reactive oxygen species production and calcium-mediated signaling. *The Journal of experimental medicine*. 2007;204:2913-23.
- [248] Hamada T, Torikai M, Kuwazuru A, Tanaka M, Horai N, Fukuda T, et al. Extracellular high mobility group box chromosomal protein 1 is a coupling factor for hypoxia and inflammation in arthritis. *Arthritis and rheumatism*. 2008;58:2675-85.
- [249] Scaffidi P, Misteli T, Bianchi ME. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. *Nature*. 2002;418:191-5.

- [250] Lamkanfi M, Sarkar A, Vande Walle L, Vitari AC, Amer AO, Wewers MD, et al. Inflammasome-dependent release of the alarmin HMGB1 in endotoxemia. *Journal of immunology*. 2010;185:4385-92.
- [251] Sha Y, Zmijewski J, Xu Z, Abraham E. HMGB1 develops enhanced proinflammatory activity by binding to cytokines. *Journal of immunology*. 2008;180:2531-7.
- [252] Urbonaviciute V, Furnrohr BG, Meister S, Munoz L, Heyder P, De Marchis F, et al. Induction of inflammatory and immune responses by HMGB1-nucleosome complexes: implications for the pathogenesis of SLE. *The Journal of experimental medicine*. 2008;205:3007-18.
- [253] Wahamaa H, Schierbeck H, Hreggvidsdottir HS, Palmblad K, Aveberger AC, Andersson U, et al. High mobility group box protein 1 in complex with lipopolysaccharide or IL-1 promotes an increased inflammatory phenotype in synovial fibroblasts. *Arthritis research & therapy*. 2011;13:R136.
- [254] Schiraldi M, Raucci A, Munoz LM, Livoti E, Celona B, Venereau E, et al. HMGB1 promotes recruitment of inflammatory cells to damaged tissues by forming a complex with CXCL12 and signaling via CXCR4. *The Journal of experimental medicine*. 2012;209:551-63.
- [255] Harris HE, Andersson U, Pisetsky DS. HMGB1: a multifunctional alarmin driving autoimmune and inflammatory disease. *Nature reviews Rheumatology*. 2012;8:195-202.
- [256] Hori O, Brett J, Slattey T, Cao R, Zhang J, Chen JX, et al. The receptor for advanced glycation end products (RAGE) is a cellular binding site for amphoterin. Mediation of neurite outgrowth and co-expression of rage and amphoterin in the developing nervous system. *The Journal of biological chemistry*. 1995;270:25752-61.
- [257] Chavakis E, Hain A, Vinci M, Carmona G, Bianchi ME, Vajkoczy P, et al. High-mobility group box 1 activates integrin-dependent homing of endothelial progenitor cells. *Circulation research*. 2007;100:204-12.
- [258] Meng E, Guo Z, Wang H, Jin J, Wang J, Wu C, et al. High mobility group box 1 protein inhibits the proliferation of human mesenchymal stem cells and promotes their migration and differentiation along osteoblastic pathway. *Stem Cells Dev*. 2008;17:805-13.
- [259] Kang R, Tang D, Schapiro NE, Livesey KM, Farkas A, Loughran P, et al. The receptor for advanced glycation end products (RAGE) sustains autophagy and limits apoptosis, promoting pancreatic tumor cell survival. *Cell Death Differ*. 2010;17:666-76.
- [260] Kokkola R, Andersson A, Mullins G, Ostberg T, Treutiger CJ, Arnold B, et al. RAGE is the major receptor for the proinflammatory activity of HMGB1 in rodent macrophages. *Scand J Immunol*. 2005;61:1-9.
- [261] van Zoelen MA, Yang H, Florquin S, Meijers JC, Akira S, Arnold B, et al. Role of toll-like receptors 2 and 4, and the receptor for advanced glycation end products in high-mobility group box 1-induced inflammation in vivo. *Shock*. 2009;31:280-4.
- [262] Venereau E, Casalgrandi M, Schiraldi M, Antoine DJ, Cattaneo A, De Marchis F, et al. Mutually exclusive redox forms of HMGB1 promote cell recruitment or proinflammatory cytokine release. *The Journal of experimental medicine*. 2012;209:1519-28.
- [263] Huttunen HJ, Fages C, Rauvala H. Receptor for advanced glycation end products (RAGE)-mediated neurite outgrowth and activation of NF-kappaB require the cytoplasmic domain of the receptor but different downstream signaling pathways. *The Journal of biological chemistry*. 1999;274:19919-24.
- [264] Andersson U, Tracey KJ. HMGB1 is a therapeutic target for sterile inflammation and infection. *Annual review of immunology*. 2011;29:139-62.
- [265] Park JS, Svetkauskaite D, He Q, Kim JY, Strassheim D, Ishizaka A, et al. Involvement of toll-like receptors 2 and 4 in cellular activation by high mobility group box 1 protein. *The Journal of biological chemistry*. 2004;279:7370-7.
- [266] Tsung A, Sahai R, Tanaka H, Nakao A, Fink MP, Lotze MT, et al. The nuclear factor HMGB1 mediates hepatic injury after murine liver ischemia-reperfusion. *The Journal of experimental medicine*. 2005;201:1135-43.

- [267] Fiuza C, Bustin M, Talwar S, Tropea M, Gerstenberger E, Shelhamer JH, et al. Inflammation-promoting activity of HMGB1 on human microvascular endothelial cells. *Blood*. 2003;101:2652-60.
- [268] Park JS, Arcaroli J, Yum HK, Yang H, Wang H, Yang KY, et al. Activation of gene expression in human neutrophils by high mobility group box 1 protein. *Am J Physiol Cell Physiol*. 2003;284:C870-9.
- [269] Nogueira-Machado JA, Volpe CM, Veloso CA, Chaves MM. HMGB1, TLR and RAGE: a functional tripod that leads to diabetic inflammation. *Expert Opin Ther Targets*. 2011;15:1023-35.
- [270] Yu M, Wang H, Ding A, Golenbock DT, Latz E, Czura CJ, et al. HMGB1 signals through toll-like receptor (TLR) 4 and TLR2. *Shock*. 2006;26:174-9.
- [271] Conti L, Lanzardo S, Arigoni M, Antonazzo R, Radaelli E, Cantarella D, et al. The noninflammatory role of high mobility group box 1/toll-like receptor 2 axis in the self-renewal of mammary cancer stem cells. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. 2013.
- [272] Kawai T, Akira S. Innate immune recognition of viral infection. *Nature immunology*. 2006;7:131-7.
- [273] Kariko K, Buckstein M, Ni H, Weissman D. Suppression of RNA recognition by Toll-like receptors: the impact of nucleoside modification and the evolutionary origin of RNA. *Immunity*. 2005;23:165-75.
- [274] Ahmad-Nejad P, Hacker H, Rutz M, Bauer S, Vabulas RM, Wagner H. Bacterial CpG-DNA and lipopolysaccharides activate Toll-like receptors at distinct cellular compartments. *European journal of immunology*. 2002;32:1958-68.
- [275] Tian J, Avalos AM, Mao SY, Chen B, Senthil K, Wu H, et al. Toll-like receptor 9-dependent activation by DNA-containing immune complexes is mediated by HMGB1 and RAGE. *Nature immunology*. 2007;8:487-96.
- [276] Ivanov S, Dragoi AM, Wang X, Dallacosta C, Louten J, Musco G, et al. A novel role for HMGB1 in TLR9-mediated inflammatory responses to CpG-DNA. *Blood*. 2007;110:1970-81.
- [277] Porto A, Palumbo R, Pieroni M, Aprigliano G, Chiesa R, Sanvito F, et al. Smooth muscle cells in human atherosclerotic plaques secrete and proliferate in response to high mobility group box 1 protein. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. 2006;20:2565-6.
- [278] Inoue K, Kawahara K, Biswas KK, Ando K, Mitsudo K, Nobuyoshi M, et al. HMGB1 expression by activated vascular smooth muscle cells in advanced human atherosclerosis plaques. *Cardiovasc Pathol*. 2007;16:136-43.
- [279] Kalinina N, Agrotis A, Antropova Y, DiVitto G, Kanellakis P, Kostolias G, et al. Increased expression of the DNA-binding cytokine HMGB1 in human atherosclerotic lesions: role of activated macrophages and cytokines. *Arteriosclerosis, thrombosis, and vascular biology*. 2004;24:2320-5.
- [280] Nin JW, Ferreira I, Schalkwijk CG, Jorsal A, Prins MH, Parving HH, et al. Higher plasma high-mobility group box 1 levels are associated with incident cardiovascular disease and all-cause mortality in type 1 diabetes: a 12 year follow-up study. *Diabetologia*. 2012;55:2489-93.
- [281] Yan XX, Lu L, Peng WH, Wang LJ, Zhang Q, Zhang RY, et al. Increased serum HMGB1 level is associated with coronary artery disease in nondiabetic and type 2 diabetic patients. *Atherosclerosis*. 2009;205:544-8.
- [282] Skrha J, Jr., Kalousova M, Svarcova J, Muravska A, Kvasnicka J, Landova L, et al. Relationship of soluble RAGE and RAGE ligands HMGB1 and EN-RAGE to endothelial dysfunction in type 1 and type 2 diabetes mellitus. *Experimental and clinical endocrinology & diabetes : official journal, German Society of Endocrinology [and] German Diabetes Association*. 2012;120:277-81.
- [283] Han J, Zhong J, Wei W, Wang Y, Huang Y, Yang P, et al. Extracellular high-mobility group box 1 acts as an innate immune mediator to enhance autoimmune progression and diabetes onset in NOD mice. *Diabetes*. 2008;57:2118-27.

- [284] Matsuoka N, Itoh T, Watarai H, Sekine-Kondo E, Nagata N, Okamoto K, et al. High-mobility group box 1 is involved in the initial events of early loss of transplanted islets in mice. *The Journal of clinical investigation*. 2010;120:735-43.
- [285] Volz HC, Seidel C, Laohachewin D, Kaya Z, Muller OJ, Pleger ST, et al. HMGB1: the missing link between diabetes mellitus and heart failure. *Basic research in cardiology*. 2010;105:805-20.
- [286] Taniguchi N, Kawahara K, Yone K, Hashiguchi T, Yamakuchi M, Goto M, et al. High mobility group box chromosomal protein 1 plays a role in the pathogenesis of rheumatoid arthritis as a novel cytokine. *Arthritis and rheumatism*. 2003;48:971-81.
- [287] af Klint E, Grundtman C, Engstrom M, Catrina AI, Makrygiannakis D, Klareskog L, et al. Intraarticular glucocorticoid treatment reduces inflammation in synovial cell infiltrations more efficiently than in synovial blood vessels. *Arthritis and rheumatism*. 2005;52:3880-9.
- [288] Goldstein RS, Bruchfeld A, Yang L, Qureshi AR, Gallowitsch-Puerta M, Patel NB, et al. Cholinergic anti-inflammatory pathway activity and High Mobility Group Box-1 (HMGB1) serum levels in patients with rheumatoid arthritis. *Molecular medicine*. 2007;13:210-5.
- [289] Pullerits R, Urbonaviciute V, Voll RE, Forsblad-D'Elia H, Carlsten H. Serum levels of HMGB1 in postmenopausal patients with rheumatoid arthritis: associations with proinflammatory cytokines, acute-phase reactants, and clinical disease characteristics. *The Journal of rheumatology*. 2011;38:1523-5.
- [290] Kokkola R, Sundberg E, Ulfgren AK, Palmblad K, Li J, Wang H, et al. High mobility group box chromosomal protein 1: a novel proinflammatory mediator in synovitis. *Arthritis and rheumatism*. 2002;46:2598-603.
- [291] Palmblad K, Sundberg E, Diez M, Soderling R, Aveberger AC, Andersson U, et al. Morphological characterization of intra-articular HMGB1 expression during the course of collagen-induced arthritis. *Arthritis research & therapy*. 2007;9:R35.
- [292] Zetterstrom CK, Jiang W, Wahamaa H, Ostberg T, Aveberger AC, Schierbeck H, et al. Pivotal advance: inhibition of HMGB1 nuclear translocation as a mechanism for the anti-rheumatic effects of gold sodium thiomalate. *Journal of leukocyte biology*. 2008;83:31-8.
- [293] Schierbeck H, Lundback P, Palmblad K, Klevenvall L, Erlandsson-Harris H, Andersson U, et al. Monoclonal anti-HMGB1 (high mobility group box chromosomal protein 1) antibody protection in two experimental arthritis models. *Molecular medicine*. 2011;17:1039-44.
- [294] Ostberg T, Kawane K, Nagata S, Yang H, Chavan S, Klevenvall L, et al. Protective targeting of high mobility group box chromosomal protein 1 in a spontaneous arthritis model. *Arthritis and rheumatism*. 2010;62:2963-72.
- [295] Kokkola R, Li J, Sundberg E, Aveberger AC, Palmblad K, Yang H, et al. Successful treatment of collagen-induced arthritis in mice and rats by targeting extracellular high mobility group box chromosomal protein 1 activity. *Arthritis and rheumatism*. 2003;48:2052-8.
- [296] Van de Wouwer M, Plaisance S, De Vriese A, Waelkens E, Collen D, Persson J, et al. The lectin-like domain of thrombomodulin interferes with complement activation and protects against arthritis. *Journal of thrombosis and haemostasis : JTH*. 2006;4:1813-24.
- [297] Lappalainen T, Kolehmainen M, Schwab U, Pulkkinen L, de Mello VD, Vaittinen M, et al. Gene expression of FTO in human subcutaneous adipose tissue, peripheral blood mononuclear cells and adipocyte cell line. *J Nutrigenet Nutrigenomics*. 2010;3:37-45.
- [298] Peinado JR, Quiros PM, Pulido MR, Marino G, Martinez-Chantar ML, Vazquez-Martinez R, et al. Proteomic profiling of adipose tissue from *Zmpste24*<sup>-/-</sup> mice, a model of lipodystrophy and premature aging, reveals major changes in mitochondrial function and vimentin processing. *Mol Cell Proteomics*. 2011;10:M111 008094.
- [299] Feng D, Tang Y, Kwon H, Zong H, Hawkins M, Kitsis RN, et al. High-fat diet-induced adipocyte cell death occurs through a cyclophilin D intrinsic signaling pathway independent of adipose tissue inflammation. *Diabetes*. 2011;60:2134-43.

- [300] Wagner M, Bjerkvig R, Wiig H, Dudley AC. Loss of adipocyte specification and necrosis augment tumor-associated inflammation. *Adipocyte*. 2013;2.
- [301] Chen Y. Exposure of Human Macrophages to Tobacco Smoke Induces Hmgb1 Release on Microvesicles That Cause Monocyte Recruitment and Impairment of Insulin Signaling in Adipocytes. *Arteriosclerosis, thrombosis, and vascular biology* 2013. p. A162.
- [302] Chen Y. HMGB1 on Microvesicles Mediates Macrophage-Adipocyte Crosstalk and Impairs Insulin Signaling in Adipocytes. *Circulation* 2012. p. A16541.
- [303] Franchi L, Munoz-Planillo R, Nunez G. Sensing and reacting to microbes through the inflammasomes. *Nature immunology*. 2012;13:325-32.
- [304] Wen H, Gris D, Lei Y, Jha S, Zhang L, Huang MT, et al. Fatty acid-induced NLRP3-ASC inflammasome activation interferes with insulin signaling. *Nature immunology*. 2011;12:408-15.
- [305] Arrigo T, Chirico V, Salpietro V, Munafo C, Ferrau V, Gitto E, et al. High-mobility group protein B1: a new biomarker of metabolic syndrome in obese children. *European journal of endocrinology / European Federation of Endocrine Societies*. 2013;168:631-8.
- [306] Gunasekaran MK, Viranaicken W, Girard AC, Festy F, Cesari M, Roche R, et al. Inflammation triggers high mobility group box 1 (HMGB1) secretion in adipose tissue, a potential link to obesity. *Cytokine*. 2013.
- [307] Roche R, Hoareau L, Bes-Houtmann S, Gonthier MP, Laborde C, Baron JF, et al. Presence of the cannabinoid receptors, CB1 and CB2, in human omental and subcutaneous adipocytes. *Histochemistry and cell biology*. 2006;126:177-87.
- [308] Murumalla RK, Gunasekaran MK, Padhan JK, Bencharif K, Gence L, Festy F, et al. Fatty acids do not pay the toll: effect of SFA and PUFA on human adipose tissue and mature adipocytes inflammation. *Lipids in health and disease*. 2012;11:175.
- [309] Meir O, Dvash E, Werman A, Rubinstein M. C/EBP-beta regulates endoplasmic reticulum stress-triggered cell death in mouse and human models. *PloS one*. 2010;5:e9516.
- [310] Evankovich J, Cho SW, Zhang R, Cardinal J, Dhupar R, Zhang L, et al. High mobility group box 1 release from hepatocytes during ischemia and reperfusion injury is mediated by decreased histone deacetylase activity. *The Journal of biological chemistry*. 2010;285:39888-97.
- [311] Lu B, Nakamura T, Inouye K, Li J, Tang Y, Lundback P, et al. Novel role of PKR in inflammasome activation and HMGB1 release. *Nature*. 2012;488:670-4.
- [312] Nystrom S, Antoine DJ, Lundback P, Lock JG, Nita AF, Hogstrand K, et al. TLR activation regulates damage-associated molecular pattern isoforms released during pyroptosis. *The EMBO journal*. 2013;32:86-99.
- [313] Wei W, Chen M, Zhu Y, Wang J, Zhu P, Li Y, et al. Down-regulation of vascular HMGB1 and RAGE expression by n-3 polyunsaturated fatty acids is accompanied by amelioration of chronic vasculopathy of small bowel allografts. *The Journal of nutritional biochemistry*. 2012;23:1333-40.