Charles University
Third Faculty of Medicine

Université Toulouse III Paul Sabatier

Regulation of lipogenesis in human adipose tissue: Effect of metabolic stress, dietary intervention and aging.

Doctoral thesis

Veronika Šrámková

Supervisors:
Lenka Rossmeislová, M.Sc., Ph.D.
Prof. Dominique Langin, D.V.M., Ph.D.

Specialization: Molecular and cell biology, genetics and virology
Génétique moléculaire (École Doctorale Biologie-Santé-Biotechnologies)

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Declaration

Hereby I declare that this doctoral thesis is based on experiments performed during my Ph.D. studies at the Department for the Study of Obesity and Diabetes, at Third Faculty of Medicine, Charles University, in Prague and in Obesity Research *Laboratory*, INSERM, Institut des Maladies Métaboliques et Cardiovasculaires (I2MC - UMR1048), in Toulouse. Also, I declare that this thesis was written by me, that I cited properly all mentioned sources and literature and that this work was not used to obtain any other or same degree.

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Klíčová slova: *tuková tkáň, lipogeneze, adipogeneze, endoplazmatické retikulum, kalorická restrikce, stárnutí*

Key words: *adipose tissue, lipogenesis, adipogenesis, endoplasmic reticulum, calorie restriction, aging.*

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Contents

List of abbreviations

ACC	Acetyl-CoA-carboxylase
ACLY	ATP citrate lyase
ACSL	Acyl-CoA synthetase
AGPAT	AcylCoA acylglycerol-3-phosphate acyltransferase
AKT	AKT Serine/Threonine Kinase
ASC	Adipose tissue-derived stem cell
ASK1	Apoptosis signal-regulating kinase 1
AT	Adipose tissue
ATF	Activating transcription factor
ATGL	Adipose triglyceride lipase
ATM	ATM serine/threonine Kinase or Ataxia Telangiectasia Mutated
BAT	Brown adipose tissue
BCL-2	B-cell lymphoma 2
BCL-XL	B-cell lymphoma-extra large
BMI	Body mass index
BrdU	Bromodeoxyuridine
C/EBP	CCAAT/enhancer-binding protein
cAMP	Cyclic adenosine monophosphate
c-Cbl	c-Cbl Proto-Oncogene
CD36	Fatty acid translocase
CRP	C-reactive protein
CS	Citrate synthase
DAG	Diacylglycerol
DGAT	Diacylglycerol acyl-transferase
DHAP	Dihydroxyacetone-3P
DNA	Deoxyribonucleic acid
DNL	De novo lipogenesis
ECM	Extracellular matrix
EDEM	ER degradation-enhancing α-mannosidase-like protein
eIF2α	Eukaryotic translational factor 2α
ELOVL	Elongase
ER	Endoplasmic reticulum/Endoplazmatické retikulum
ERS	Endoplasmic reticulum stress
ERAD	ER-associated degradation
ERO1	Endoplasmic reticulum oxidoreductase 1
FABP4	Fatty acid transport protein 4
FABP5	Fatty acid transport protein 5
FABPpm	Plasma membrane fatty acid binding protein
FAHFA	Fatty acid-hydroxy-fatty acid
FAS	Fatty-acid synthase
FATP	Fatty acid transport protein
G3P	Glycerol-3-phosphate

GADD34	Growth arrest and DNA damage-inducible protein
GK	Glycerokinase
GLUT	Glucose transporter
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPAT	Glycerol-3-phosphate acyltransferase
GPDH	Glycerophosphate dehydrogenase
GRP78	Glucose regulated protein 78
GRP94	Glucose regulated protein 94
HDL	High-density lipoproteins
HOMA-IR	Homeostasis model assessment for insulin resistance
HSL	Hormone-sensitive lipase
HSPA5	Heat shock protein family A member 5
CHOP	C/EBP homologous protein
ChREBP	Carbohydrate response element binding protein
IDL	Intermediate-density lipoproteins
IKK	Nuclear factor-κB-IκB kinase
IL	Interleukin
INF	Interferon
IRE1	Inositol-requiring enzyme-1
IRS	Insulin receptor substrate
IκB	Inhibitor of κB
JNK	c-Jun-N-terminal kinase
KLF	Krüppel-like factor
LCD	Low-calorie diet
LDL	Low-density lipoproteins
LPL	Lipoprotein lipase
MAG	Monoacylglycerol
MAPK	Mitogen-Activated Protein Kinase
MCP1/CCL2	Monocyte chemoattractant protein 1
MGL	Monoacylglycerol lipase
mTOR	Mammalian target of rapamycin
NF-κB	Nuclear factor-κB
NK cells	Nature killer cells
NKT cells	Nature killer T-cells
PAHSA	Palmitic acid and stearic acid
PAP	Phosphohydrolase
PDK	Pyruvate Dehydrogenase Kinase
PERK	PKR-like eukaryotic initiation factor 2a kinase
PH	Pleckstrin homology
PI3K	Phosphatidylinositol 3-kinase
PIP_3	Phosphatidylinositol-2,4,5-triphosphate
PKA	Protein-kinase A
PKB	Protein kinase B
PKC	Protein kinase C

PPAR	Peroxisome proliferator-activated receptor
QUICKI	Quantitative insulin sensitivity check index
Rab-GAB	Rab-GTPase activating protein
ROS	Reactive oxygen species
SAAT	Subcutaneous abdominal adipose tissue
SASP	Senescence-associated secretory phenotype
SAβgal	Senescence-associated β-galactosidase
SCD	Stearoyl-CoA-desaturase
SDHA	Succinate Dehydrogenase Complex Flavoprotein Subunit A
SHC	Src-homology-2-containing protein
SREBP1c	Sterol regulatory element binding protein 1c
SVF	Stromal vascular fraction
TAG	Triglycerides
TG	Thapsigargin
TLR4	Toll-like receptor 4
TM	Tunicamycin
TNF	Tumor necrosis factor
TRAF2	Tumor necrosis factor receptor-associated factor 2
T_{REG}	T regulatory cells
TT	Tuková tkáň
UCP1	Uncoupling protein 1
UPR	Unfolded protein response
UQCRC2	Ubiquinol-Cytochrome C Reductase Core Protein II
VEGF	Vascular Endothelial Growth Factor
VLCD	Very-low calorie diet
VLDL	Very-low density lipoproteins
WAT	White adipose tissue
WHO	World Health Organisation
XBP1	X-box protein 1
XBP1s	X-box protein 1 spliced
γH2AX	Histone H2AX phosphorylation

Summary

Adipose tissue (AT) is a complex organ specialised in safe storage and release of energy as lipids. The adipose organ is therefore essential for the maintenance of energy homeostasis. The prototypical cells of AT are adipocytes, emerging from the precursors in a process called adipogenesis. Adipogenesis itself is tightly connected with lipogenesis, i.e. with the synthesis of fatty acids and triglycerides. Various stimuli can disturb adipocyte differentiation and lipogenesis and thus contribute to AT dysfunction and development of associated metabolic diseases.

This thesis was focused on the investigation of lipogenesis in the context of endoplasmic reticulum stress (ERS), calorie restriction and aging.

In **Project A**, we showed that exposition of adipocytes to high acute ERS inhibits expression of lipogenic genes and glucose incorporation into lipids. Moreover, chronic exposure of preadipocytes to ERS impaired both, lipogenesis and adipogenesis. On the other hand, chronic low ERS had no apparent effect on lipogenesis in adipocytes. These effects of ERS could therefore contribute to the worsening of AT function seen in obesity.

The capacity of AT to store lipids decreases in aging, possibly due to the accumulation of senescence cells or higher ERS. In **Project B**, we investigated lipogenic capacity of human AT in relation to senescence and markers of ERS. AT and adipose cells from young and elderly women were investigated. While mRNA expression of major senescent markers was increased in AT from the elderly compared to young individuals, mRNA expression of lipogenic enzymes and chaperones was decreased in AT from elderly individuals. These results were also partly observed *in vitro* in differentiated adipocytes from AT of the same individuals suggesting the reduced capability to cope with ERS in aging.

Very-low calorie diet (VLCD) is first line lifestyle intervention to achieve rapid weight loss. The improvement of whole body insulin sensitivity can be seen as soon as after 2 days of VLCD. However, little is known about AT metabolic changes in those early days. Thus, in **Project C**, we compared metabolic and inflammation-related characteristics of subcutaneous AT in the early (2 days) and later (28 days) phase of a VLCD. In the early phase of VLCD, the expression of lipolytic genes was increased, whereas the expression of lipogenic genes was suppressed. The inflammatory markers remained unchanged in AT. The changes in AT gene expression in the early phase of VLCD could not explain the effect of short calorie restriction on the improvement of insulin sensitivity. At the later phase, expression of genes involved in lipogenesis and β-oxidation was markedly suppressed,

whereas the expression of inflammatory markers was increased. Thus, we found that the early and later phases of VLCD differ with respect to metabolic and inflammatory responses in subcutaneous AT.

In **Project D**, we compared and defined the effects of moderate calorie restriction on preadipocytes and *in vitro* differentiated adipocytes in two groups of obese men: juniors and seniors. We did not observe any effect of the intervention on metabolism of preadipocytes in either group. However, we observed an intervention-driven improvement in adipocyte metabolism selectively in the group of seniors. Therefore, our data suggest that moderate calorie restriction could initiate positive changes in metabolism of adipocytes in seniors.

In conclusion, this thesis brought several pieces of evidence that lipogenesis in human AT can be inhibited by ER stress, severe caloric restriction and aging.

Résumé

Le tissue adipeux (TA) est un organe complexe specialisé dans le stockage et la libération d´énergie sous forme de lipides. Cet organe adipeux est essentiel pour le maintien de l´homéostasie énergétique. Les adipocytes sont les cellules prototypiques du TA. Elles se forment durant la différenciation de précurseurs, un processus appelé adipogenèse. L'adipogenèse est intimement associée à la synthèse des acides gras et de triglycérides lors de la lipogenèse. Néanmoins, divers facteurs peuvent perturber l'adipogénèse et la lipogenèse, contribuant au dysfonctionnement du TA et au développement des maladies métaboliques.

Le but de cette thèse a été d´étudier la lipogenèse dans le contexte du stress du réticulum endoplasmiques (SRE), de la restriction calorique et du vieillissement.

Dans **le projet A**, nous avons montré que l´exposition d'adipocytes à un SRE aigu inhibe l´expression des gènes liés à la lipogenèse et empêche l´incorporation du glucose dans les lipides. En plus, l´exposition des préadipocytes à un SRE chronique, détériore à la fois la lipogenèse et l´adipogenèse. Par contre, pour les adipocytes, un SRE chronique mais modéré n´a pas d´effet évident sur la lipogenèse. Ces effets du SRE pourraient contribuer à la détérioration de la fonction de TA vue dans l´obésité.

La capacité du TA à stocker des lipides diminue avec l´âge, probablement à cause de l´accumulation de cellules sénescentes ou un SRE plus élevé. Dans **le projet B**, nous avons étudié la capacité lipogénique du TA humain en relation à la sénescence et aux marqueurs du SRE au sein d'une cohorte de femmes obèses jeunes ou âgées. Tandis que l´expression des principaux marqueurs de la sénescence était augmentée dans le TA des femmes âgées, l´expression génique des enzymes de lipogenèse et des chaperonnes était diminuée dans le TA des personnes âgées. Ces résultats étaient partiellement retrouvés dans les adipocytes differenciés *in vitro* des mêmes individus ce qui suggère une moins bonne capacité à faire face au SRE lors du vieillissement.

Le régime à très basses calories (VLCD) est souvent prescrit en première intention pour une rapide perte de poids. L'amélioration de la sensibilité à l'insuline se voit dès 2 jours de VLCD. Néanmoins, on ne sait quasiment rien des modifications métaboliques du TA survenant durant les premiers jours. Dans **le projet C**, nous avons donc comparé les réponses métaboliques et inflammatoires du TA sous-cutané précocément (2 jours) et plus tardivement (28 jours) lors d'un VLCD. A 2 jours de régime, l´expression des gènes lipolytiques était augmentée, alors que l´expression des gènes lipogéniques était diminuées. Les marqueurs d´inflammation n´étaient pas changés dans le TA. Néanmoins, les changements d´expression

dans le TA lors de la phase précoce du régime ne pouvait pas expliquer l´effet de ce régime court à l´amélioration de la sensibilité à l´insuline. Dans la phase tardive, l´expression des gènes impliqués dans la lipogenèse et la β-oxydation était largement réduite, tandis que l´expression des marqueurs inflammatoires était augmentée. Nous avons donc montré que les réponses métaboliques et inflammatoires du TA sous-cutané à 2 jours et 28 jours de VLCD sont différentes.

Dans **le projet D**, nous avons comparé et défini les effets de la restriction calorique modérée sur la physiologie des préadipocytes et des adipocytes différenciés *in vitro* chez des jeunes obèses ou des personnes âgées obèses. De façon surprenante, on n´a observé aucun effet de l´intervention sur le métabolisme des préadipocytes dans les deux groupes. Par contre, un effet bénéfique de l'intervention sur le métabolisme adipocytaire n'a été observé que chez les personnes âgées. Nos données montrent donc qu'une restriction calorique modérée peut avoir un effet positif sur le métabolisme adipocytaire des séniors.

Pour conclure, cette thèse montre que la lipogenèse dans le TA humain peut être inhibée par le SRE, la restriction calorique sévère et le vieillissement.

Shrnutí

Tuková tkáň (TT) je komplexní orgán specializovaný pro bezpečné skladování a uvolňování energie ve formě lipidů. TT je tedy nezbytná pro udržování energetické homeostázi. Základní funkční jednotky TT se nazývají adipocyty a vznikající z prekurzorových buněk procesem adipogeneze. Adipogeneze jako taková je velmi úzce spojena s lipogenezí, neboli syntézou mastných kyselin a triglyceridů. Nejrůznější faktory mohou ovšem narušit diferenciaci a lipogenezi adipocytů a přispívat tak k dysfunkci TT a rozvoji metabolických onemocnění.

Proto byla tato dizertační práce zaměřena na zkoumání lipogeneze v kontextu stresu v endoplasmatickém retikulu (ER), kalorické restrikce a stárnutí.

V **projektu A** jsme ukázali, že vystavení adipocytů silnému akutnímu stresu ER snižuje expresi lipogenních genů a inkorporaci glukózy do lipidů. Chronický stres ER negativně ovlivňoval jak lipogenezi, tak vlastní diferenciaci preadipocytů, i když v již maturovaných adipocytech neměl chronický stres ER na lipogenezi zjevný efekt. Tyto efekty stresu ER na lipogenezi a adipogenezi tak mohou přispívat ke zhoršení funkce TT pozorované u obézních jedinců.

Kapacita TT skladovat lipidy se snižuje s věkem, pravděpodobně kvůli akumulaci senescentních buněk nebo zvýšenému stresu v ER. V **projektu B** jsme zkoumali lipogenní kapacitu lidské TT ve vztahu k senescenci a markerům stresu ER. K analýze byly použity vzorky TT a adipocyty mladších žen a seniorek. Zatímco mRNA exprese hlavních senescentních markerů byla zvýšená v TT seniorek ve srovnání s mladšími ženami, mRNA exprese lipogenních enzymů a šaperonů ER byla v TT u seniorek snížena. Tyto výsledky byly částečně potvrzeny v *in vitro* diferencovaných adipocytech z TT identických žen. Tyto výsledky naznačují sníženou odpověď na stres v ER ve stáří.

Velmi přísná nízkoenergetická dieta (VLCD, z anglického *very low-calorie diet*) patří mezi primární intervence používané k rapidnímu poklesu hmotnosti u obézních. Zlepšení celotělové inzulinové sensitivity je možno pozorovat již po 2 dnech VLCD. Nicméně o změnách probíhajících v TT během těchto prvních dnech diety se neví prakticky nic. V **projektu C** jsme proto srovnávali metabolické a zánětlivé charakteristiky subkutánní TT během rané (2 dny) a pozdější (28 dní) fáze VLCD. Během rané fáze VLCD došlo ke zvýšení exprese lipolytických genů, kdežto exprese lipogenních genů byla potlačena. Zánětlivé markery zůstaly v TT nezměněny. Změny na úrovni genové exprese v TT v rané fázi VLCD nicméně nevysvětlily efekt krátké kalorické restrikce na zlepšení inzulinové sensitivity. V

pozdější fázi byla exprese genů zapojených do lipogeneze a β-oxidace markantně snížena, zatímco exprese zánětlivých markerů byla zvýšena. Tento projekt ukázal, že raná a pozdější fáze VLCD se liší s ohledem na metabolickou a zánětlivou odpověď subkutánní TT.

V **projektu D** jsme srovnávali a definovali efekty mírné kalorické restrikce na preadipocyty a *in vitro* diferencované adipocyty u dvou skupin obézních mužů: mladších mužů a seniorů. Zatímco jsme nepozorovali žádný efekt intervence na metabolismus preadipocytů v žádné ze dvou skupin, ve skupině seniorů jsme po intervenci zaznamenali zlepšení metabolismu adipocytů. Naše výsledky tedy naznačují, že mírná kalorická restrikce může vest k zahájení pozitivních změn v metabolismu adipocytů u seniorů.

Závěrem je možné shrnout, že tato dizertační práce přinesla několik důkazů o tom, že lipogeneze v lidské TT může být inhibována stresem ER, přísnou kalorickou restrikcí a stárnutím.

1 Introduction into biology of adipose tissue

Traditional perception of adipose tissue (AT) as a passive organ dedicated to energy storage, insulation and thermoregulation has changed dramatically in the last decades, when the extraordinary complex role of AT in various physiological processes started to be recognised and appreciated. Nowadays, it is known that apart from the regulation of whole body energy homeostasis AT is involved in inflammation, angiogenesis, reproduction, atherogenesis or regeneration (**Figure 1**). These pleiotropic functions of AT rely not only on the paracrine communication between various cell types within AT itself but also on the cross-talk with distant organs through secreted factors, called adipokines and lipokines.

In the introductory section of this thesis, two elementary types of AT and its cellular composition will be described at first. Next, the most important physiological functions of AT and adipocytes will be explained. Lipogenesis, the process of lipid synthesis, will be described in detail, as it is the main subject of this thesis. Third part of the theoretical introduction will be dedicated to the description of processes which contribute to AT dysfunction in obesity and aging. The last part will briefly outline the possibilities of fight against obesity through lifestyle and dietary interventions.

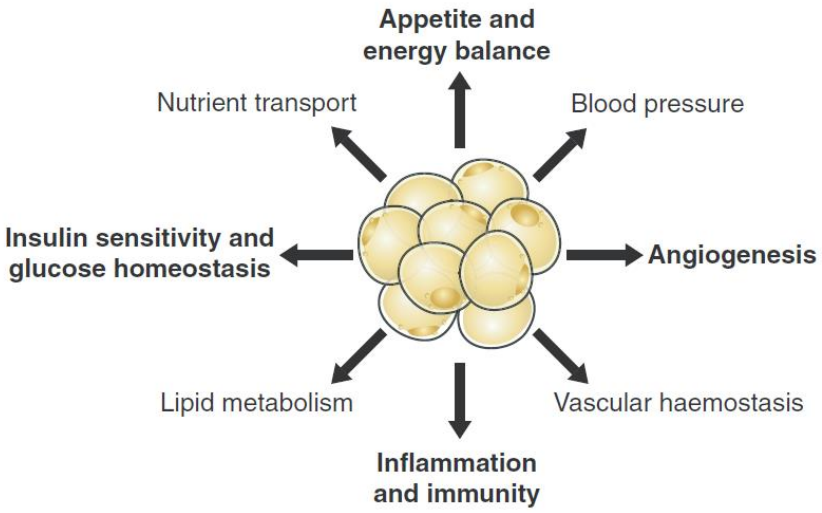


Figure 1: Adipose tissue is an organ with a plethora of functions. This picture illustrates some of principal physiological and metabolic processes with which adipose tissue is involved through the secretion of various adipokines from adipocytes. The interactions may be autocrine, paracrine, or endocrine. Adapted from [7].

1.1. Adipose tissue

1.1.1 White adipose tissue

AT organ, in some individuals the largest organ in the body, is distributed in many different depots throughout the body. Different cellular characteristics and anatomical location predetermine specific properties of each depot and its particular function. In mammals, the AT pool is composed of at least two functionally and histologically distinct types of fat: white and brown. Major white AT (WAT) depots are situated in subcutaneous region in both, the upper (superficial and deep abdominal) and lower (gluteal-femoral) body, as well as in the visceral region (omental, mesenteric, mediastinal and epicardial depot) (**Figure 2**) [2].

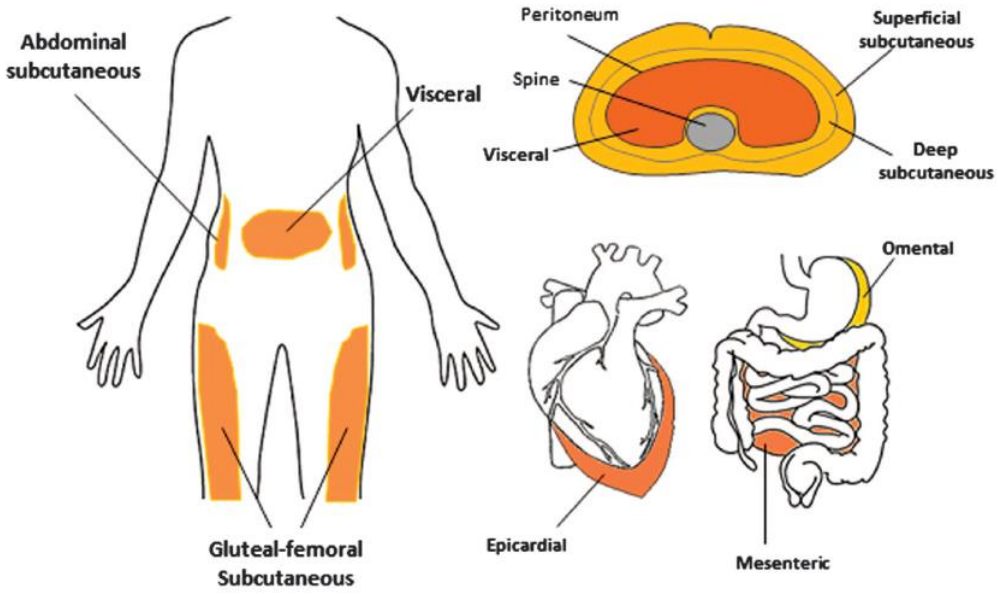


Figure 2: White adipose tissue depots in humans, shown in orange. Major subcutaneous white adipose tissue includes superficial and deep abdominal depots and gluteal-femoral depot. Major visceral white adipose tissue includes epicardial, omental and mesenteric. Adapted from [2, 3].

Subcutaneous WAT, a major energy storing depot, is located under the skin to provide a layer of insulation preventing heat loss and protecting against mechanical stress. On the other hand, visceral WAT coats vital organs within the peritoneum and rib cage. In addition, WAT can be found in many other areas, including retro-orbital space, on the face and extremities, and within the bone marrow [8].

Fat distribution is markedly altered by many factors, such as sex, hormonal status, age and disease state [9]. Female body type tends to be a *pear* shape, because subcutaneous fat is

preferentially deposited around the hips and thighs [10, 11]. Pregnancy often emphasizes this sexually dimorphic fat distribution [12]. In contrast, men (and postmenopausal women) accumulate fat around the waist (so called *apple* shape) and tend to accumulate more visceral fat [13, 14]. Gradually, these gender-based differences in the AT deposition become less prominent later in life due to decreasing influence of steroid hormones.

Also, the aging *per se* affects the distribution of body fat mass. The peak of fat depot sizes is reached by middle or early old age (40-70 years), followed by a substantial decline in advanced old age (>70 years) [15]. The volume of subcutaneous fat declines first, followed much later by loss of fat in visceral depots. However, the observed decrease in total body fat with old age does not coincide with a decline in percent body fat, which may remain constant or even increase. The age-associated decline in sizes of adipose depots is accompanied by the accumulation of fat outside AT and loss of lean body mass (particularly muscle). Ectopic fat accumulation occurs in bone marrow, muscle, liver and at other sites. This ectopic fat deposition causes lipotoxicity and worsens age-dependent dysfunction of these tissues.

1.1.2 Brown and brite adipose tissue

In comparison with WAT, that is present in humans throughout whole lifetime, brown AT (BAT) is in human present mainly in newborns, predominantly in the interscapular region. BAT uses the chemical energy from lipids and glucose to produce heat through non-shivering thermogenesis via mitochondrial uncoupling [16]. This is possible by the presence of uncoupling protein 1 (UCP1) that uncouples electron transport from ATP production, leading to the generation of heat [17]. Because of the high mitochondria content and dense vascularisation, BAT appears *brown* compared to WAT.

For many years it was generally accepted that BAT postnatally disappears and that BAT depots are absent in human adults. Nevertheless, a few years ago, the use of ^{18}F-fluorodeoxyglucose led to discovery of small areas of tissue functionally resembling BAT in the thorax region, chest and abdomen in adult humans (**Figure 3**) [1]. This metabolically active tissue responds to temperature similarly as BAT, however it is still distinct from the dorsal interscapular BAT in children. Subsequent studies suggested that brown fat cells might be interspersed within the WAT (brown in white, i.e. *brite*, or *beige* AT).

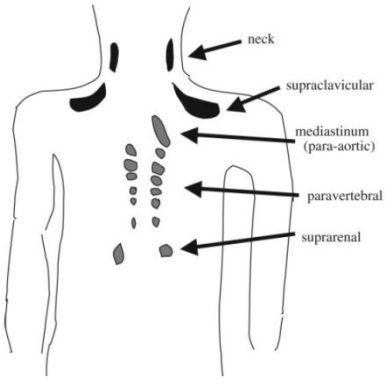


Figure 3: Sites of ^{18}F-fluorodeoxyglucose uptake corresponding to BAT in adult humans. The black areas are those that are most frequently described; the gray areas are not always found, even in humans positive in the black areas. Adapted from [1].

This brite AT is considered as a subtype of WAT that has adopted features of BAT upon the stimulation by low temperatures in a process known as *browning*. Brite AT occurrence and activity in adult decrease with age and higher adiposity [18]. These results suggest that the decreased BAT activity might be associated with the accumulation of classical WAT with age.

1.1.3 Cellular composition of white adipose tissue

Histologically, AT is composed of adipocytes, i.e. the mature fat cell, and stromal-vascular fraction (SVF), comprising stem cells, preadipocytes, immune cells, endothelial cells and extracellular matrix (ECM).

1.1.3.1 Adipocytes

White adipocytes are rounded cells containing a single large lipid droplet that occupies usually over 90% of the cell volume. The cytoplasm and organelles, such as nucleus and mitochondria, are displaced to the periphery of the cell. Lipids stored in the droplet are primarily triglycerides (or triacylglycerols, TAG) and cholesteryl esters. The degree of lipid accumulation determines adipocyte size which is in average 80-90 μm but can reach up to 200 μm [19].

1.1.3.2 Stromal vascular fraction

1.1.3.2.1 Adipose tissue stem cells

Adipose tissue stem cells (ASCs) are precursors of adipocytes, and as such they are prerequisite to hyperplastic growth of AT mainly in the early childhood and puberty. In adulthood, when the total number of adipocytes remains relatively constant [20], they ensure replenishment of aged non-functional adipocytes. In fact, lifespan of adipocytes was estimated to be approximately 10 years. ASCs tend to be associated with blood vessels and may be derived from AT pericytes (cells that wrap around endothelial cells) [21-23]. Since ASCs are multipotent cells, they are capable to differentiate not only into brown and white adipocytes through the precursor stage of preadipocytes but also to other cell types, including macrophages, muscle or bone progenitors [24-28].

1.1.3.2.1.1 From ASC to mature adipocyte: An insight into adipocyte differentiation

The process of adipocyte differentiation from ASC to mature adipocytes includes many cellular intermediates. Although there have been efforts to describe these distinct intermediates, they have been difficult to characterise at the molecular level. Therefore,

adipogenesis is generally presented as a two-phase process including *determination* and *terminal differentiation phase*.

Determination involves the commitment of a pluripotent stem cell to the adipocyte lineage [29]. This stage results in the conversion of the stem cell to a preadipocyte. Preadipocyte cannot be distinguished morphologically from its precursor cell but has lost the potential to differentiate into other cell types.

During *terminal differentiation*, the preadipocyte acquires the characteristics of mature adipocyte. These include building machinery necessary for lipid transport and synthesis, insulin sensitivity and the secretion of adipocyte-specific proteins.

The molecular regulation of terminal differentiation is more extensively characterised than determination. It is known that differentiation requires the activation of numerous transcription factors that are responsible for coordinated expression or silencing of more than 2000 genes related to the regulation morphology and physiology of adipocyte [30].

Two members of CCAAT/enhancer-binding proteins (C/EBPs), C/EBPβ and C/EBPδ, are dramatically induced during the first stage of adipogenesis, at least *in vitro*, in response to the exposition of cells to hormonal differentiation cocktail [31]. Their primary function in adipogenesis is to provoke expression of peroxisome proliferator-activating receptor γ (PPARγ) and C/EBPα, the key transcriptional regulators of adipocyte differentiation [32, 33]. PPARγ and C/EBPα initiate a positive feedback loop in which they induce their own expression and expression of a large number of adipocyte specific genes.

PPARγ, the *master regulator* of adipogenesis, is a member of the nuclear-receptor superfamily. It was shown that PPARγ is both necessary and sufficient for adipogenesis, as C/EBPα or other transcription factors cannot promote adipogenesis in its absence [34, 35].

In addition to PPARγ and C/EBPs, several other transcription factors are likely to play an important role in the molecular control of adipogenesis. In general, pro-adipogenic factors seem to function at least in part by inducing PPARγ expression or enhancing its activity. These include certain Krüppel-like factors (KLFs), such as KLF4, 5, 6, 9 and 15. On the other hand, KLF2, 3 and 7 are anti-adipogenic [36]. GATA2 and GATA3 also belong to anti-adipogenic factors. They are expressed in preadipocytes and downregulated during terminal maturation [37]. Constitutive expression of GATA2 and GATA3 blunts adipocyte differentiation and trap cells at the stage of preadipocyte. Therefore, the process of adipocyte differentiation is a result of a balance between these intervening factors. Their expression can be influenced by the present cellular state. For example, in the experimental part A of this thesis, we show that adipocyte differentiation is blunted by stress of endoplasmic reticulum

(ER). If the milieu for adipogenesis is favourable, newly formed adipocyte gain a rounded-cell shape with one lipid droplet inside and the whole machinery for handling and synthesis of lipids.

1.1.3.2.2 Immune cells

Immune cells, which reside in AT, include almost the full spectrum of known immune cell types. Their primary physiological role is to maintain AT homeostasis. This includes ECM remodelling, angiogenesis, activation of inflammatory response, removal of molecular debris and apoptotic cells [38, 39].

The most abundant population of AT immune cells are macrophages [40]. Although the spectrum of macrophage phenotypes is continuous, there is no nomenclature that could provide all of the required definitions. Thus investigators generally accept the simplified consensus which distinguishes two principal phenotypes: M1-like (classically activated) and M2-like (alternatively activated). The function of both phenotypes is different: resident M2-like AT macrophages have a role in AT homeostasis, whereas recruited M1-like macrophages contribute to inflammation and insulin resistance, described later. Nevertheless, it should be noted that this M1-/M2-like distinction is artificial and macrophages can possess features of both phenotypes [41].

Neutrophils are myeloid cells with short lifetime that are essential for the initial response to bacterial infections and injury [42, 43]. This is because they facilitate the recruitment of macrophages, dendritic cells and lymphocytes into the site of infection. In metabolically healthy animals, neutrophils represent less than 1 % of total AT immune cells [43]. Dendritic cells are the major antigen presenting cells and can induce proliferation of lymphocyte population. Their gene expression profile is very similar to that one of macrophages [43], yet dendritic cells are still probably the least characterized myeloid cells of AT. Mast cells are known to mediate allergic reactions [44]. They can modulate AT inflammation and possibly fibrotic state found in obesity and diabetes [45]. Eosinophils are classic effectors of anti-helminth responses. In the context of AT, they can assist the induction into M2-like macrophages via interleukin 4 (IL-4) [46]. Other cells originating from lymphoid lineage found in AT are B cells, T cells, nature killer (NK) cells and their numerous subtypes. Activities of T and B lymphocytes rely on the antigen recognition and include diverse populations of cells with proinflammatory or regulatory functions. In human AT, T cells, B cells, NK cells, NKT cells and innate lymphoid cells, group 2 account for up to 10% of non-adipocyte cells [47].

The dynamic nature of immune cells in AT during the progression of obesity is briefly mentioned in *1.3.2.4 Inflammation*.

1.1.4 Extracellular matrix

Each adipocyte is surrounded by a thick ECM, also called basal lamina. Basal lamina can decrease the mechanical stress by spreading the forces over a larger area of the tissue and therefore protect adipocyte/lipid droplet from disruption.

The composition of ECM depends on the developmental stage of preadipocyte and/or adipocyte, viability and subtype of the adipocyte, but the two main classes of ECM proteins are proteoglycans and fibrous proteins. Major component of basal lamina is collagen IV [48]. Compared to other ECM components, collagen VI seems to be more specific for adipocytes and there is evidence of its contribution to the pathology of obesity-related disease [49, 50]. Importantly, even in mature adipocyte, ECM is under constant turnover. ECM remodelling is an energy demanding process mediated by a balance between various remodelling enzymes and their enhancers or inhibitor. It is regulated not only by mechanical forces but also by insulin, redox status of the cell and activity of ER [51].

This chapter summarised various function and anatomical location of adipose tissue. Two main types of adipose tissue, white and brown, were introduced. White adipose tissue has plethora of functions, but primarily it is dedicated to safe storage and release of lipids. Brown adipose tissue uses the chemical energy from lipids and glucose to produce heat. Adipose tissue is composed from adipocytes and stromal-vascular fraction which includes stem cells, preadipocytes, immune cells, endothelial cells and extracellular matrix. The physiological function of AT will be the subject of the next chapter.

1.2 Physiology of adipocytes and adipose tissue

Besides the thermogenic and mechanical protection, AT serves as a biological reservoir of calories that expands in response to overnutrition and releases lipids in response to lack of energy. Thus, two main metabolic processes – lipogenesis and lipolysis – help to maintain energetic demands of organism.

1.2.1 Lipogenesis

High fat and/or carbohydrate intake stimulate lipogenesis, a process of fatty acid and TAG synthesis. Fatty acids are synthesised from acetyl-CoA and TAGs are formed by esterification of free fatty acids to glycerol. The majority of fatty acids used for TAG synthesis in AT are derived from the diet.

1.2.1.1 Fatty acid transport

Dietary fat (TAG), when ingested with food, is absorbed by the gut. Because of hydrophobic nature of lipids, they are transported in plasma mostly as parts of specific lipoprotein complexes. Dietary lipids are transported by chylomicrons, whereas endogenous fat and cholesterol are carried by lipoproteins of different densities (very-low density lipoproteins: VLDL, intermediate-density lipoproteins: IDL, low-density lipoproteins: LDL, high-density lipoproteins: HDL). At the site of AT beds, fatty acids are liberated from these complexes through the action of lipoprotein lipase (LPL) that is secreted by adipocytes. Released fatty acids are bound by albumin and in this form they are available for the uptake by adipocytes where the TAG are resynthesized and stored in cytoplasmic lipid droplets (**Figure 4**).

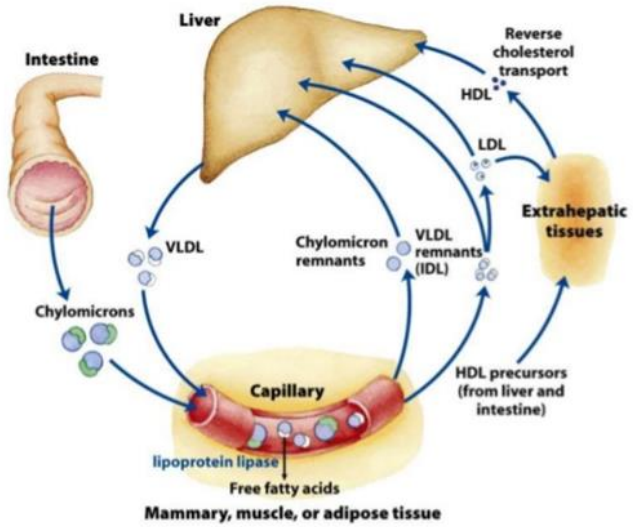


Figure 4: Systemic transport of lipids and lipoproteins. Dietary lipids are transported by chylomicrons, whereas lipids from liver are trasported to other tissues via VLDL. At the site of mammary, muscle or AT beds, fatty acids are liberated from lipoprotein complexes by the action of lipoprotein lipase. Then, free fatty acid bind to albumin (not shown) and can be uptaken inside the cell. Remnants of VLDL may transform to IDL or LDL, travelling back to the liver or became (in the case of LDL) another source of lipids for extragepatic tissues. HDL deliver cholesterol to the liver. Adapted from [4].

The transport of fatty acids across the adipocyte plasma membrane appears to be highly complex but still somehow elusive process. There is an evidence for two different (but not mutually exclusive) processes: diffusion and protein-mediated uptake. Proteins implicated in fatty acid transport are plasma membrane fatty acid binding protein (FABPpm) and fatty acid translocase (CD36). FABPpm is a 43 kDa protein which is expressed on the surface of various cell types, including adipocytes [52]. Evidence supporting the role of FABPpm in adipocyte fatty acid uptake came from the finding that anti-FABPpm antibodies selectively inhibit uptake of oleate in 3T3-L1 adipocyte without alteration of 2-deoxyglucose uptake [53]. CD36 is a transmembrane glycoprotein which belongs to a family of class B scavenger receptor. In addition to fatty acids, it is thought to bind a wide variety of hydrophobic molecules such as thrombospondin, sickle cell erythrocytes, collagen, apoptotic cells and oxidized LDLs [54, 55]. Except for FABPpm and CD36, also other proteins like caveolin-1, fatty acid transport protein (FATP) or acyl-CoA synthetase (ACSL) have been found to be functionally linked to fatty acid transport [56-58].

Once inside the adipocyte, fatty acids can be converted into acyl-CoA by acyl-CoA synthetase [56, 57], bound and sequestered by intracellular fatty-acid binding proteins, FABP4 and FABP5, and oriented into diverse metabolic pathways, including restoration of TAG.

1.2.1.2 Triglyceride formation

TAG synthesis involves the activation of three molecules of fatty acids through formation of acyl-CoA and then the synthesis of monoacylglycerol (MAG) and diacylglycerol (DAG) by reacting with glycerol-3-phosphate (G3P) [59]. In AT, the main source of G3P is catabolism of glucose via glycolysis since the activity of glycerokinase (GK), the enzyme that transforms glycerol into G3P, is low.

1.2.1.3 De novo lipogenesis

Biochemical process of fatty acid and TAG synthesis from non-lipid precursors is called *de novo* lipogenesis (DNL). DNL takes place primarily in the liver and AT [60, 61]. The contribution of AT to DNL was considered for a long time negligible, but newer methods have shown that DNL contributes to the synthesis of approximately 20% TAG [61].

Insulin, a peptide hormone secreted by the β cells of the pancreatic Langerhans islets, is a potent regulator of carbohydrate, lipid and protein metabolism [62]. At the level of adipocytes, insulin promotes all steps necessary for lipid synthesis. Therefore, before going further, insulin signalling will be described in brief. Insulin acts through insulin receptor that

becomes autophosphorylated and thus forms binding sites for docking proteins such as IRS1 [63]. Tyrosine phosphorylation of IRS1 is then necessary for the activation of phosphatidylinositol 3-kinase (PI3K)-AKT/protein kinase B (PKB) pathway, resulting in the translocation of glucose transporter 4 (GLUT4)-containing vesicles to the cell surface and glucose transport inside the cells [64]. Glucose can be then used in both, fatty acid as well as in TAG synthesis. Because carbohydrate metabolism, especially that of glucose, is the most commonly involved in the supplementation of carbon units for DNL, we will use glucose as an example of a primary source to describe DNL pathway.

Following glucose transport into adipocyte, glucose is converted via glycolysis into pyruvate. Pyruvate from cytosol enters mitochondria where it is converted into citrate by citrate synthase (CS). Citrate then passes through the inner mitochondrial membrane by the citrate transporter into the cytosol. There, citrate cleavage by ATP citrate lyase (ACLY) regenerates acetyl-CoA. Thus, ACLY represents a first enzyme of DNL, linking the metabolism of carbohydrates and the production of fatty acids. Acetyl-CoA then serves as a substrate for acetyl-CoA-carboxylase (ACC). This enzyme, containing a biotin prosthetic group, catalyses the conversion of acetyl-CoA into malonyl-CoA in two-step reaction. Malonyl-CoA is a substrate for fatty acid synthase (FAS), a multienzyme complex responsible for the formation of long carbon chains of fatty acids by a repetition of four-step sequence. A saturated acyl group produced by this set of reactions becomes the substrate for subsequent condensation with an activated malonyl group. With each passage through the cycle, the fatty acyl chain is extended by two carbons until the chain reaches 16 carbons length. Then the product (palmitate, 16:0) leaves the cycle.

The further destiny of palmitate can vary. In ER, palmitate can be elongated by fatty acid elongases (ELOVLs) and desaturated at the Δ9 position by stearoyl-CoA desaturase (SCD). In mice, four SCD isoforms (SCD1-SCD4) have been found, whereas only two SCD isoforms have been identified in human (SCD1 and SCD5) [65]. In human AT, only SCD1 isoform is expressed [66]. The presence of at least one functional isoform is critical, as SCD is a rate-limiting enzyme in the production of monounsaturated fatty acids, specifically oleate and palmitoleate [67]. Importantly, the degree of fatty acid unsaturation in cell membrane lipids determines membrane fluidity whose alteration has been implicated in a variety of diseases [68]. SCD1 has been found to be located in proximity with another DNL enzyme – diacylglycerol acyltransferase (DGAT) [69]. DGAT is enzyme that catalyses the final reaction in the synthesis of TAG. Two DGAT genes, *DGAT1* and *DGAT2*, have been identified [70, 71]. Although these enzymes catalyse similar reactions, their nucleotide and

amino acid sequence differ [72]. Studies performed on mice lacking DGAT1 (*DGAT1*$^{-/-}$) have suggested that DGAT1 does not have a profound effect on TAG metabolism in general and is not essential for life [73]. In contrast, mice with a disruption of the DGAT2 gene (*DGAT2*$^{-/-}$) have severely reduced TAG content in their tissues and survive only early postnatal periods [72]. These results demonstrate that DGAT2 is essential for the fundamental synthesis of TAG in mammals and is crucial for survival.

The overall pathway of DNL is depicted on **Figure 5**. The analysis of mRNA expression changes of major DNL enzymes in response to calorie deficit is described in the result part C of this thesis.

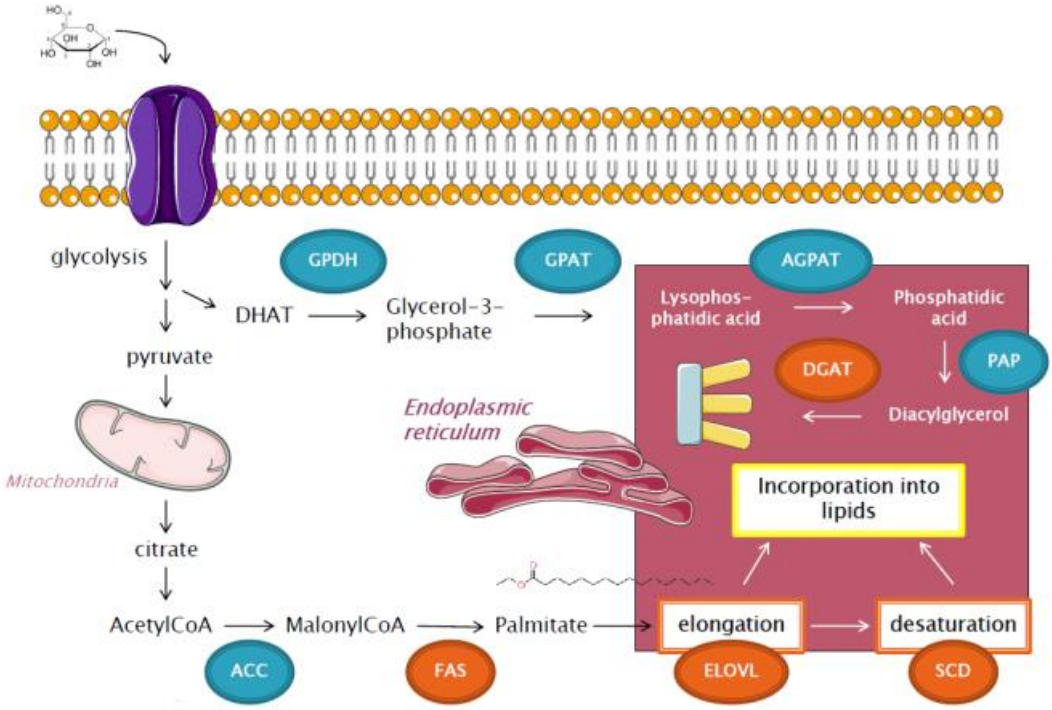


Figure 5: Simplified pathway of *de novo* lipogenesis. Glucose enteres inside the cell and undergoes glycolysis. Pyruvate enters mitochondria, where it is metabolised to citrate. In cytoplasm, citrate is converted to acetyl-CoA, metabolised by acetyl-CoA-carboxylase (ACC) into malonylCoA. MalonylCoA is utilised by fatty-acid synthase (FAS) to create palmitate, which enters into endoplasmic reticulum where it can be modified by elongase (ELOVL) or stearoyl-CoA-desaturase (SCD) and finally incorporated into various kind of lipids, including TAG. Glucose also serves as a main source of glycerol-3-phosphate, which subsequently provides backbone of TAG. TAG (triacylglycerides), DGAT (diacylglycerol acyl-transferase), GPDH (glycerophosphate dehydrogenase), GPAT (glycerol-3-phosphate acyltransferase), AGPAT (acylCoA acylglycerol-3-phosphate acyltransferase), PAP (phosphohydrolase).

1.2.1.3.1 Transcriptional regulation of de novo lipogenesis

As *master regulators* of DNL, two transcriptional factors have been identified – sterol regulatory element binding protein 1c (SREBP1c) and carbohydrate response element binding protein (ChREBP).

SREBP1c belongs to the basic-helix-loop-helix leucine zipper (bHLH/LZ) family of transcription factors and is synthesized as an inactive precursor bound to the membrane of the ER [74]. To become a mature transcription factor, SREBP1c must undergo proteolytic cleavage to liberate its N-terminal domain from the membrane. The cleaved fragment then moves to the nucleus to initiate transcription of target genes.

ChREBP (also known as MondoB or MLXIPL), similarly as SREBP1c, belongs to the family of bHLH/LZ transcription factor. Recently, a novel ChREBPβ isoform have been discovered [75]. ChREBPβ more potently induces DNL genes and its expression in human AT correlates with insulin sensitivity.

Interestingly, although both of these DNL transcriptional factors regulate expression of key lipogenic genes such as *FASN* or *ACC* [76], they are activated by different mechanisms. Experiments showed that SREBP1c gene expression is strongly stimulated by insulin [74, 77, 78], whereas ChREBP activity is regulated by glucose and other carbohydrates [79, 80]. Nevertheless, the distinct regulation and functional roles of SREBP1c and ChREBP in AT are still being worked out.

1.2.1.3.2 Relevance of de novo lipogenesis

Considering the huge quantities of lipids stored in adipocytes, DNL is unlikely to contribute essentially to the lipid mass of AT. Thus the intrinsic engagement of adipocytes in fatty acid synthesis raises the possibility that in comparison with their dietary counterparts, adipocyte-derived fatty acids may have unique functions beyond energy storage and may be involved in important biological processes.

Indeed, recent research revealed that lipids have wide-ranging actions as signalling molecule and that particularly the products of DNL can be essential for metabolic homeostasis.

Work of Cao et al. led to the identification of AT-derived lipokine palmitoleate (C16:1n7) which directly links DNL to beneficial systemic effects [81]. Similarly, another independent group has shown insulin-sensitizing effects of palmitoleate on muscle [82]. Considering the clinical research, numerous studies which assessed serum palmitoleate in humans have revealed both positive and negative associations with metabolically adverse

conditions [83-86]. These discrepant results could be related to the measurement of various forms of palmitoleate: while the free fatty acid form of palmitoleate seems to act as a lipokine with systemic beneficial effects, esterified palmitoleate probably loses this distinct function or may simply reflects the hepatic output [87].

Notably, palmitoleate is not the only mediator which provides systemic beneficial effects of elevated AT DNL. Recently, a novel class of AT-derived lipids was described: fatty acid-hydroxy-fatty acids (FAHFAs). FAHFAs were found to be elevated 16-18 fold mice which overexpress GLUT4 transporter in adipocytes and have elevated lipogenesis [88]. The concentration of one specific isomer of FAHFAs, consisting of palmitic acid and stearic acid (PAHSA), was correlated with improved insulin sensitivity. Remarkably, exogenous PAHSA treatment improved glucose tolerance and overall glucose metabolism in mice. In addition, PASHA administration exerted anti-inflammatory effects on AT-resident immune cells. Finally, PAHSA levels were reduced in the serum and subcutaneous AT of insulin resistant human subjects.

In addition to DNL products, many other lipid species as sphingolipids [89], cardiolipins [90] and prostaglandins [91, 92] also act as mediators of metabolism with both beneficial and deleterious effects and undoubtedly many more wait to be discovered.

1.2.2 Lipolysis

Lipolysis is a catabolic pathway that promotes fat mobilization from AT to peripheral tissues. Lipolysis involves hydrolysis of TAG that results in the release of fatty acids and glycerol into the circulation. Complete hydrolysis of TAG requires actions of three different lipases [93]. The first step, involving TAG hydrolysis into diacylglycerol (DAG) and one free fatty acid, is catalysed by adipose triglyceride lipase (ATGL). Next, hormone-sensitive lipase (HSL) cleaves DAG into MAG. Complete hydrolysis accomplishes monoacylglycerol lipase (MGL) by the conversion of MAG into fatty acid and glycerol.

1.2.2.1 Regulation of lipolysis

In response to changing metabolic conditions and nutrient intake, nutritional regulation of lipolysis occurs at multiple levels. Insulin inhibits lipolysis [94], whereas fasting and exercise acutely stimulate lipolysis and the primary activators are catecholamines (norepinephrine, epinephrine) [95, 96]. Norepinephrine binds to β-adrenergic receptors on the plasma membrane of adipocytes. These receptors are coupled with G_s-proteins that transmit a

stimulatory signal to adenylyl cyclase in order to generate cyclic adenosine monophosphate (cAMP). cAMP activates protein-kinase A (PKA) [97]. PKA phosphorylates HSL on multiple sites, which causes activation and subsequent translocation of HSL from cytosol to lipid droplet [98-100]. Except for HSL, PKA also phosphorylates perilipin, a major lipid droplet coating protein in adipocytes. As a result, perilipin moves away from the lipid droplet [101]. This increases the area on the surface available for lipolytic attack of the droplet [102]. Moreover, phosphorylated perilipin assists in the translocation of HSL from cytosol to lipid droplet [103].

Insulin is a suppressor of lipolysis and this regulation involves cAMP-dependent, as well as cAMP-independent mechanisms. cAMP-dependent suppression of lipolysis involves activation of phosphodiesterase 3B which degrades cAMP in adipocytes [104]. The second mechanism involves the stimulation of protein phosphatase-1. This enzyme, when activated, rapidly dephosphorylates and deactivates HSL, causing a fall in the rate of lipolysis [100, 105-107].

1.2.3 Secretory function of adipose tissue

AT functions as an active endocrine organ and releases multiple bioactive molecules known as lipokines [87] and adipokines [5, 108, 109]. Via these molecules, AT is able to communicate with distinctly located metabolically active organs and influence the systemic metabolism. As lipokines were already mentioned in the section below (1.2.1.3.2), the text will continue directly with adipokines.

1.2.3.1 Adipokines

A group of cytokines secreted by AT, i.e. adipokines, comprises today hundreds of molecules, including both anti-inflammatory (f.e. adiponectin) and pro-inflammatory mediators (f.e.tumor necrosis factor α: TNFα, monocyte chemoattractant protein 1: MCP1, interleukin 6: IL6). Key adipokines, their source and function are depicted in **Table 1**. Because issue of adipokines exceeds the scope of this thesis, only selected adipokines will be mentioned in more detail.

Adipokine	Primary source	Function
Adiponectin	Adipocytes	Insulin sensitizer, anti-inflammatory
Angiopoietin-like protein 2	Adipocytes, other cells	Local and vascular inflammation
C-X-C motif chemokine ligand 5	SVF cells (macrophages)	Antagonism of insulin signalling through the JAK-STAT pathway
Interleukin 18	SVF cells	Broad-spectrum inflammation
Interleukin 6	Adipocytes, SVF cells, liver, muscle	Changes with source and target tissue
Leptin	Adipocytes	Appetite control through the central nervous system
Lipocalin 2	Adipocytes, macrophages	Promotes insulin resistance and inflammation through TNF secretion from adipocytes
Monocyte chemotactic protein 1	Adipocytes, SVF cells	Monocyte recruitment
Nicotinamide phosphoribosyltransferase	Adipocytes, macrophages, other cells	Monocyte chemotactic activity
Retinol Binding Protein 4	Liver, adipocytes, macrophages	Implicated in systemic insulin resistance
Resistin	Monocytes/macrophages (human), adipocytes (rodent)	Promotes insulin resistance and inflammation through IL6 and TNF secretion from macrophages
Secreted Frizzled Related Protein 5	Adipocytes	Suppression of pro-inflammatory WNT signalling
Tumor necrosis factor	SVF cells, adipocytes	Inflammation, antagonism of insulin signalling

Table 1: Adipokines, their primary source and function. SVF (stromal vascular fraction). Adapted from [5].

Adiponectin

Adiponectin is almost exclusively produced by adipocytes and is present at high levels in blood (3-30 μg/ml) [108]. It has a collagen-like domain followed by a globular domain [5]. Adiponectin forms trimers, through collagen-like domain interactions, that can further associate to form stable multimeric oligomers (hexamers and a high molecular weight form). All three forms are detectable in the blood. Plasma levels of adiponectin are negatively associated with the accumulation of body fat, particularly visceral fat [110] and with plasma levels of IL6 and C-reactive protein (CRP) [111, 112], i.e. adiponectin levels in plasma are lowered in obese individuals with signs of systemic inflammation. On the other hand, bodyweight reduction in obese women through calorie restriction and lifestyle changes is associated with an increase in adiponectin levels. Thus, adiponectin is a unique adipokine expressed at the highest levels by the functional and insulin-sensitive adipocytes and downregulated in dysfunctional adipocytes frequently found in obese body [113].

Leptin

Leptin is the product of the obese gene (*ob*; also known as *lep*), which was identified in *ob/ob* mice by the method of positional cloning [114]. This adipokine is important in regulating feeding behaviour through central nervous system. Leptin levels, contrary to adiponectin levels, correlate positively with adiposity and thus, leptin functions as a measure of long term energy reserves. The *ob/ob* mice, which lack leptin, suffer from hyperphagia leading to obesity and insulin resistance [115]. Administration of recombinant leptin in leptin deficient animals or humans reverses these changes. Nevertheless, in diet induced obesity, increased leptin levels do not exert the expected anorexic responses that would be capable to prevent further weight gain which indicates the occurrence of leptin resistance [116].

In addition, leptin has multiple roles in the immune system. It is an inflammatory molecule that is capable of activating both adaptive and innate immunity [117].

Interleukin 6

IL6 belongs to a group of pro-inflammatory cytokines. It is estimated that AT produces approximately one-third of total circulating IL6 [118]. Clinically, plasma IL6 levels positively correlate with the degree of adiposity in human populations [118] and weight loss leads to a reduction in IL6 levels [112, 119]. Plasma levels of IL6 are also increased in type 2 diabetic patients and elevated IL6 plasma concentration predicts the development of type 2 diabetes [120]. Increased secretion of IL6 may disturb proper insulin signalling [121] and

thus diminish not only glucose uptake by insulin-sensitive tissues but also increase AT lipolysis, which can contribute to ectopic lipid accumulation and lipotoxicity [122].

In healthy AT, the expression of anti-inflammatory and pro-inflammatory adipokines is balanced. However, in most obese humans, the expression of pro-inflammatory adipokines predominate and promote insulin resistance which is manifested by decreased insulin-stimulated glucose transport and metabolism in adipocytes and skeletal muscle and by impaired suppression of hepatic glucose output [123]. In addition to pro-inflammatory adipokines, various pathological conditions may disturb well-ordered AT milieu. Next chapter will introduce the global problem of obesity and analyse some of the possible culprits AT dysfunction.

Adipocytes are specialised to safely energy storage and release. This is due to lipogenesis and lipolysis. Lipogenesis is the process of fatty acid and triglyceride synthesis. The term ***de novo lipogenesis*** *means fatty acid formation from non-lipid precursors. These precursors come from a various cellular pathways, most commonly from carbohydrate metabolism. Lipolysis is the opposite process, responsible for the breakdown of triglycerides stored in lipid droplets. Newly synthetised and released lipids can serve as signalling molecules. In addition to lipokines, adipocytes and others cells of adipose tissue secrete plethora of molecules called adipokines with anti-inflammatory or pro-inflammatory properties. However, pro-inflammatory environment, which is often seen in obesity, can disrupt proper functions of adipocytes and adipose tissue.*

1.3 Pathophysiology of adipocytes and adipose tissue

1.3.1 Obesity

Conditions of excess calorie intake and prolonged food abundance result in the excessive storage of fat, eventually leading to obesity, defined as *body mass index (weight in kilograms divided by the square of the height in m2)* over 30 kg/m^2 [124, 125].

World Health Organisation (WHO) reported that since 1980 obesity has doubled and now kills more people than undernourishment. In 2014, more than 1.9 billion adults were overweight (BMI 25-30 kg/m2) and over 600 million were obese. The alarming point is that obesity spreads rapidly not only among adults, but also in children and the elderly. This poses a major public health issue, since obesity is a major contributor to the global burden of chronic diseases [126]. In fact, obesity is associated with an increased risk of developing insulin resistance [127] and is a major risk factor for many diseases such type 2 diabetes (which is predicted to become the 7^{th} cause of death in 2030) [128, 129], atherosclerosis, hypertension, stroke, depression, infertility, obstructive sleep apnoea and several types of cancer [130, 131].

Several decades of research have brought a huge amount of evidence that a cornerstone in the pathogenesis of obesity-related diseases is indeed a dysfunction of AT.

1.3.2 Adipose tissue dysfunction

1.3.2.1 Insulin resistance

AT and adipocyte dysfunctions play a crucial role in the pathogenesis of obesity-related insulin-resistance, the most commonly known metabolic complication of obesity [132]. Insulin resistance is defined clinically as the inability of a known quantity of exogenous or endogenous insulin to increase glucose uptake and utilisation in an individual as much as it does in a normal population [133]. Disability to react on insulin stimuli leads inevitably to deterioration of adipocyte metabolic function. On the body level, insulin resistance manifests itself as a hyperinsulinemia and dyslipidaemia, accompanied by hyperglycaemia in postprandial state.

1.3.2.2 Adipocyte hypertrophy

In obesity, AT expands in order to safely store lipids. This can occur through two processes: recruitment of new adipocytes (*hyperplasia*) or/and expansion of existing

adipocytes in size (*hypertrophy*). Hypertrophic, rather than hyperplastic, obesity is known to be related to insulin resistance and metabolic syndrome [134-136] and is an independent predictor for development of type 2 diabetes [137, 138].

Therefore, it was hypothesized that adipocytes are able to effectively and safely store lipids only until they reach certain size. This "critical" size is probably related to the degree of various stress conditions affecting expanding adipocytes, including increased mechanical stress, hypoxia, mitochondria stress and associated production of reactive oxygen species (ROS) and ER overload [139]. It was suggested that these stresses contribute to the deterioration of basic physiologic function of adipocytes, i.e. lipogenesis, and at the same time they initiate processes leading to higher production of pro-inflammatory adipokines and eventually to cell death by apoptosis [126]. Furthermore, enlarged adipocytes of obese subjects are resistant to antilipolytic effect of insulin. Deregulated lipolysis leads to an increased release of fatty acids into the circulation [140], resulting in the ectopic deposition of lipids in nonadipose tissues (where lipids cause so called "lipotoxicity") and the promotion of systemic insulin resistance.

As one of the aims of this thesis is dedicated to the effects of ER stress on adipocytes, ER stress is described in greater details in following subchapter.

1.3.2.3 Endoplasmic reticulum stress

The ER is a type of specialized cytosolic organelle in which various metabolic signals and pathways are integrated to regulate lipid, glucose, cholesterol and protein metabolism. The ER is a principal site of synthesis of all secretory and integral membrane proteins. Within the lumen of the ER, protein chaperones such as BiP, known also as glucose regulated protein 78 (GRP78), or heat shock protein family A member 5 (HSPA5), calnexin and calreticulin assist in the proper folding of *de novo* peptides and prevent the aggregation of unfolded or misfolded precursors. Once folded into the right conformation, the proteins are released to the Golgi for final modifications and transported to their cellular destinations. In addition to protein synthesis, the ER is also the site of lipid biosynthesis and triglyceride droplet formation [141-143].

When the ER function becomes insufficient or is chronically disturbed, the accumulation of unfolded proteins creates ER stress. Various factors have been reported to contribute to ER stress [144]. For example, disturbances in cellular redox regulation (as a consequence of oxidants), hypoxia or reducing agents affect the formation of protein disulphide bonds in the ER lumen, which can lead to improper protein folding. Deprivation of

glucose disrupts N-linked protein glycosylation in the ER and disruption of Ca^{2+} impairing the functions of Ca^{2+}-dependent chaperons, including HSPA5. Importantly, high fat diet was also shown to cause ER stress [145].

In obesity, the capacity of adipocyte for protein and lipid synthesis is challenged and has to be enhanced to meet the increased demands. ER stress activates a stress response signalling network known as the unfolded protein response (UPR). The overall goal of the UPR is to restore the normal functions of the ER and therefore the functions of the cell [146]. The UPR acts via three signalling arms or branches, denoted by three stress-sensing proteins found in the ER membrane: PKR-like eukaryotic initiation factor 2a kinase (PERK), inositol-requiring enzyme 1 (IRE1) and activating transcription factor 6 (ATF6).

These transmembrane proteins are normally bound in ER by the chaperone HSPA5 in their intraluminal domains. When client (i.e. unfolded proteins) bound by HSPA5 with higher affinity than UPR sensors accumulates within ER, less HSPA5 is available for binding UPR sensors. Without HSPA5 anchor, free PERK and IRE1 auto-oligomerize and undergo autophosphorylation, which leads to the activation of downstream signalling. ATF6 is released to the Golgi where it undergoes two subsequent proteolytic cleavages, thereby producing an active transcription factor.

Immediate result of PERK activation is repression of protein translation through inhibitory phosphorylation of eukaryotic translational factor 2α (eIF2α) (at serine 51). This phosphorylation also results in a selectively increased translation of several target genes including *ATF4*, which induces expression of many genes including those involved in ER redox control [endoplasmic reticulum oxidoreductase 1 (*ERO1*)] [147], inflammation [148], apoptosis [C/EBP homologous protein (*CHOP*)] [149], and the negative feedback release of eIF2α inhibition [growth arrest and DNA damage-inducible protein (*GADD34*)] [150].

In addition to nonselective inhibition of *de novo* protein synthesis that minimizes the acute ER burden, the UPR induces the production of chaperones to assist with the unfolded protein load. Activated ATF6 translocates to the nucleus where it enhances transcription of chaperones genes such as *HSPA5*, *CALR*, and *GRP94* [151, 152]. At the same time, the process of ER-associated degradation (ERAD) is upregulated to facilitate the clearance and degradation of excess client proteins from the ER lumen. This is mediated by ATF6 induced expression of ER degradation-enhancing α-mannosidase-like protein (EDEM), which is involved in this process. Importantly, ATF6 upregulates X-box protein 1 (*XBP1*) mRNA, which is further processed and specially regulated by the IRE1 response arm [153].

IRE1 activation by the UPR contributes to the increase in protein chaperone content, ER biogenesis and enhanced secretory capacity through the activation of XBP1. IRE1 acts as an endoribonuclease and cleaves a 26bp segment out of the mRNA of XBP1, creating a spliced mRNA which is translated into an active form of the transcription factor (XBP1s) [153]. XBP1s, in turn, induces the expression of chaperones and proteins involved in ER biogenesis and secretion. Thus, XBP1s is a key element of one of the major pathways regulating ER function and folding capacity.

The process of the UPR activation and its consequences on cell function is depicted in **Figure 6**.

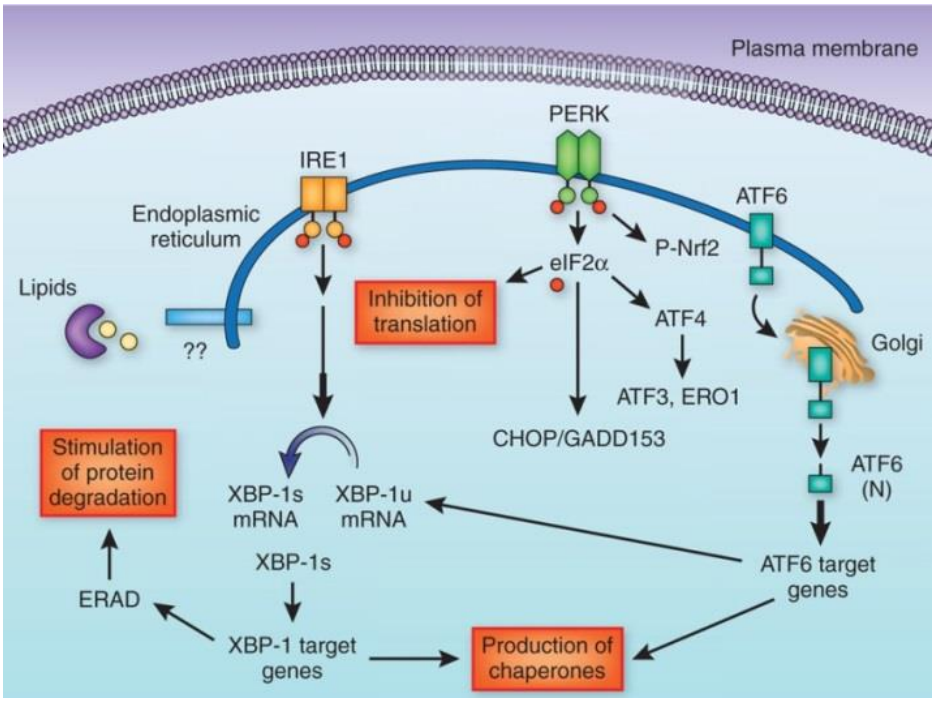


Figure 6: Activation of unfolded protein response. UPR is initiated through three paralelle signaling arms: IRE-1, PERK and ATF6. UPR activation stimulates protein degradation, selectively attenuates protein synthesis and increases the production of chaperons. When the ER stress is not resolved, the cell can undergo apoptosis. Adapted from [6].

In addition to the effort to restore the ER homeostasis, ER stress-induced IRE1 phosphorylation leads to the recruitment of tumor necrosis factor receptor-associated factor 2 (TRAF2) and apoptosis signal-regulating kinase 1 (ASK1) to the cytosolic leaflet of the ER membrane [154]. This leads to the phosphorylation and activation of c-Jun-N-terminal kinase (JNK). JNK activity may lead to a variety of downstream effects depending on the cellular context, some of which include apoptosis, cell survival, insulin resistance and inflammation.

Since the effect of ER stress on differentiation of preadipocytes and lipogenesis remains unknown, we have tried to shed more light into this topic in **Project A and B**.

1.3.2.4 Inflammation

Obesity is accompanied by chronic low-grade inflammation. This inflammation differs from "classical" inflammation, as there are no typical signs of inflammation as rubor (heat), calor (pain), dolor (redness) and tumor (swelling). On the other hand, the pro-inflammatory mediators and signalling pathways are the same for both types of inflammation [155].

In obesity, stressed or damaged cells release damage-associated molecular patterns which are sensed by pattern-recognition receptors, thereby inducing inflammation [156]. For example, free fatty acids released from hypertrophied adipocytes can report, as a danger signal, their diseased state to macrophages via Toll-like receptor 4 (TLR4) complex. Similarly, production of pro-inflammatory cytokines by dysfunctional adipocytes attracts and activates immune cells. Stressed hypertrophic adipocytes produce TNFα that stimulates preadipocytes and endothelial cells to secrete MCP1 [157]. MCP1 belongs to one of the critical factors attracting macrophages to adipocytes [158]. Thus, as obesity develops, AT became progressively infiltrated by macrophages [157, 159]. Increased secretion of leptin (and/or decreased production of adiponectin) by adipocytes may also stimulate adhesion of macrophages to endothelial cells [160] and infiltration of macrophages into AT [161]. Local proliferation of macrophages also contributes to obesity-associated AT inflammation [162]. Whatever the initial stimulus to recruit macrophages into AT is, once these cells are present and active, they could perpetuate along with adipocytes and other cell types a vicious cycle of macrophages recruitment, production of inflammatory cytokines and impairment of adipocyte function [163].

Macrophage population and function are highly heterogeneous, depending on the surrounding environment. This has led to their characterization and classification. As noted earlier (chapter *1.1.3.2.2 Immune cells*), macrophages can exert M1 phenotype, identified as the pro-inflammatory or "classically"-activated state, secreting various pro-inflammatory cytokines (TNFα, IL6, IL1b) and the M2 phenotype referred as to the anti-inflammatory or "alternatively"-activated state, which is characteristic by the production of IL10, remodelling capacities and lipid handling [164]. It has been shown that macrophages recruited during a diet-induced obesity exhibit an inflammatory M1 profile compared to resident AT macrophages [165].

The majority of these macrophages aggregate in so-called "crown-like structure", which surround dead adipocytes and serve to scavenge adipocyte debris [166]. In rodents,

these macrophages can comprise up to 40% of the cells in obese adipose tissue [159]. These accumulated macrophages are the further source of pro-inflammatory cytokines [167, 168].

Although the rise in macrophage population was recognized as first, the metabolic state of AT alters the composition of other immune cell populations. Neutrophils participate in the progression of immune response by facilitating the recruitment of macrophages, dendritic cells and lymphocytes [43]. Contrary, obesity is linked with the reduction in eosinophil numbers [46].

Pro-inflammatory cytokines (such as IL6 and TNFα) and saturated free fatty acids belong to the major contributors to the establishment of insulin resistance [169, 170]. These factors activate various ser/thr kinases such as nuclear factor-κB (NF-κB) signalling pathway, JNK or PKC [170, 171].

NF-κB is normally kept in the cytoplasm by the inhibitor of κB (IκB). Activation of IKK leads to phosphorylation of IκB and subsequent release of NF-κB which translocates to the nucleus. As a transcriptional factor, NF-κB binds to the promoters of pro-inflammatory genes and initiates transcription of its target genes [172].

Activation and phosphorylation of JNK leads to the phosphorylation of c-Jun homodimers. Upon phosphorylation, c-Jun binds c-Fos in order to form c-Jun-c-Fos heterodimeres. These heterodimers bind to promoter sequences of proinflammatory genes and activate their transcription. JNK activation also directly inhibits insulin signalling pathways through serine-threonine phosphorylation of IRS1 [173]. The consequences of this phosphorylation event are numerous. Interaction of IRS1 with the insulin receptor is blocked, tyrosine phosphorylation is prevented and IRS1 can be marked for proteasome-mediated degradation [174]. The simplified pathway from macrophages accumulation in obese AT to insulin resistance is depicted on **Figure 7**.

Notably, proinflammatory cytokines not only interfere with insulin signalling, but also induce cellular senescence. TNFα, interferon (IFN) γ and β induce cellular senescence in epithelial cells by producing ROS and activating the ATM/p53/p21 signalling pathway [175]. C-X-C Motif Chemokine Receptor 2, a chemokine receptor, induces cellular senescence of fibroblast [176]. Thus, imbalance of pro- and anti-inflammatory factors in obese AT additionally supports the accumulation of senescent cells, which is considered as a hallmark of aging [177, 178].

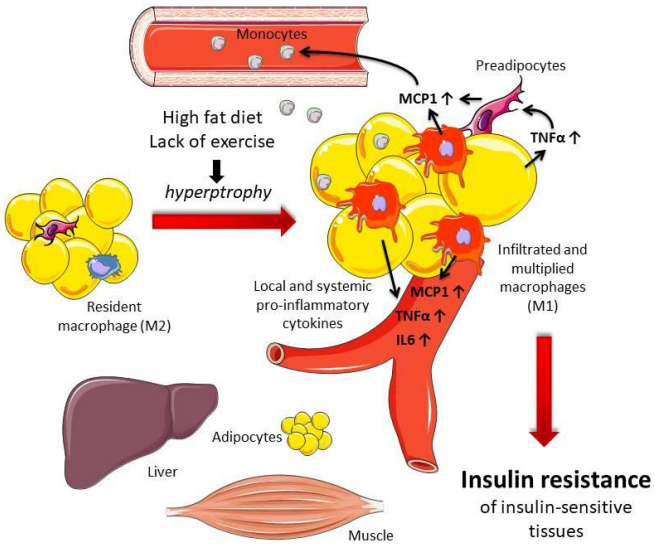


Figure 7: Obesity-induced macrophage infiltration into AT causes insulin resistance. In AT in a lean state, most resident macrophages are M2 macrophages that contribute to insulin sensitivity. High fat diet and/or lack of exercise cause hypertrophy of adipocytes. These produce TNFα making preadipocytes to secrete MCP1 into the circulation, leading to the recruitment of circulating monocytes to AT. These infiltrated monocytes differentiate into activated M1 macrophages, which robustly secrete proinflammatory cytokines such as TNFα, IL6, and MCP1, thus contributing to local low-grade inflammation. At the same time, these cytokines are transported through the bloodstream causing insulin resistance in insulin-sensitive tissues.

1.3.2.5 Senescence

Cellular senescence is defined as an irreversible growth arrest that occurs in response to various cellular stressors, such as telomere shortening, DNA damage, oxidative stress or oncogenic activation [179]. Thus, depending on the type of stressor, several "subtypes" of cellular senescence have been identified [178]. These include:

- Oncogene-induced senescence
- Stress-induced premature senescence
- Replicative senescence

In obesity, senescent cells could be classified within stress-induced premature senescence. Irrespective of the origin, all senescent cells adopt several unique characteristics (**Figure 8**), which includes large and flattened morphology in culture, upregulation of cell cycle inhibitors such as p16, p21 and p53, accumulation of DNA damage foci, higher production of ROS and the shift of pH optimum for lysosomal senescent associated β-galactosidase (SAβgal) activity [180, 181]. This activity is based on the increased lysosomal content of senescent cells, which enables the detection of lysosomal βgal at a suboptimal pH (pH 6.0) [182]. In fact, histochemical detection of β-galactosidase activity at pH 6.0 (referred to as senescence associated-βgal) is probably the most widely used assay for senescence detection [183]. Nevertheless, to define senescence both in culture cells and in tissues, a collection of markers in combination should be used [184].

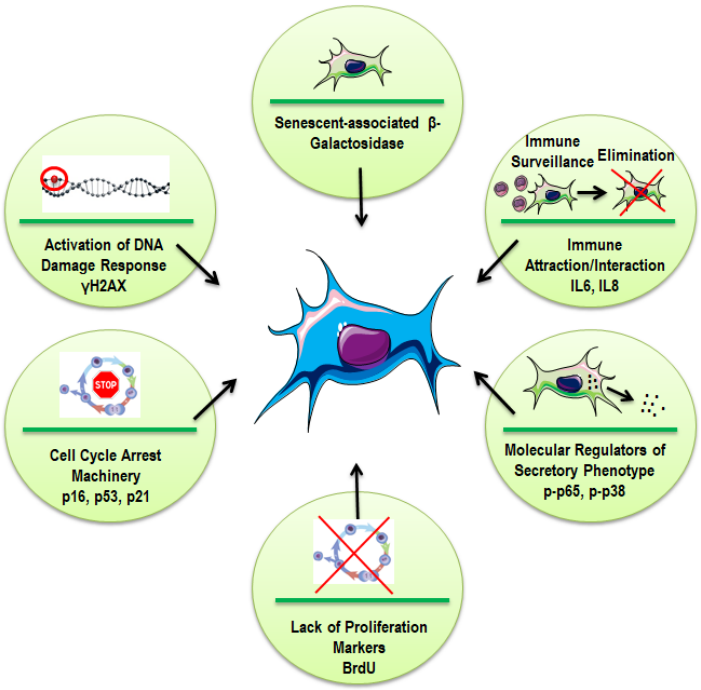


Figure 8: Characteristics of senescent cell are represented by the use of several molecular markers. Such molecular markers can represent expression of senescent-associated β-galactosidase, expression of secretory factors (e.g. IL6, IL8), the activation of immune surveillance-related genes and possible regulators for their pro-survival response (p-Akt, p-Erk, not shown), the activation of the pathways that regulate the secretory phenotype (e.g. p-p65 or p-p38), lack of cellular proliferation [e.g. lack of bromodeoxyuridine (BrdU) incorporation], the cell cycle arrest machinery (e.g. p16, p53, p21), activation of the DNA damage response [e.g. histone H2AX phosphorylation (γH2AX)]. Inspired by [3].

Although senescent cells are unable to divide, they are metabolically active. This high metabolic activity is pointed to a complex pro-inflammatory response known as senescence-associated secretory phenotype (SASP) [185-188] The SASP is mediated by the transcription factors NF-κB and C/EBPβ and includes the secretion of pro-inflammatory cytokines (IL6, IL8), chemokines and macrophage inflammatory proteins (MCP1, macrophage migration inhibitory factor), growth factors (TGFβ), granulocyte-macrophage colony-stimulating factor (GM-CSF) and proteases [178]. The secretion of these pro-inflammatory agents by senescent cells causes inflammation that, at least in some cases, may be pivotal for the clearance of senescent cells by phagocytosis [189, 190].

1.3.2.5.1 Senescence in the context of adipose tissue

Interestingly, even a low absolute number of senescent cells in a tissue may be able to exert systemic effects through the SASP [179]. In this way, senescent cells within AT could contribute to chronic low-grade inflammation in obesity. Furthermore, senescent cells can also trigger senescence in neighbouring cells through SASP component, most notably TGFβ, through a mechanism that generates ROS and DNA damage [191-193]. Thus, the accumulation of senescent cells within obese AT could represent another important step in development and progression of type 2 diabetes [194, 195].

Preadipocytes belong to the cells known to be susceptible to the development of cellular senescence [196, 197]. Intriguingly, it was shown that the abundance of senescent preadipocytes is greater in obese subjects compared with lean age-matched counterparts, even

in young individuals and this burden can be over 30-fold more in extremely obese subjects [196]. Moreover, AT SA-βgal activity and p53 increase with BMI [196]. Abundance of SA-βgal+ cells also increases in fat tissue in diabetes. Importantly, mRNA and protein expression of p53 and mRNA expression of p21 are increased in the fat cell fraction from subjects with diabetes [198]. Also, it was found that a higher level of DNA oxidation and a reduction in telomere length in AT of mice on a high-fat diet leads to the activation of the p53 pathway in adipocytes [199].This suggests that a senescent-like state might occur in also differentiated adipocytes, even though these cells are post mitotic and therefore would not fit the usual definition of senescence [196]. The exact functional consequences of the senescence in adipocytes remain rather elusive. However, when the adipocytes *in vitro* were exposed to doxorubicin, the drug frequently used to experimentally induce premature senescence, they exhibited deteriorated glucose uptake and increased lipolysis. All this suggests that obesity is linked with accelerated cellular aging within AT that could partly explain worsened AT functions. Therefore, a relationship of possible senescence-like state in adipocytes/AT and DNL was investigated in **Project B**.

1.3.2.5.2 Therapeutic clearance of senescent cells

Based on the above discussed facts, it was hypothesized that the clearance of senescent cells might provide a new possibility to treat obesity-associated dysfunctions. As noted earlier (*1.3.2.5.1 Senescence in the context of adipose tissue*), SASP itself is believed to attract immune cells, which carry out the clearance of senescent cells [189, 200]. However, the functions of immune system decline with aging, which lead to progressive accumulation of senescent cells [201-203]. Similarly, the effectivity of immune cells might be affected by components of the metabolic syndrome, including abdominal obesity, diabetes, hypertension, and atherosclerosis, which may be the reason for increased senescent cell burden [196, 198, 204]. Nevertheless, the role of immune cells in the clearance of senescent cells in these diseases is less clear. Up to now, clearance of senescence cells has been studied mainly on mice models of aging and this approach shows promising results. For example, the clearance of p16+ senescent cells (including those in AT) delayed ageing-associated disorders in a progeroid mouse model [205]. In humans, any possible treatment to remove senescent cells has not been approved yet. Nevertheless, new senolytic agents, which are small molecules selectively inducing death in senescent cells, are currently being investigated [206-208]. For example, ABT263 showed to be a potent senolytic agent and a specific inhibitor of the anti-apoptotic proteins BCL-2 and BCL-XL [207]. Oral administration of ABT263 to irradiated or

normally aged mice led to the effective depletion of senescent cells, including senescent bone marrow hematopoietic stem cells and senescent muscle stem cells.

Nevertheless, one should be careful with complete depletion of senescence cells, because although cellular senescence plays a negative role in the function of various tissue including AT, senescence is also involved in normal embryogenesis, development, wound repair and protection from the cancer [178]. Elimination of senescent cells generally remove only a portion of senescent cells, and the effect of removing all senescent cells from the tissue is unknown [209]. However, reduction of 30-70% of senescent cells does not appear to have detrimental effects on health in experimental animals [205, 210]. Thus, strategies targeting senescent cells could improve metabolic function of obese AT, as well as aged AT.

1.3.2.6 Aging

Similarly as obesity, chronological aging is associated with ectopic fat accumulation causing lipotoxicity [211], chronic-low grade inflammation [212] and indeed, with the accumulation of senescent cells.

With aging, extensive changes in preadipocyte function occur [196]. The *in vitro* replicative potential of preadipocytes declines, as well as subsequent adipogenic potential. Preadipocytes are more susceptible to lipotoxicity, because of reduced expression of enzymes required for fatty acids processing [213] and show pro-inflammatory phenotype [196]. Interestingly, these preadipocyte changes progress at different rates and to different extends depending on the fat depot. Age-dependent decline in replication and differentiation remains evident in most clones derived from single preadipocyte cultured in parallel from animals of different ages [214, 215].

Preadipocytes from old individuals have lower expression of C/EBPα, PPARγ and their target genes than in preadipocytes from young individuals [216-218]. Expression of these adipogenic transcription factors is also lower in serially passaged human preadipocytes when the cells are exposed to differentiation-inducing medium [219]. Interestingly already six population doublings is sufficient to detectably impair adipogenesis [220, 221]. Adipocyte metabolism (e.g. lipolysis) is negatively affected by aging as well [222].

Although processes triggered by obesity may accelerate aging of AT, aging per se can drive mechanistic pathways leading to aggravating of age-related dysfunction both independently and synergistically with obesity [223]. As mentioned, aging is associated with a pro-inflammatory state in metabolic tissues. However, age-related insulin resistance may be also differentially regulated. Bapat et al. performed comparative adipo-immune profiling that

revealed accumulation of T regulatory cells (T_{REG}) in aged AT, but not in obese AT [224]. Furthermore, mice deficient in T_{REG} specifically in fat area were protected against age-associated insulin resistance, but remained susceptible to obesity-associated insulin resistance. Finally, selective depletion of AT T_{REG} increased AT insulin sensitivity, although the exact mechanism remains unclear.

Together, many classical aging mechanisms, such as cellular senescence or chronic inflammation, occur in AT and are accelerated in obese AT. Therefore, interventions that target fundamental aging mechanisms should have beneficial effect on AT. One of possible interventions includes lifestyle changes. Next chapter is devoted to the problematic of diets and lifestyle interventions.

Obesity usually leads to pathophysiological behaviour of cells within adipose tissue. Adipocytes that expand by hypertrophy are exposed to various stressors. Unability to cope with calorie overload leads to endoplasmic reticulum stress and unfolded protein response. The activation of unfolded protein response through three arms (PERK, ATF6 and IRE1α) should resolute the endoplasmic reticulum stress. However, the outcomes may vary, depending on the severity of the stress. Usual consequence is the activation of inflammatory pathway. Adipocytes and surrounding cells start to release many pro-inflammatory cytokines to attract macrophages and other immune cells into adipose tissue. Inflammation, reactive oxygen species and other stressors can induce in neighbouring cells senescence, an irreversible growth cell arrest. Senescent cells are characterised, among others, by increased mRNA expression of p16 and p21, detection of β-galactosidase at pH 6.0 and senescence-associated secretory phenopyte. Adipose tissue of obese individuals favors the accumulation of senescent cells and especially preadipocytes are very susceptible to the development of senescence. Recent data suggest that senescent-like state might occur even in differentiated adipocytes. Today, no methods enabeling the removal of senescent cells have been approved in human. Therefore, senescent cells accumulate with age in many tissues, including adipose tissue. Hence, interventions that target fundamental aging mechanisms should have an effect on adipose tissue.

1.4 Obesity management

Typically, strategies for obesity treatment can be divided into the following categories:

- Non-pharmacological (diet and lifestyle intervention)
- Pharmacological (anti-obesity drugs such as orlistat and others)
- Surgical (bariatric surgeries)

Pharmacological treatment is recommended just for short-term use, because of the frequent side effects and rather low efficacy [225]. Today, Food and Drug Administration allowed only five pharmacological agents, which influence absorption of neurotransmitters or lipids [226]. Therefore, another option for the treatment of obese patients may be bariatric surgery. The success rate (usually defined as >50% excess weight loss, i.e. total weight loss divided by baseline excess weight, that is maintained for at least five years from the surgery) from surgical treatment ranges from 40% to >70% in the complex operations [227]. Nevertheless, because of inherent complications from surgeries and high cost, this option is usually considered only for morbidly obese patients or moderately obese individuals with comorbidities [228-230]. Moreover, patients undergoing any kind of bariatric surgery need lifelong medical monitoring and nutritional supplements. Therefore, hypocaloric diets and lifestyle modifications remain the key components of weight-reducing treatment of obesity and obesity-related disorders.

1.4.1 Dietary and lifestyle interventions

1.4.1.1 Hypocalorie diets

Very-low calorie diet (VLCD) is defined as a diet comprising the daily energy intake of 400-800 kcal per day (1600-3500 kJ). VLCD is usually administrated for 4-8 weeks and can result in significant weight loss [231-234]. The most striking decrease of weight is during the first week (2-8 kg), the later weight loss is moderate (maximally 2 kg/week) [235]. There is a number of commercially available VLCDs (Cambridge Weight Plan$_{TM,}$ Modifast, Optifast, Ultra Eat and Loose, Ultra Fit and Slim, Slim fast), frequently in the form of liquid diet. VLCDs are considered safe and effective when used under careful medical surveillance [236]. The diets are designed for patients with a BMI $\geq$30kg/m^2,i.e. subjects with increased risk of cardiovascular morbidity and mortality.

In contrast to VLCD, low-calorie diet (LCD) comprises of 800-1500 kcal per day (3500-5000 kJ), consists of natural foods and is commonly administrated for 2-3 months.

To avoid regaining the weight after the diet, weight-maintenance phases are important and represent the stabilization of body adaptation to a new energy balance [237]. This adaptation may be reached and evaluated in several months after the end of calorie restriction phase of diet.

It is worthy to mention that these diets performed under professional guidance are designed to reduce mainly the amount of fat mass without the reduction (or with the minimal reduction) of lean mass. While acute energy deficit of VLCD leads to the improvement of whole body metabolic parameters, AT demonstrate higher inflammation and decrease expression of metabolic genes [238-240]. However, after the long-term adaption to VLCD, the inflammation decreases under the prior values and expression of metabolic genes is normalised. However, no information is available on the very early reaction of AT to severe energy deficit. Therefore, in **Project C**, we compared AT response after 2 and 28 days of VLCD.

1.4.1.2 Lifestyle modification

Lifestyle modification includes three primary components: diet, exercise and behaviour therapy. The instructions are provided by trained interventionist, typically health professionals. Complex interventions are designed to induce a weight loss of approximately 0,5-1 kg/week for the first 12 weeks, with more gradual weight loss thereafter, until weight loss stagnates at 6-9 months [241].

1.4.1.2.1 Diet

The Obesity Guidelines recommend a deficit of 500-750 kcal per day (i.e. LCD as described above in *1.4.1.1 Hypocalorie diets*) in a diet to get loss of 0.5-1 kg per week [228]. Therefore, diets with 1200-1500 kcal/day and 1500-1800 kcal/day are often prescribed for women and men, respectively. The calorie intake can be adjusted accordingly if patients do not achieve the expected weight loss. Interestingly, the Guidelines also acknowledge the benefit of restricted consumption of a specific macronutrient to induce an energy deficit.

1.4.1.2.2 Physical activity

Lifestyle interventions typically prescribe 150-180 min per week of aerobic activity, such as brisk walking, or other types of moderate-intensity aerobic exercise [228]. Regular

aerobic activity is associated with many health benefits such as improvements in lipid levels, decrease of blood pressure and visceral fat [242] and improved fitness [243].

As physical activity per se (without dietary restriction) has no or only marginally effect on weight loss [244, 245], obese individuals should engage in physical activity for its cardiovascular benefits or as a prevention of sarcopenia in the elderly rather than its effects on weight [246].

1.4.1.2.3 Behaviour therapy

Behaviour therapy provides a set of strategies and techniques to modify diet and patters of physical activity [228]. The cornerstone of behaviour change is self-monitoring of food and calorie intake, along with recording physical activity and weight [241]. Individuals who engage in frequent self-monitoring of eating and weight achieve the largest weight losses [247-249].

1.4.1.3 Weight loss in the elderly

The general recommendations for weight loss and maintenance of a healthy body weight in order to improve quality of life and decrease obesity-related health risks are clear and definite in the young and middle age groups. However, there is a little evidence about application of these guidelines in the group of elderly. This represents a new challenge, because increased life expectancy and declining fertility has dramatic shifted the age structure worldwide [250]. Large populations are getting old and this has become a global social and major health burden. As a consequence of aging, a progressive decline in muscle mass and strength, collectively termed *sarcopenia*, develop [251]. The reported prevalence of sarcopenia varies widely depending on the definition and methods of assessment: it ranges from 8% to 40% of people aged over 60 years [250]. Sarcopenia results in frailty, loss of independence, physical disability and increased mortality in older adults [252, 253]. The combination of sarcopenia and obesity led to a term of *sarcopenic obesity* which is associated with more physical functional decline than simple obesity [254]. People with sarcopenic obesity may be more insulin resistant, and have a higher risk for metabolic syndrome and atherosclerosis than simply obese.

Today, there is a little evidence for outweigh of benefits over the risks from weight loss in the obese and possible sarcopenic elderly. Weight loss could aggravate further sarcopenia and frailty. Therefore, weight loss prescription should ensure treatment that avoid loss muscle mass and bone in the elderly [255, 256]. Comorbidities, frailty, sarcopenia, mobility and functional limitations and influence of social and living environments make the

practical recommendation for weight management very complex and challenging. Thus, further research should be done in this group of population. Indeed, a clinical study described in **Project D** was designed with the aim to clarify the effects of weight loss in the elderly.

Non-pharmacological strategies to obesity treatment include diets and lifestyle interventions. Very-low calorie diet comprises 400-800 kcal per day, wheather low-calorie diet 800-1500 kcal per day. Weight maintenance phase is aimed to stabilise adaptation of the body to new weight. Lifestyle interventions are more complex: apart from a diet include physical activity and behavioural therapy. Better recommendations for weight loss and maintenance of health body weight should be defined for the elderly, because little is known about the ratio benefits/risk from weight loss in this group liable to sarcopenia.

2 Aims

The overall aim of this thesis was to assess lipogenesis, the fundamental metabolic pathway in the adipocyte differentiation and physiology, in the context of ER stress, calorie restriction and aging. Therefore, four specific projects dedicated to this problematic were established, each with specific scientific aim (below).

PROJECT A:

Aim: To assess the impact of ER stress on differentiation and lipogenic capacity of human adipocytes.

PROJECT B:

Aim: To evaluate the effect of aging on lipogenic potential of human subcutaneous AT and adipocytes in relation to senescence and ER stress markers.

PROJECT C:

Aim: To compare the effects of 2 days and 28 days of VLCD on metabolic and inflammation-related indices in subcutaneous AT and their possible relationship with systemic inflammatory and metabolic status in moderately obese women.

PROJECT D:

Aim: To investigate and compare the effects of moderate calorie restriction on preadipocytes and adipocytes from young and elderly obese men.

3 Material and methods

3.1 Material

3.1.1 Cohorts, clinical investigation and intervention protocols

In all projects, volunteers were informed on the corresponding study and written informed consent was obtained before participation in the study. The studies were performed according to the Declaration of Helsinki and approved by the respective Ethical Committees.

Project A: Cells were derived from obese women that were recruited at the Third Faculty of Medicine of Charles University, University Hospital Kralovske Vinohrady, Czech Republic, and Toulouse University Hospitals, France.

Project B: Two groups of women (aged 36.6±1.8 and 72.1±1.32, n=15 in each group) were recruited at the Third Faculty of Medicine of Charles University and University Hospital Kralovske Vinohrady, Prague, Czech Republic. Exclusion criteria were diagnosed cancer, diabetes, cardiovascular diseases, liver and renal diseases. Long term medications to lower inflammation (anti-rheumatics and analgesics affecting cyclooxygenases, 100 mg of anopyrin daily was acceptable in the group of elderly). Subjects taking medication to lower cholesterol levels and blood pressure (representing 70-90 % of elderly population) were admitted to the study. Clinical investigation was performed after an overnight fasting. Anthropometric measurements and blood sampling were performed as previously described in [257].

Project C: Seventeen metabolically healthy obese women (aged 35±7.1, mean BMI 32.6±3.6 kg/m^2) were recruited for the study. All subjects were drug free and healthy, as determined by medical history and laboratory findings, and had a stable weight for at least 3 months prior to inclusion. All subjects underwent a VLCD intervention program, during which they received 800 kcal/d (liquid formula diet Redita; representing an intake of 52.0 g of protein, 118.0 g of carbohydrates, and 12.9 g of fat per day). Patients consulted a dietitian once a week. The evaluation of physical activity was performed before the start of the study by the International Physical Activity Questionnaire, and the subjects were recommended not to change their habitual activity during the study. Clinical investigation was carried out at day 0 (baseline), day 2 (2 d of the VLCD), and day 28 of the VLCD. During these investigations the subjects were examined at 8:00 AM after overnight fasting. Body weight and waist and hip circumferences were measured, and body composition was assessed by bioimpedance (QuadScan 4000; Bodystat). In addition to needle biopsy of subcutaneous AT, the samples of peripheral blood were taken.

Project D: Twenty two men (n=11 aged 35.8±0.7 and n=12 aged 64.5±1.1) were recruited by Centre d'Investigation Clinique Inserm – Hôpitaux de Toulouse (CIC). Exclusion criteria was obesity less than 2-3 years, type 1 or 2 diabetes, previous cardio-vascular disease or bariatric surgery, pathologies interfering with the realisation of planned physical activity, treatment by GLP1 agonists or insulin secretagogues and medication interfering with autonomous nervous system and lipid metabolism (diuretics, thiazides, beta-blockers and fibrates). Both groups underwent a dietary intervention, consisting of moderate calorie restriction (at least 20 % of everyday energetic needs) and 30-60 min of physical activity led by professional trainer least 5 times per week. The subjects consulted clinical specialist once a week by telephone.

3.1.2 List of used chemicals

List of used chemicals is depicted in **Table 2**.

Table 2: List of used chemicals. **Used in Project D, **used in Projects A, B, C.*

Chemical	Supplier	Country of origin	Used for
2-deoxy-D-[3H] glucose	PerkinElmer	USA	Transport of glucose
3,3,5-Triiodo-L-thyronine sodium salt (T3)	Sigma Aldrich	USA	Cell culture
Acetic acid sodium salt	Sigma Aldrich	USA	De novo lipogenesis
acetic acid sodium salt [1-14C]	PerkinElmer	USA	De novo lipogenesis
Bafilomycin A	Santa Cruz Biotechnology	USA	Induction of pH 6.0
BSA	Sigma Aldrich	USA	De novo lipogenesis, transport of glucose
C12 FDG	Thermo Fisher Scientific	USA	Analysis of βgal activity
Collagenase I	Serva	Germany	Isolation of primary cultures
Cortisol	Sigma Aldrich	USA	Cell culture
deoxy-D-glucose, 2-[14C(U)	PerkinElmer	USA	De novo lipogenesis
Dexamethasone	Sigma Aldrich	USA	Cell culture
DMEM/F12	Lonza Std	Switzerland	Cell culture
DMEM-no glucose medium*	Gibco	USA	De novo lipogenesis
FBS qualified for MSC	Thermo Fisher Scientific	USA	Cell culture
Gentamycin	Lonza Std	Switzerland	Isolation of primary culture
Glucose	Sigma Aldrich	USA	Transport of glucose
H2O2	Provided by hospital pharmacy	Czech Republic	Induction of senescence
Hepes	Lonza Std	Switzerland	Cell culture
Insulin	Sigma Aldrich	USA	Cell culture
Isobutylmethylxanthine	Sigma Aldrich	USA	Cell culture
Oil Red O	Sigma Aldrich	USA	Cell proliferation
Phosphate buffer saline (PBS)*	Gibco	USA	Washing or isolation of cells
Phosphate buffer saline (PBS)**	Lonza Std	Switzerland	Washing or isolation of cells
Quiazol Lysis Reagent	Qiagen	Germany	Isolation of RNA
rhEGF	Immunotools	Germany	Cell culture
rhFGF	Immunotools	Germany	Cell culture
Rosiglitazone	Cayman	Estonia	adipogenesis
Thapsigargin	Alexis	Switzerland	ER stress induction
Transferrin	Sigma Aldrich	USA	Cell culture
Trypsin in EDTA (0,05%)	Gibco	USA	Trypsinisation
Tunicamycin	LKT Laboratories, Inc.	USA	ER stress induction
Wst-1	Sigma Aldrich	USA	Cell proliferation

3.2 Methods

3.2.1 Explant cultures of adipose tissue

AT samples obtained by needle biopsy were washed in PBS and an aliquot of approx. 400 mg was cut into small pieces. The explants were incubated in 4 ml Krebs Ringer buffer (pH 7.4) supplemented with 20 g/l of BSA and 1 g/l of glucose at 37°C in a shaking water bath with air as the gas phase. After 4 hours of incubation the conditioned medium was collected, cellular debris was removed by centrifugation and the cell free supernatant was stored at -80°C until further analysis. IL6, IL8, IL10, MCP1 and TNFα were measure in conditioned media with Multiplex immunoassay at the MagPIX or Luminex 200 and leptin was measured with Human Adipocyte Kit according to manufacturer instructions (Merck-Millipore, USA).

3.2.2 Analysis of metabolites and cytokines in plasma

Plasma samples were prepared from uncoagulated peripheral blood by centrifugation. Plasma glucose and insulin were assessed in certified laboratories as a paid service. β-hydroxybutyrate, glycerol and free fatty acids were analysed by enzymatic colorimetric assays according to manufacturer guidelines (Randox Laboratories Ltd., Crumlin, UK). Multiplex immunoassay at the MagPIX or Luminex 200 was used to analyse plasma levels of IL6, IL8, IL10 and TNFα (High Sensitivity Human Cytokine Milliplex panel; Merck-Millipore, USA). Circulating levels of MCP1, leptin, FGF21, and CRP were quantified by ELISA kits according to manufacturer protocols (eBioscience, USA; RaD, USA).

3.2.3 Cell culture

3.2.3.1 Isolation and culture of stromal-vascular cells

Subcutaneous abdominal AT obtained by needle biopsy technique was cleaned from blood vessels and fibrous material, cut into small pieces (≤2 mm^3) and digested in 1.5 volume of collagenase (from Serva for **Projects A, B** and from Sigma Aldrich for **Project D**) diluted on 300 units/ml for 60 min in 37°C shaking water bath. Digested tissue was filtered through 250 μm strainer to get rid of undigested scraps, diluted with PBS/gentamycin and centrifuged at 1300 rpm for 5 min. Pellet containing cells from the stromal vascular fraction was incubated in erythrocyte lysis buffer for 10 min at room temperature. Cells were then filtrated through 100 μm strainer, centrifuged, resuspended in PM4 medium [258] with 132 nmol/L

insulin and plated into 35 mm Petri dish. PM4 was replaced every second day. Cells were subcultivated at 70% confluence. Experiments were performed at passage 3 or 4. Differentiation of 2-day post-confluent cells was induced by Dulbecco's modified Eagle's/F12 medium supplemented with 66 nmol/L insulin, 1 mol/L dexamethasone, 1 nmol/L T3, 0.1 µg/ml transferrin, 0.25 mmol/L isobutylmethylxanthine, and 1 µmol/L rosiglitazone. After 6 days, rosiglitazone and isobutylmethylxanthine were omitted and dexamethasone was replaced

with 0.1 µmol/L cortisol. The procedure is depicted on **Figure 9.**

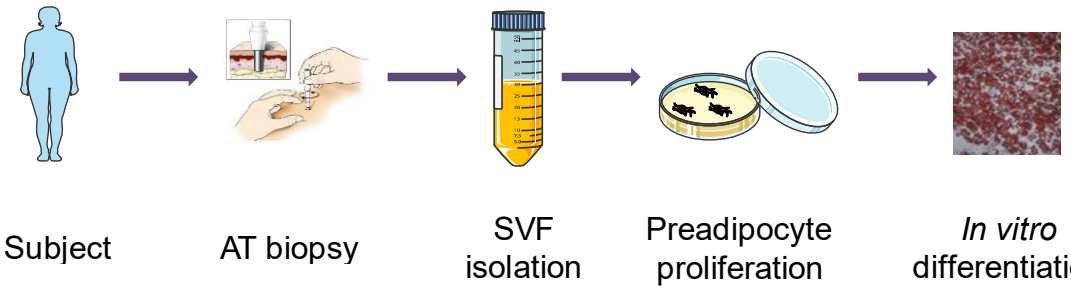


Figure 9: Isolation of stromal-vascular cells and culture. AT obtained by needle biopsy technique was digested with collagenase, enabeling dissociation of adipocytes and SVF. Culture media allowed the proliferation of preadipocytes and specific cocktail of factors their differentiation into adipocytes.

3.2.3.2 Wst-1 assay

Preadipocytes were seeded at the density of 2000 cells/cm^2 in 100µl PM4/well in 96-well plate in quadruplicates. After 6 hours of seeding, 10µl of *Wst-1* reagent was added into each well. Formazan formation was measured after 2 hours at 440 nm on spectrophotometer (Spectra MD, Dynex) and subsequently 48, 96 and 144 hours after seeding. Results are presented as a fold change of formazan production related to the day of seeding.

3.2.3.3 Sensitivity of preadipocytes to proliferative stimuli

Preadipocytes were seeded at the density of 10 000 cells/cm^2 and let grow for 2 days, when the confluence reached 20-40%. Then, cells were washed twice with PBS and serum/insulin starved in basal media with 0.1mg/mL transferrin overnight. The next day, preadipocytes were exposed to basal medium and PM4 for 10 min and collected for Western blot analysis.

3.2.3.4 Analysis of senescence

Preadipocytes were seeded at the density of 100 000 cells/well (for control/autofluorescence and P3/P4) or 300 000 cells/well (for peroxide treatment and P11).

The next day, cells were treated with 100 μM H_2O_2 for 2.5 hours. After the treatment, medium was replaced by fresh PM4 in all wells for 72 hours. At the day of experiment, cells were pretreated with 100 nM bafilomycin A1 for 1 hour in fresh medium supplemented with 2.5% serum. Next, C12FDG was added in the final concentration of 33 μM for 2 hours. Then, cells were washed twice with PBS, trypsinised, spinned at 300g for 5 min and resuspended in 500 μl of PBS. Cells were analysed on BD FACS Verse cytometer and BD FACSuite Software (BD Biosciences, USA). Two parameters were taken into account during the analysis: % of β-galactosidase positive cells (determined on FITC channel) and cellular size (determined by Forward Scatter). The threshold was determined according to a random subject (cells at P3/4 *after* the intervention) and the same threshold was then applied to all other subjects. Four populations were obtained: βgal negative cells, βgal positive cells, BIG βgal positive cells and BIG cells. Non-treated cells were used as a negative control and preadipocytes treated with H_2O_2 and preadipocytes at passage 11 served as positive controls. The number of non-senescent and senescent populations was expressed as percentage of gated events. Background was set up to 2.5 % of positive autofluorescence cells.

3.2.3.5 Sensitivity of adipocytes to insulin

Experiment was performed on differentiated adipocytes at day 13. Cells were washed twice with PBS and serum/insulin starved in basal media with 0.1mg/mL transferrin overnight. The next day, adipocytes were exposed to basal medium supplemented with or without 100nM insulin for 10 min and collected for Western blot analysis.

3.2.3.6 Oil-Red-Oil staining

Differentiated adipocytes were fixed directly by adding 10% formaldehyde in culture media for 10 min, washed once with PBS and fixed for another 20 min. Cells were washed properly with PBS and 60% isopropanol and stained for 20 min with 60% Oil Red O. Then the excessive Oil Red O stain was removed by washing with tap water and cells were photographed. Oil Red O was then eluted with 100% isopropanol and absorbance of eluates was measured at 500 nm (Spectra MD, Dynex). To normalize the data, standard curve from Oil Red O stock solution was used.

3.2.4 Gene expression

3.2.4.1 RNA isolation

AT samples obtained by needle biopsy were washed in PBS and separated to several aliquots. Two aliquots (200-500 mg) were snap frozen in liquid nitrogen for subsequent gene

expression analysis. RNeasy lipid tissue RNA minikit (Qiagen, Germany) was used to isolated total RNA from AT and RNeasy Mini kit (Qiagen, Germany) was used to isolate total RNA from *in vitro* cultivated cells. The RNA concentration was measured using Nanodrop1000 or 2000 (Thermo Fisher Scientific, USA).

3.2.4.2 Gene expression analysis

To remove genomic DNA, deoxyribonuclease I (Invitrogen, USA) treatment was applied. Total RNA was reverse transcribed using a high-capacity cDNA reverse transcription kit (Applied Biosystems, USA). Equivalent of 5 ng of RNA was then used for Real Time PCR reactions using TaqMan® Fast Advanced Master Mix (Applied Biosystems, USA) or by Power Sybr Green Master Mix (Applied Biosystems, USA). All samples were run in duplicates. TaqMan Gene expression assays (Applied Biosystems, USA) are listed in **Table 3**. For aP2, XBP1 full-length and spliced variants, sequences of primers are depicted in **Table 4**.

Table 3: List of TaqMan Gene expression assays. Names of proteins coded by detected mRNA, their function and involvement in the Projects A, B, C or D are depicted.

Gene symbol	Assay ID	Protein	Function, notes	Project
Adipogenesis				
ADIPOQ	Hs00605917_m1	Adiponectin	Control of fat metabolism and insulin sensitivity	D
APOM	Hs00219533_m1	Apolipoprotein M	Involved in lipid transport	D
CEBPA	Hs00269972_s1	CCAAT/Enhancer Binding Protein Alpha	Transcription factor, coordinates adipocyte differentiation	D
CEBPB	Hs00270923_s1	CCAAT/Enhancer Binding Protein Beta	Transcription factor, coordinates adipocyte differentiation	D
CEBPD	Hs00270931_s1	CCAAT/Enhancer Binding Protein Delta	Transcription factor, coordinates adipocyte differentiation	D
DLK1	Hs00171584_m1	Delta Like Non-Canonical Notch Ligand 1	Adipocyte differentiation	D
FABP4	Hs01086177_m1	Fatty acid protein binding 4	Lipid transport in adipocytes	D
GPD1	Hs00193386_m1	Glycerol-3-Phosphate Dehydrogenase 1	Links carbohydrate metabolism and lipid metabolism	D
LEP	Hs00174877_m1	Leptin	Regulation of energy balance and body weight control	C, D
PPARγ	Hs01115513_m1	Peroxisome proliferator-activated receptor gamma	Master regulator of adipogenesis and glucose and lipid metabolism of adipocytes	A, C
PPARγ	Hs00234592_m1	Peroxisome proliferator-activated receptor gamma	Master regulator of adipogenesis and glucose and lipid metabolism of adipocytes	D
Lipogenesis				
ACC=ACACA	Hs01046047_m1	Acetyl-CoA carboxylase alpha	Synthesis of malonyl-CoA	B, D
ACLY	Hs00982738_m1	ATP citrate lyase	Synthesis of acetyl-CoA	D
DGAT1	Hs01017541_m1	Diacylglycerol O-Acyltransferase 1	Re-esterification of fatty acids	C
DGAT1	Hs00201385_m1	Diacylglycerol O-Acyltransferase 1	Re-esterification of fatty acids	D
DGAT2	Hs01045913_m1	Diacylglycerol O-Acyltransferase 2	Triglyceride synthesis	A, B, D
ELOVL6	Hs00225412_m1	Elongation of very long chain fatty acids protein 6	Elongation of 2 carbons to the chain of long- and very long-chain fatty acids	B, C
ELOVL6	Hs00907564_m1	Elongation of very long chain fatty acids protein 6	Elongation of 2 carbons to the chain of long- and very long-chain fatty acids	D
FASN	Hs01005622_m1	Fatty acid synthase	Synthesis of palmitate	A, B, C
FASN	Hs00188012_m1	Fatty acid synthase	Synthesis of palmitate	D
CHREBP	Hs00263027_m1	Carbohydrate-Responsive Element-Binding Protein	Transcription factor regulating de novo lipogenesis and glucose metabolism	C
ChREBP	Hs00975714_m1	Carbohydrate-Responsive Element-Binding Protein	Transcription factor regulating de novo lipogenesis and glucose metabolism	D
SCD1	Hs01682761_m1	Stearoyl-CoA desaturase 1	Catalyses the insertion of a double bond into fatty acyl-CoA substrates	A, B, D
SREBP1c	Hs01088691_m1	Sterol regulatory element binding protein 1c	Transcription factor regulating de novo lipogenesis and glucose metabolism	A, C
SREBP1c	Hs01088679_g1	Sterol regulatory element binding protein 1c	Transcription factor regulating de novo lipogenesis and glucose metabolism	D

Oxidation				
ACADM	Hs00936580_m1	Acyl-CoA Dehydrogenase	First step of β-oxidation pathway of medium long chain-fatty acids	C
ACOX1	Hs01074241_m1	Acyl-CoA Oxidase 1, Palmitoyl	First step of β-oxidation pathway of very long chain-fatty acids	C, D
COX4I1	Hs00971639_m1	Cytochrome C Oxidase Subunit IV Isoform 1	Terminal enzyme of the mitochondrial respiratory chain	D
CPT1B	Hs03046298_s1	Carnitine Palmitoyltransferase 1B	Conversion of the long-chain acyl-CoA to long-chain acylcarnitine	C
CPT1b	Hs00993896_g1	Carnitine Palmitoyltransferase 1B	Conversion of the long-chain acyl-CoA to long-chain acylcarnitine	D
CS	Hs02574374_s1	Citrate Synthase	Synthesis of citrate from oxaloacetate and acetyl coenzyme A	D
CYC1	Hs00357717_m1	Complex III Subunit IV	Part of Complex III in electron transport chain	D
FABP3	Hs00997360_m1	Fatty acid protein binding 3	Intracellular transport of long-chain fatty acids and their acyl-CoA esters	D
NDUFA11	Hs00418300_m1	NADH:Ubiquinone Oxidoreductase Subunit A11	Part of the membrane-bound mitochondrial complex I	D
PLIN5	Hs00965990_m1	Perilipin 5	Protection of lipid droplets in tissues with high lipid oxidative metabolism	D
PPARGC1A=PGC1	Hs01016719_m1	Peroxisome proliferator-activated receptor gamma coactivator 1 alpha	Cofactor of PPARγ, involved in thermogenesis and energy metabolism	C
PPARα	Hs00947539_m1	Peroxisome Proliferator Activated Receptor Alpha	Transcription factor regulating glucose and lipid metabolism	C
PPARα	Hs00231882_m1	Peroxisome Proliferator Activated Receptor Alpha	Transcription factor regulating glucose and lipid metabolism	D
SDHA	Hs00188166_m1	Succinate Dehydrogenase Complex Flavoprotein Subunit A	Part of complex II of the mitochondrial respiratory chain	D
UQCRC2	Hs00996395_m1	Ubiquinol-Cytochrome C Reductase Core Protein II	Complex III, part of the mitochondrial respiratory chain	D
Lipolysis				
ATGL	Hs00982040_g1	Adipose triglyceride lipase	Triglyceride hydrolysis	C, D
CGI58=ABHD5	Hs01104373_m1	Lipid Droplet-Binding Protein CGI-58=Abhydrolase Domain Containing 5	Cofactor of ATGL	C, D
FAT/CD36	Hs00169627_m1	Fatty acid translocase	Transport and regulation of fatty acid transport across the cell membranes	C, D
G0S2	Hs00274783_s1	G0/G1 Switch 2 (cofactor ATGL)	ATGL inhibitor (lipolysis inhibitor)	C, D
HSL=LIPE	Hs00943404_m1	Hormone sensitive lipase=Lipase E, Hormone Sensitive Type	Diglyceride hydrolysis	C, D
MGLL	Hs00200752_m1	Monoglyceride lipase	Monoglyceride hydrolysis	C, D
PLIN1	Hs00160173_m1	Perilipin 1	Coating of lipid droplets, inhibitor of lipolysis	A, C, D
Senescence				
BRINP1=DBC1	Hs01089686_m1	BMP/Retinoic Acid Inducible Neural Specific 1	Unknown function, possible marker of adipocyte senescence	D
DLC1	Hs01089686m1	Deleted In Liver Cancer 1 Protein	Regulation of small GTP-binding proteins	B
E2F7	Hs00403170_m1	E2F Transcription Factor 7	Transcription factor, upregulated by p53	D
GDF15	Hs01379108m1	Growth Differentiation Factor 15	Secreted ligand of the TGFβ, universal marker of senescence	B
GLB1	Hs01035168_m1	Galactosidase beta 1	Hydrolysis of a terminal beta-linked galactose residue	D

NOX4	Hs01379108_m1	NADPH Oxidase 4	Generates reactive oxygen species (ROS) from molecular oxygen, mRNA marker of senescence	D
NOX4	Hs00171132	NADPH Oxidase 4	Oxygen sensor, catalyses the reduction of molecular oxygen to various ROS	B
p16=CDKN2A	Hs00923894_m1	Cyclin Dependent Kinase Inhibitor 2A (=CDKN2A)	Inhibits the activity of cyclin-CDK4 and -CDK6 complexes (-> cell cycle arrest in G1 and G2 phases)	B, D
p21=CDKN1A	Hs00355782_m1	Cyclin Dependent Kinase Inhibitor 1A	Inhibits the activity of cyclin-CDK2, -CDK1, and -CDK4/6 complexes (-> regulation at G1 and S phase)	D
p27=CDKN1B	Hs00153277_m1	Cyclin Dependent Kinase Inhibitor 1B	Inhibits the activity of cyclin-CDK2 or -CDK4 complexes (-> regulation at G1)	B, D
SIRT1	Hs01009006_m1	Sirtuin 1	NAD+-dependent protein deacetylase, downregulated in senescent cells	D
Inflammation				
ACP5	Hs00356261_m1	Acid phosphatase 5, tartrate resistant	Belongs to the metallophosphoesterase superfamily	C
CCR2	Hs00356601_m1	MCP1 receptor	Mediates chemotactic perception for monocytes and basophils	C
CD163	Hs00174705_m1	Cluster of differentiation 163	Scavenger receptor	C
CD68	Hs00154355_m1	Cluster of differentiation 68	Scavenger receptor	C
FCGBP	Hs00175398_m1	IgGFc-binding protein	Function unknown	C
IL10	Hs00961622_m1	Interleukin 10	Regulation of inflammatory response	C
IL1RN	Hs00893626_m1	Interleukin 1 receptor antagonist	Natural inhibitor of the pro-inflammatory IL1 cytokine	C
IL1ß	Hs00174097_m1	Interleukin 1 beta	Mediator of inflammatory response	D
IL6	Hs00985639_m1	Interleukin 6 (interferon, beta 2)	Cytokine stimulating immunologic response and regulating energy metabolism	C, D
IL8	Hs00174103_m1	Interleukin 8	Causes chemotaxis of immune cells and induce phagocytosis	C, D
INHBA	Hs01081598_m1	Inhibin Beta A Subunit (=Activin A)	Negative regulator of adipocyte differentiation	D
IRF5	Hs00158114_m1	Interferon regulatory factor 5	Transcription factor regulating induction of inflammatory cytokines	C
ITGAX	Hs00174217_m1	Integrin, alpha X (complement component 3 receptor 4 subunit) (CD11C)	Mediates cell-cell interaction during inflammation	C
MCP1	Hs00234140_m1	Monocyte chemoattractant protein 1	Attracts inflammatory cells to the inflammatory site	C, D
MMP3	Hs00968305_m1	Matrix Metalloproteinase 3	Breakdown of extracellular matrix	D
MSR1	Hs00234007_m1	Macrophage scavenger receptor 1	Class A macrophage scavenger receptor	C
TNFα	Hs00174128_m1	Tumor necrosis factor alpha	Proinflammatory cytokine regulating also lipid and carbohydrate metabolism	C
Endoplasmic reticulum stress				
ATF4	Hs00909569_g1	Activating Transcription Factor 4	Transcription factor, influences autophagy, amino acid metabolism, anti-oxidant machinery and apoptosis	A, B, D
CALR	Hs00189032_m1	Calreticulin	Calcium-binding chaperone	D
DNAJC13	Hs00967069_m1	DnaJ Heat Shock Protein Family (Hsp40) Member C13	Chaperon cofactor	D
DNAJC3	Hs00534483_m1	DnaJ Heat Shock Protein Family (Hsp40) Member C3	Co-chaperone of HSPA8/HSC70	B
EDEM1	Hs00976004_m1	ER Degradation Enhancing Alpha-Mannosidase Like Protein 1	Targets misfolded glycoproteins for degradation	A, B, D

GADD34=PPP1R15A	Hs00169585_m1	Growth Arrest And DNA-Damage-Inducible 34	Dephosphorylation of p-eIF2α	B, D
HSPA5	Hs99999174_m1	Heat Shock Protein Family A (Hsp70) Member 5 (=GRP78)	Folding and assembly of proteins in the endoplasmic reticulum	A, B, D
HYOU1	Hs00197328_m1	Hypoxia Up-Regulated 1	Chaperon from heat shock protein 70 family	B, D
CHOP	Hs01090850_m1	C/EBP homologous protein (=GADD153)	Transcription factor: Pro-inflammatory response and apoptosis	B, D
PERK	Hs00984006_m1	PRKR-Like Endoplasmic Reticulum Kinase	Initiate PERK arm of the UPR pathway	B, D
Antioxidant genes				
NQO1	Hs00168547_m1	NAD(P)H dehydrogenase, quinone 1	Reduces quinones to hydroquinones	D
SOD2	Hs00167309_m1	Mitochondrial superoxide dismutase 2	Binds to the superoxide byproducts of oxidative phosphorylation	D
Specific WAT markers				
DPT	Hs00355056_m1	Dermatopontin	Extracellular matrix protein with possible functions in cell-matrix interactions and matrix assembly	D
TCF21	Hs00162646_m1	Transcription Factor 21	Transcription factor, manages cell-fate specification during development	D
Specific "Brown-brite" markers				
CIDEA	Hs00154455_m1	Cell Death-Inducing DFFA-Like Effector A	Transcriptional coactivator, thermoregulatory marker	D
CITED1	Hs00918445_g1	Cbp/P300-Interacting Transactivator 1	Transcriptional coactivator, thermoregulatory marker	D
CKMT1a	Hs00179727_m1	Creatine Kinase, Mitochondrial 1A	Transfer of high energy phosphate from mitochondria to creatine	D
CKMT2	Hs00176502_m1	Creatine Kinase, Mitochondrial 2	Transfer of high energy phosphate from mitochondria to creatine	D
ELOVL3	Hs00537016_m1	Elongation Of Very Long Chain Fatty Acids Protein 3	Elongation of long chain fatty acids for the synthesis of sphingolipids and ceramides	D
GK	Hs02340010_g1	Glycerol kinase	Formation of glycerol-3-phosphate	D
PDK4	Hs01037712_m1	Pyruvate Dehydrogenase Kinase 4	Regulates glucose metabolism	D
PGC1alpha	Hs00173304_m1	PPARG Coactivator 1 Alpha	Transcriptional coactivator regulating genes involved in energy metabolism	D
TMEM26	Hs00415619_m1	Transmembrane Protein 26	Selective surface protein, marker of brite/beige adipocytes	D
UCP1	Hs00222453_m1	Uncoupling protein1	Key function in thermogenesis	D
Specific BAT marker				
SIRT3	Hs00202030_m1	Sirtuin 3	Influences mitochondrial energy metabolism and function	D
Insulin sensitization				
FFAR4=GPR120	Hs00699184_m1	Free Fatty Acid Receptor 4	Anti-inflammatory and insulin-sensitizing effects of omega 3 fatty acids	D
GLUT1	Hs00892681_m1	Glucose transporter 1	Basal glucose uptake	C, D
GLUT4	Hs00168966_m1	Glucose transporter 4	Stimulated glucose uptake	C, D
IRS1	Hs00178563_m1	Insulin receptor substrate 1	Transmits signal from insulin and IGF-1 receptor downstream	C, D

Fibrosis				
COL1A1	Hs00164004_m1	Collagen type I alpha 1	Major component of type I collagen	C, D
COL6A3	Hs00365098_m1	Collagen, type VI, alpha 3	Constitute alpha chain of type VI collagen	C
COL6A3	Hs00915126_m1	Collagen, type VI, alpha 3	Constitute alpha chain of type VI collagen	D
LOX	Hs00942480_m1	Lysyl oxidase	Cross-links collagens and elastins	C, D
LUM	Hs00929860_m1	Lumican	May regulate collagen fibril organization and tissue repair	D
MMP9	Hs00234579_m1	Matrix metallopeptidase 9	Catalysis breakdown of ECM (in normal physiological processes)	C, D
TGFb1	Hs00171257_m1			D
TGFβ1	Hs00998133_m1	Transforming growth factor beta 1	Pro-fibrotic factor	C
TLR4	Hs01060206_m1	Toll-like receptor 4	Endotoxin receptor mediating the development of AT fibrosis	C, D
TNC	Hs01115665_m1	Tenascin C	Extracellular matrix protein, myriad of functions	D
Others				
HP	Hs00978377_m1	Haptoglobin	Binds free plasma haemoglobin	D
ORM1	Hs01590791_m1	Orosomucoid 1	Transport protein in the blood stream, increased during inflammation	D
ORM2	Hs01037491_m1	Orosomucoid 2	Key acute phase plasma protein with non-specific function	D
Reference genes				
GUSB	Hs00939627_m1	Glucuronidase Beta	Hydrolysis of β-D-glucuronic acid	A, C, D
HPRT1	Hs02800695_m1	Hypoxanthine-Guanine Phosphoribosyltransferase	Converts hypoxanthine to inosine monophosphate and guanine to guanosine monophosphate	D
LRP10	Hs01047362_m1	LDL Receptor Related Protein 10	Involved in the internalization of lipophilic molecules and/or signal transduction	D
PPIA	Hs04194521_s1	Peptidylprolyl isomerase A (cyclophilin A)	Cis-trans isomerization of proline imidic peptide bonds	C, D
PUM1	Hs00472881_m1	Pumilio homolog 1	Translational regulator	C, D
RPS13	Hs01011487_g1	Ribosomal Protein S13	Component of the 40S subunit of ribosome	A, B

Table 4: List of genes and primer sequences of mRNA analysed by SYBR Green technology.

Gene symbol	Protein	Function	Project
aP2/FABP4	Fatty acid protein binding 4	Lipid transport in adipocytes	A
XBP1t	X-box binding protein 1, total	Non-active transcription factor, part of unfolded protein response	A, B
XBP1s	X-box binding protein 1, spliced	Active transcription factor, part of unfolded protein response	A, B

Gene symbol	Primer	Sequence
aP2/FABP4	Forward:	5´-GCATGGCCAAACCTAACATGA-3´
	Reverse:	5´-CCTGG CCCAGTATGAAGGAAA-3´
XBP1t	Forward:	5´-CGCTGAGGAGGAAACTGAA-3´
	Reverse:	5´-CACTTGCTGTTCCAGCTCACTCAT-3´
XBP1s	Forward:	5´-GAGTCCGCAGCAGGTGCA-3´
	Reverse:	5´-ACTGGGTCCAAGTTGTCCAG-3´

Project A: Gene expression of target genes was normalized to expression of GUSB or RPS13 and fold change of expression was calculated using ΔΔ Ct method.

Project B: Because of lack of RNA from AT, 2 ng of cDNA was preamplified (as in **Project C**) to detect expression of p16, p27, NOX4, GDF15 and DLC1. Gene expression of target genes was normalized to expression of RPS13 and fold change of expression was calculated using ΔΔ Ct method.

Project C: For microfluidics, 4 ng of cDNA was preamplified within 16 cycles to improve detection of target genes during subsequent real-time quantitative PCR (qPCR; TaqMan Pre Amp master mix kit; Applied Biosystems, USA). For the preamplification, 20xTaqMan gene expression assays of all target genes were pooled together and diluted with water to the final concentration 0.2 for each probe. The real-time qPCR was performed in duplicates on Biomark real-time qPCR system using 96.96 array (Fluidigm). In addition, the mRNA expression of CD36, PPARγ, ATGL, HSL, DGAT-2, IL-6, IL-8, IL-10, MCP-1, TNF-α, and leptin was quantified by qPCR without preamplification on an ABI PRISM 7500 (Applied Biosystems). Data were normalized to reference gene PPIA, which proved to be superior over two other measured reference genes, PUM1 and GUSB. The method of $2^{(-\Delta Ct)}$ was calculated for statistical analysis, and the final values for the figures were expressed as fold change related to mean basal value.

Project D: Expression of mRNA for p16 in preadipocytes was quantified by qPCR on an ABI PRISM 7500 (Applied Biosystems). Gene expression from adipocytes differentiated *in vitro* was assessed using microfluidics. cDNA was preamplified as a paid service by the technical facility for Transcriptomics at the Institute of Cardiovascular and Metabolic

Diseases (I2MC, UMR1048) in Toulouse. The real-time qPCR using 96.96 array (Fluidigm) was performed as a paid service as well. Five genes (GUSB, HPRT1, LRP10, PPIA and PUM1) were used as the reference genes. Data were normalized to reference gene PPIA, which proved to be superior over four other measured reference genes. Results are expressed as a fold change calculated using ΔΔ Ct method.

3.2.4.3 Western blot

Cells were washed twice with cold PBS, collected in RIPA buffer (hand-made or Sigma Aldrich, USA) supplemented with proteases and phosphorylases inhibitors (Complete, PhosStop from Roche, Mannheim, Germany or Protease Inhibitor Cocktail, Phosphatase Inhibitor Cocktail 2 and 3, Sigma Aldrich, USA) and frozen in liquid nitrogen. Protein content was determined by Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, USA) according to manual instructions. Denaturized proteins were separated on 4-20% gradient SDS-PAGE gels (Bio-Rad), blotted onto nitrocellulose membranes and incubated with following primary antibodies: pAkt S473 (4060L, Cell Signaling, USA), Akt (C67E7, Cell Signaling, USA), pIRS-1 Y612 (44-816G, Invitrogen, USA), IRS-1 (D23G12, Cell Signaling, USA), pERK Thr202/Tyr204 (4370P, Cell Signaling, USA), ERK (4695P, Cell Signaling, USA), GAPDH (7074S, Cell Signaling, USA), eIF2α (2103S, Cell Signaling, USA) and peIF2α (3398, Cell Signaling, USA). Antigen-antibody complexes were detected using secondary anti-rabbit antibody coupled with horseradish peroxidase and the ECL detection system (Pierce) via ChemiDoc MP System (Bio-Rad). For quantification of the signal, Image Lab software (Bio-Rad) was used.

3.2.5 Analysis of metabolites

3.2.5.1 De novo lipogenesis

To determine rate of DNL, cells were deprived from insulin overnight. Next day, cells were washed twice and incubated for 3 hours in Krebs Ringer buffer supplemented with 2% BSA, 10 mM HEPES, 66 nM insulin, 2 mM glucose and 2 mCi D-[^{14}C(U)]glucose or 5 mM acetic acid and 2 mCi [1-^{14}C]-Acetic Acid Sodium Salt. After incubation, cells were washed twice in PBS and scraped into 0.1% SDS in PBS. Neutral lipids were isolated using methanol/chloroform (1:2) extraction. Organic phase was dried under nitrogen and hydrolysed in 1 mL 0.25N NaOH in methanol/chloroform (1:1) for 1 h at 37°C. Next, 500 µL 0.5N HCl in methanol was added to neutralize the solution. Organic and aqueous phases were separated by adding 1.7 mL chloroform, 860 µL water, and 1 mL methanol/chloroform (1:2).

Incorporation of ^{14}C into fatty acids was determined by liquid scintillation counting on Tri-Carb2100TR (Packard). Specific activity was counted and used to determine the quantity of incorporated radioisotope equivalent. Results from metabolic measurements were normalized to total protein content of cell extracts.

3.2.5.2 Separation of lipid species by thin layer chromatography

Neutral lipids were isolated as described above. Lipids were separated by thin-layer chromatography on Silica Gel plates developed in Heptane:Isopropylether:Acetic acid mixture (60:40:4) for 1 h, visualized by iodine vapour and quantitatively scraped from plate. Distribution of de novo incorporated ^{14}C among major lipid species was analysed by liquid scintillation counting on Tri-Carb2100TR (Packard). Specific activity was counted and used to determine the quantity of incorporated radioisotope equivalent. Results from metabolic measurements were normalized to total protein content of cell extracts.

3.2.5.3 Glucose transport

Adipocytes were deprived of insulin overnight. Next day, cells were washed twice with PBS and cultivated with or without 100nM insulin in Krebs Ringer buffer supplemented with 12.5 mM Hepes and 1% BSA (the pH was adjusted to 7,4) for 50 min at 37°C. Then, 125 mM 2-deoxy-D-glucose and 0.4 mCi 2-deoxy-D-[^{3}H] glucose per well were added for 10 min. After that, culture plates were placed on ice and washed with cold 10 mM glucose in PBS. Cells were collected into 0.05N NaOH. Uptake of 2-deoxy-D-[^{3}H] glucose was measured by liquid scintillation counting of cell lysate and normalized to protein level. Protein quantity was measured in duplicates using 10 µl of cell lysate and Bradford assay (Bio-Rad, USA) following the manufacture instructions.

3.2.6 Statistical analysis

Data were analysed using GraphPad Prism 6.0 software using Wilcoxon matched-pair signed rank, Mann Whitney test, one-way ANOVA with repeated measures, followed by post hoc pairwise comparisons with Bonferroni adjustment for multiple testing or two-way ANOVA with Bonferroni adjustment for multiple testing, as appropriate. Correlations were assessed by Spearman or Pearson's correlation (if the values were logaritmated). The level of significance was set at ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

4 Results

4.1 Experimental part A

4.1.1 Introduction to experimental part A

Adipocytes are cells highly specialized for storage of neutral lipids. They are equipped with dedicated receptors and transporters necessary for an uptake and transport of free fatty acids and with enzymatic cascade enabling their incorporation into TAG. Moreover, adipocytes are able to synthetize lipids *de novo*, from glucose [61]. Glucose is necessary also for the synthesis of glycerol phosphate, the backbone of TAG. Thus lipogenic activity of adipocytes directly influences fatty acid and glucose plasma levels and this homeostatic effect is regulated by many factors [259, 260]. Paradoxically, obesity impairs capacity of adipocytes to synthetize and store lipids [261, 262], which further contributes to high plasma free fatty acids levels, a putative cause of obesity-related hepatic and muscle insulin resistance [263]. The reason for the deterioration of lipogenic activity of adipocytes remains unclear. Notably, several enzymatic steps of lipogenesis and the formation of lipid droplets take place in the (ER, an organelle also essential for calcium homeostasis and protein folding [264, 265]. The situation when the folding and other metabolic capacities of ER are overwhelmed is referred to as ER stress. ER stress activates a defense mechanism called UPR in order to enhance ER capacity and restore ER homeostasis [266]. The signs of chronic ER stress have recently been found in obese and insulin resistant subjects [267, 268]. The significance of ER stress for metabolic health was confirmed by experiments on rodents corroborating ER stress as a trigger of insulin resistance and other metabolic disturbances caused by obesity [145]. Importantly, ER stress and consequently UPR were found to be important regulators of lipogenesis in liver [269]. But there is a lack of comprehensive studies that would investigate whether metabolic stress sensed through ER controls lipogenesis also in human adipose tissue. Thus, we aimed at investigating the effect of ER stress on lipogenesis in human adipose cells.

4.1.2 Results: experimental part A

Acute high intensity ER stress reduces adipogenesis and lipogenesis of human preadipocytes and adipocytes. To evaluate the effect of acute ER stress on lipogenic capacity of adipocytes, we exposed *in vitro* differentiated adipocytes from 15 donors to two commonly used ER stressors, thapsigargin (TG) and tunicamycin (TM), for 24 hours. Both, 100 nM TG and 1 μg/ml TM [270], dramatically enhanced expression of major ER chaperone HSPA5 (heat shock 70 kDa protein 5), a marker of ER stress (**Figure 10A**). The same treatment decreased mRNA levels of genes involved in lipogenesis, i.e. fatty acid synthase (FASN), stearoyl desaturase (SCD1), and diacylglycerol O-acyltransferase 2 (DGAT2) (**Figure 10A**). The suppressive effect of ER stress on lipogenesis was confirmed by a decreased capacity of adipocytes treated with TG to incorporate glucose carbon into lipids (**Figure 10B, C**). Thus, in adipocytes, acute high intensity ER stress lowers lipogenic capacity of adipocytes on both transcriptional and enzymatic level.

In addition, we tested an effect of acute ER stress on adipogenic capacity of preadipocytes. In order to limit ER stress only to preadipocytes we employed reversibly acting TM in high-dose (1μg/ml). Confluent preadipocytes were treated with TM for 4 hours, then washed by PBS several times and subjected to standard 12 day adipogenic procedure using media free of ER stress inducer. This treatment resulted in approximately 60% reduction of neutral lipid content compared to control conditions (**Figure 10D**) without apparent effect on the viability of cells. Moreover, the effect of TM-pretreatment of preadipocytes on adipogenesis was detectable already after 3 days of differentiation when mRNA levels of aP2, PPARγ and perilipin were reduced compared to control conditions (**Figure 10E**).

Chronic low ER stress impairs adipogenesis and associated lipogenesis. Obesity leads to chronic low intensity rather than acute high intensity ER stress [267, 271]. Therefore, we aimed at imitating chronic ER stress in adipose cells by the use of TG dose capable of activating the UPR without acute induction of downstream effectors [272]. To determine such a dose, we exposed both preadipocytes and mature adipocytes to 1, 2.5, 5 and 100 nM TG for 1, 4 and 24 hours and then analyzed expression of genes representing early and late markers of UPR. Neither dose of TG caused appearance of hypodiploid apoptotic preadipocytes within 24 hours (not shown). Early marker of UPR activation, i.e. phosphorylated eIF2α (PERK arm of the UPR), was induced already by 2.5 nM TG (**Figure 11A**) within 1 hour, while an

induction of expression of downstream ER stress effectors (ATF6 arm- HSPA5[151], PERK arm- ATF4 [273], IRE1 arm- EDEM1, XBP1 splicing [274]) within 4 and 24 hours required higher TG concentrations (5 or 100 nM TG) (**Figure 11B-D**). Therefore, 2.5 nM TG was selected for chronic treatments of cells.

We investigated whether low intensity but chronic ER stress reduces adipogenic conversion of preadipocytes similarly as acute high intensity ER stress. Preadipocytes were differentiated in the absence or presence of 2.5 nM TG. Chronic treatment of cells with TG led to a mild increase of mRNA levels of HSPA5, ATF4 and EDEM1 during the whole time course of differentiation (**Figure 12A**). Capacity to accumulate neutral lipids was lowered by more than 50% in TG-treated adipocytes as detected by ORO staining (**Figure 12B, C**). This was accompanied by diminished mRNA levels of differentiation markers (i.e. a key adipogenic factor, PPARγ, transcription factor SREBP-1c and late adipogenic markers, aP2 and perilipin) (**Figure 12D, E**). mRNA expression of the lipogenic genes SCD1, DGAT2 and FASN was also lowered (**Figure 12F**).

To determine a critical period of time for ER stress to exert inhibitory effect on adipogenesis, preadipocytes were differentiated in the presence of 2.5 nM TG for various days (0-6, 1-12, 3-12, 6-12, 9-12). Capacity to store neutral lipids evaluated by ORO staining was strongly impaired in adipocytes exposed to TG between days 0-6 and 1-12, mildly between days 3-12 and not between days 6-12 and 9-12 of differentiation (**Figure 12G, H**). Lipogenesis measured as ^{14}C-glucose carbon incorporation into lipids was also not altered when cells were exposed to 2.5 nM TG at day 6-12 of differentiation (not shown).

Lipogenic capacity of mature adipocytes is not influenced by chronic low ER stress. Next, we analyzed the effect of chronic (6 days) low ER stress on adipocytes differentiated for 12 days. Accumulation of neutral lipids (^{14}C-glucose carbon incorporation) was not affected by 2.5 nM TG (**Figure 13A**), similarly as seen when TG was applied between day 6 and 12 of adipogenesis. Only expression of perilipin was decreased while other lipogenic genes remained unaffected by this treatment (**Figure 13B**).

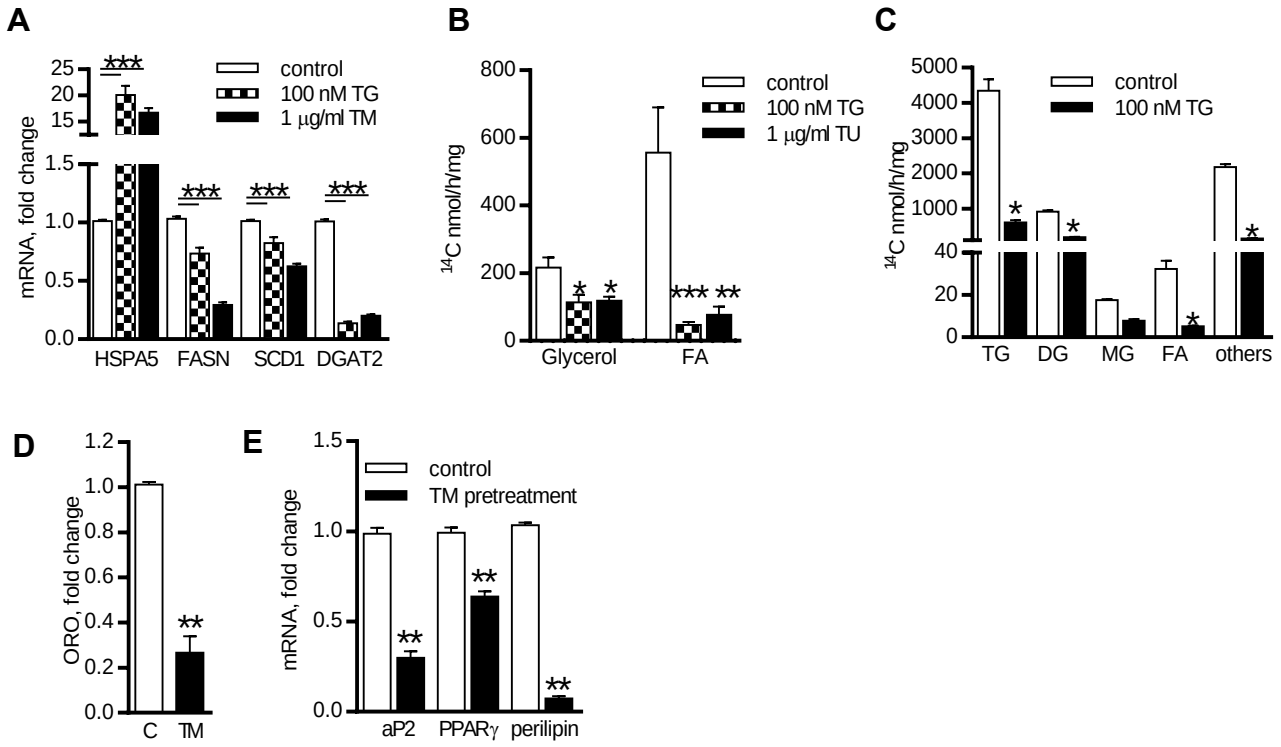


Figure 10: Acute ERS lowers lipogenesis in human adipocytes and adipogenesis of preadipocytes.
Preadipocytes were differentiated for 12 days and then incubated for 24 hours with DMSO or 100 nM TG or 1µg/ml TM. **A**. mRNA expression of HSPA5, FASN, SCD1 and DGAT2 was measured by qRT-PCR and normalized to GUSB expression (n=15). **B**. Glucose carbon (^{14}C) incorporation into lipids (hydrolyzed into fatty acids (FA) and glycerol) during 3 hour incubation was determined by liquid scintillation and normalized to protein content (n=3). **C.** Distribution of de novo incorporated ^{14}C in lipid species was analyzed after TLC separation of extracted lipids (n=2). **D.** Preadipocytes were exposed to 1µg/ml TM for 4 hours and then differentiated in the absence of TM for 12 days. Quantification of neutral lipids accumulation is expressed as a relative fold change to control (n=5). **E.** Preadipocytes were exposed to 1µg/ml TM for 4 hours and then differentiated in the absence of TM for 3 days. mRNA expression of adipogenic markers was analyzed by qRT-PCR and normalized to RPS13 (n=5). Data are means ±SE, *p<0.05, ** p<0.01, *** p<0.001.

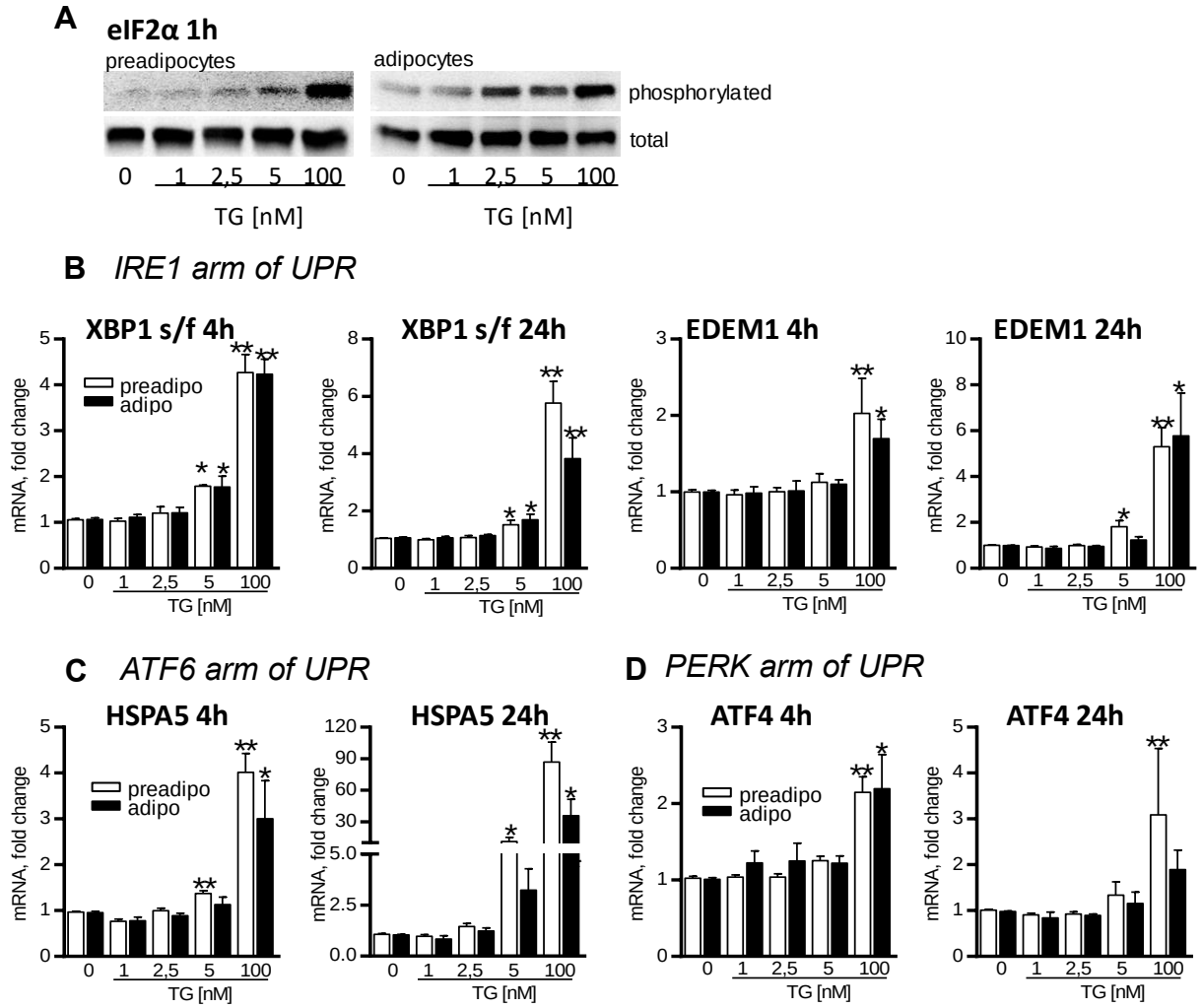


Figure 11: Determination of TG dose appropriate for experiments with chronic low ERS. Cells were incubated with DMSO or 1, 2.5, 5, 100 nM TG for indicated time. **A**. Western blotting analysis of eIF2α activation (n=3, the representative image is shown). **B-D.** mRNA expression of HSPA5, EDEM1 and ATF4 was measured by qRT-PCR and normalized to GUSB expression. mRNA expression of XBP1-spliced was normalized to XBP1-total expression (n=4). Data are means ±SE, *p<0.05, ** p<0.01.

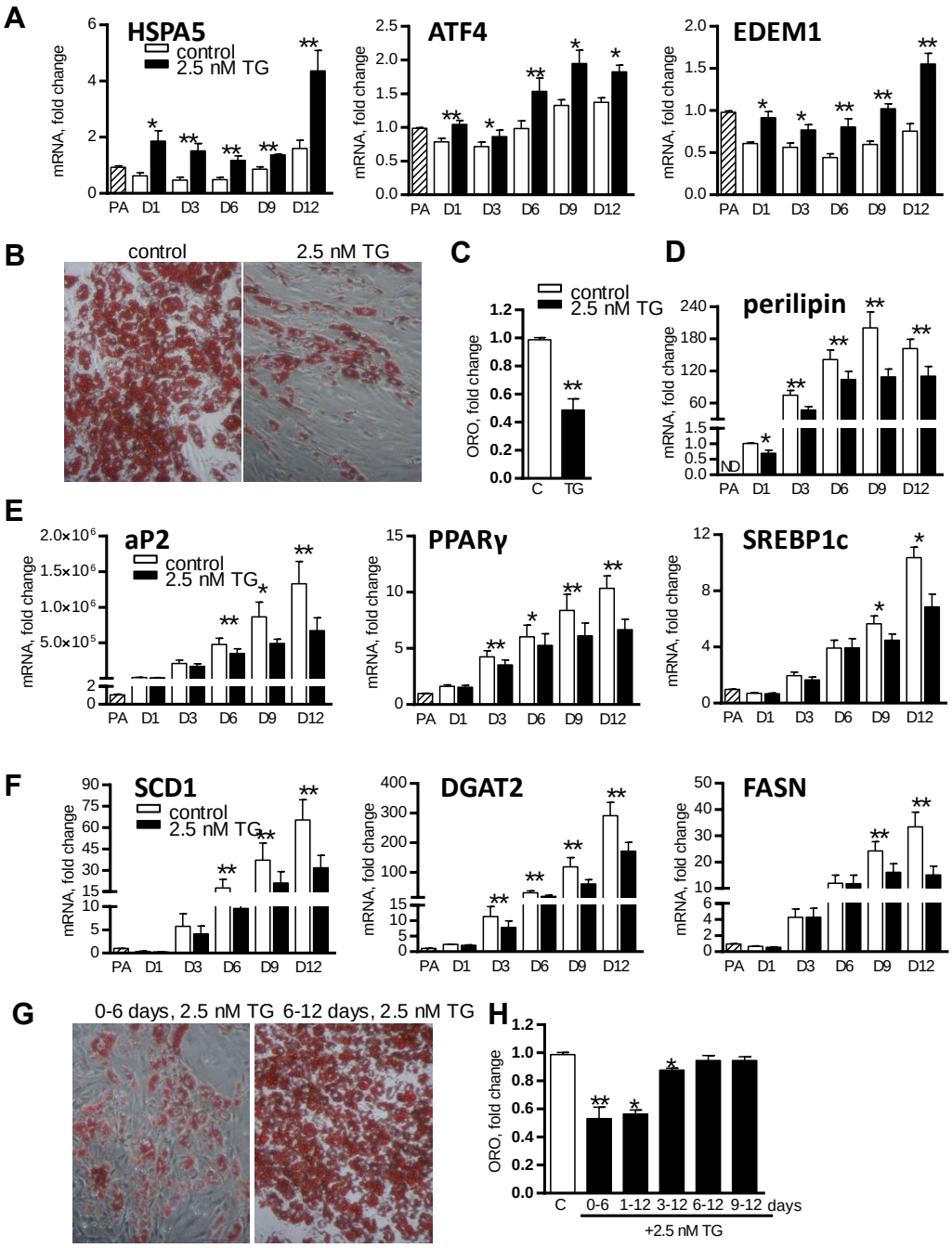


Figure 12: Chronic low ERS impairs adipogenesis and associated lipogenesis. Preadipocytes (PA) were differentiated for 12 days in the absence or presence of 2.5 nM TG. Cells were harvested upon indicated days for mRNA analysis or at day 12 for ORO staining. mRNA expression of UPR effectors (**A**), adipogenic **(D, E**) and lipogenic markers (**F**) was measured by qRT-PCR and normalized to RPS13 expression (n=4, ND-not detectable). **B**. Representative image of cells staining with ORO at day 12. **C**. Quantification of neutral lipids accumulation is expressed as a relative fold change to control (n=5). **G**. Preadipocytes were differentiated and 2.5 nM TG was added to the differentiation media for indicated days. Representative image of cells staining with ORO at day 12. **H**. Preadipocytes were differentiated and 2.5 nM TG was added to the differentiation media for indicated days. Quantification of neutral lipids accumulation is expressed as a relative fold change to control (n=5). Data are means ±SE, *p<0.05, ** p<0.01.

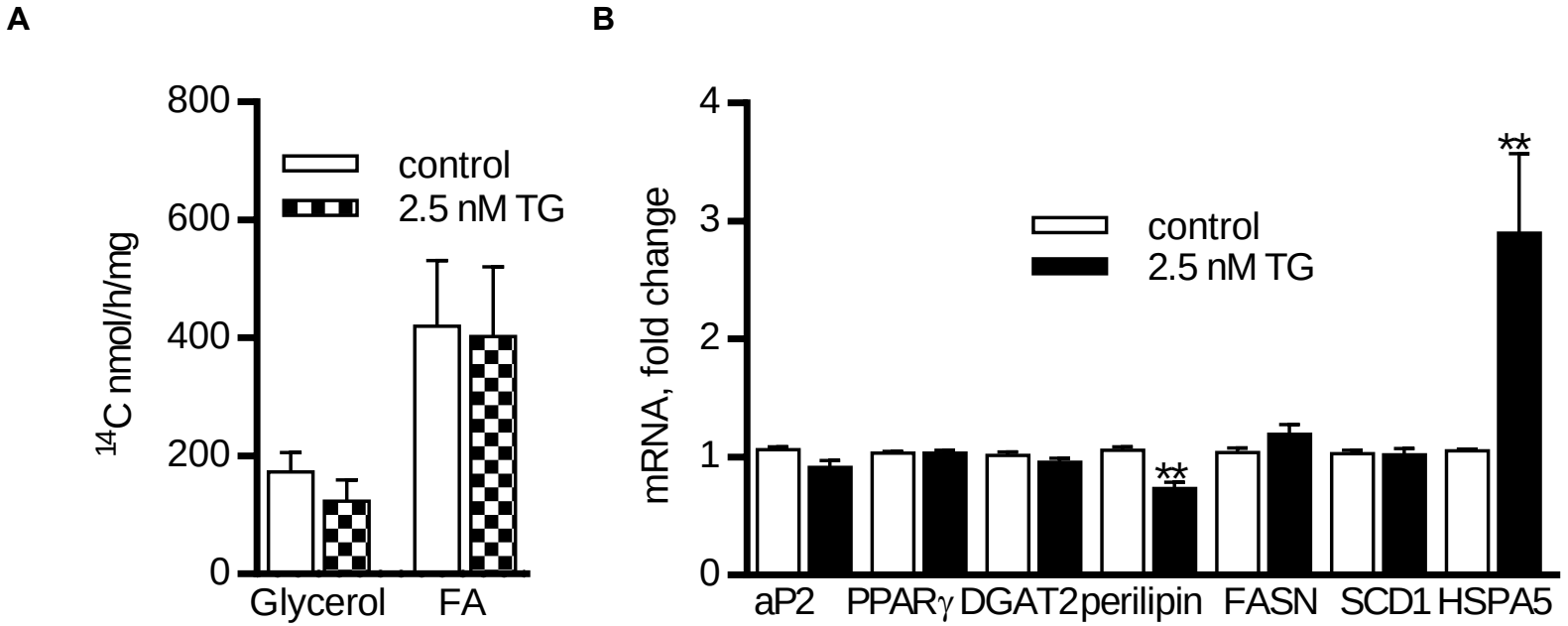


Figure 13: Effect of low ERS stress on mature adipocytes. Mature adipocytes (12 days after onset of differentiation) were exposed for next 6 days to 2.5 nM TG. **A**. Glucose (^{14}C) incorporation into lipids (hydrolyzed into fatty acids FA and glycerol) during 3 hour incubation of adipocytes treated as in Fig 1B. was determined by liquid scintillation and normalized to protein content (n=3). **B**. mRNA expression of HSPA5, adipogenic and lipogenic markers was measured by qRT-PCR and normalized to RPS13 expression (n=5). Data are means ±SE, *p<0.05, ** p<0.01.

4.2 Experimental part B

4.2.1 Introduction to experimental part B

Subcutaneous AT is an organ specialized for the synthesis and metabolically safe storage of lipids through process of lipogenesis and thus it is indispensable for the maintenance of whole-body energy homeostasis [275]. For lipogenesis, AT utilizes mainly dietary lipids but it can synthetize fatty acids de novo from glucose or other acetyl/malonyl CoA sources in the processes of DNL. Emerging data implicate DNL in the maintenance or improvement of insulin sensitivity, as DNL generates insulin sensitizing lipokines and enhances fluidity of membranes necessary for insulin signaling [88, 262, 276-278].

In aging, however, the capacity of subcutaneous AT to synthetize and store lipids progressively decreases and this may contribute to metabolically unfavorable fat redistribution, dyslipidemia, insulin resistance and metabolic syndrome [196]. Despite substantial health impact of this subcutaneous AT dysfunction in the elderly, its cellular and molecular triggers remain unclear. It has been suggested that the age-related dysfunction of various tissues can be partly related to the accumulation of senescent cells. Senescence is an irreversible cell-cycle arrest that can be induced by various stimuli, such as telomere shortening, DNA damage, oncogene activation, and/or metabolic stress [180]. Senescent cells cannot fulfil their original function and moreover, they exert highly pro-inflammatory phenotype described as SASP that can profoundly affect the function of bystander cells [179].

Another possible inhibitor of lipogenesis in aging adipocytes can be stress of ER, an organelle essential for lipid synthesis. ER stress, the condition when ER folding or synthetic capacity becomes overwhelmed, leads to the activation of a signaling network known as UPR. The general aim of the UPR is to restore the ER homeostasis mainly through the reinforcement of ER capacity by the induction of ER chaperons. At the same time, IRE-1 branch of the UPR leads to the phosphorylation and activation of JNK [279]. JNK activity may lead to a variety of downstream effects depending on the cellular context, some of which include apoptosis, cell survival, insulin resistance and inflammation [280]. Indeed, the experiments on rodents established ER stress as a trigger of insulin resistance and other metabolic disturbances caused by obesity [281]. In line with this, we showed recently that ER stress impairs DNL in adipocytes and differentiation of preadipocytes [282]. In addition, ER stress appears to be higher in subcutaneous AT from aged mice [283]. Therefore, our goal was to compare the lipogenic capacity in subcutaneous AT of young and elderly women, in relation to senescence and ER stress markers.

4.2.2 Results: experimental part B

Clinical characteristics. Clinical data of young and elderly subjects are depicted in **Table 5**. The two groups differed in age (35.3±2.0 years for the young and 70.8±1.4 years for the elderly), but were matched for the amount of fat mass (37.3% and 38.9% for the young and the elderly, respectively). The matching for fat mass was selected since this relative value related to AT mass is less biased by age-related sarcopenia than other general anthropometric measures as weight and BMI. Metabolically, both groups had similar insulin sensitivity calculated as HOMA-IR despite the differences in fasting glucose and insulin levels.

Expression of lipogenic genes in subcutaneous AT decreases with age. To compare lipogenic potential of subcutaneous AT of the young and the elderly, we analyzed mRNA expression of six major lipogenic genes. Subcutanoues AT mRNA transcripts of *FASN*, a rate limiting enzyme in DNL, and *DGAT2*, an enzyme catalyzing final step of the triglyceride synthesis, were decreased significantly in the elderly group ($p<0,05$; **Figure 14A**). Levels of mRNA for these two genes were strongly correlated (**Figure 14D**). The tendency to decreased expression was also observed for mRNA of *ACACA* and *SCD1*, even though the difference was not statistically significant. mRNA expression of *ACLY* and *ELOVL6* did not differ between the two groups.

Elderly subcutaneous AT display more senescent phenotype than subcutaneous AT from young individuals. To analyze the level of senescence in the subcutaneous AT samples, we measured expression of *p16*, an inhibitor of cell cycle progression and well-established marker of senescence, and 3 other senescent markers in both groups. Elderly subcutaneous AT expressed more *p16* and *NOX4* mRNA transcript compared to subcutaneous AT from young group ($p<0,05$; **Figure 14B**) and these two transcripts positively correlated (**Figure 14D**). The expression level of *p27* was similar in the two groups. Even though mRNA expression of an additional senescence marker, GDF15, was also not different in subcutaneous AT from the two groups of women, the negative correlation between its expression and mRNA expression of all analyzed lipogenic markers was found (**Figure 14D** and not shown).

ER chaperones are not elevated in elderly subcutaneous AT, despite increased expression of XBP-1s and PERK. To determine the level of ER stress, we measured the

mRNA expression of 9 UPR markers. Despite higher expression of *XBP1s*, an essential transcription factor activated by IRE1-UPR branch, and *PERK*, one of the stress sensors, in the elderly, the expression of ER chaperones *HSPA5*, *DNAJC3*, and *HYOU3* and phosphatase *GADD34* were significantly decreased in subcutaneous AT of the elderly group (**Figure 14C**). Expression of *HSPA5* and other chaperones correlated well with that of *FASN* and *DGAT2* (**Figure 14D** and not shown). mRNA expression of other ER genes involved in the UPR, specifically *ATF4*, *CHOP*, *EDEM1*, *CALR*, were not different between the groups.

Lower lipogenic potential of the elderly is manifested also in *in vitro* differentiated adipocytes and is negatively linked to GDF15 expression. To assess adipocyte-specific age-related differences in lipogenic, ER and senescence markers we used subcutaneous AT derived *in vitro* differentiated adipocytes. Adipose precursors were isolated from subcutaneous AT biopsies in the subgroups of volunteers (n=11 for each young and elderly), subcultivated for 3 passages and then differentiated into adipocytes. The accumulation of neutral lipids measured by Oil Red O staining was similar in adipocytes from both groups (33.9% ± 2.09 of ORO standard in the young, vs. 33.66% ± 1.626 in the elderly).

Similarly as seen in subcutaneous AT, *in vitro* differentiated adipocytes from the elderly exerted a co-regulated reduction of mRNA level for lipogenic genes (*FASN*, *DGAT2*, *SCD1*) compared to the cells from young group (**Figure 15A, D** and not shown) and the expression of all lipogenic markers was strongly correlated with the expression of GDF15 (**Figure 15D** and not shown). Also *HSPA5* and *DNAJC3* mRNA were less expressed in adipocytes from the elderly on the background of higher *XBP1s* expression. However, in adipocytes mRNA levels of chaperones did not correlate with the expression of lipogenic markers (**Figure 15C** and not shown).

In contrast to subcutaneous AT, the expression of senescent markers *p16* and *NOX4* was not different between the cells from two age-differing groups, while adipocytes from the elderly expressed 3 times more mRNA levels of *GDF15* compared to cells from the young (**Figure 15B**). Another difference between expression patterns in subcutaneous AT vs. adipocytes was higher and co-regulated expression of *ATF4*, a transcription factor important for the expression of ER chaperones, *EDEM1*, a marker of ER-associated degradation of misfolded glycoproteins (ERAD), and a proapoptotic transcription factor *CHOP*, in adipocytes from the elderly compared to cells from the young (**Figure 15C, D** and not shown).

	YOUNG	ELDERLY	*p*
	Obese	Overweight	
Number	15	15	
Age (year)	36.6 ± 1.8	72.1 ± 1.3	****
Weight (kg)	86.2 ± 2.1	70.4 ± 1.0	****
Fat mass (%)	37.2 ± 1.0	38.9 ± 1.0	*NS*
Fat mass (kg)	32.2 ± 1.5	27.5 ± 0.9	*
Waist circumference (cm)	97.1 ± 2.3	84.9 ± 1.2	***
Hip circumference (cm)	116.0 ± 1.7	104.7 ±1.2	****
Waist to Hip ratio	0.8 ± 0.0	0.8 ± 0.0	*NS*
BMI (kg/m2)	31.0 ± 0.6	27.0 ± 0.5	****
Cholesterol (nmol/l)	5.1 ± 0.2	4.9 ± 0.2	*NS*
HDL (nmol/l)	1.8 ± 0.1	1.7 ±0.1	*NS*
Triglycerides (nmol/l)	1.1 ± 0.1	0.9 ± 0.1	*NS*
Fasting glucose (nmol/l)	5.3 ± 0.1	6.0 ± 0.2	*
Fasting insulin (mU/l)	9.2 ± 0.8	6.8 ± 0.6	*
HOMA-IR	2.2 ± 0.2	1.9 ± 0.2	*NS*

Table 5: Clinical characteristics of young and elderly subjects. Abbreviations: BMI, body mass index; HDL, high-density cholesterol; HOMA-IR, homeostasis model assessment for insulin resistance; Data are presented as mean ± SEM.

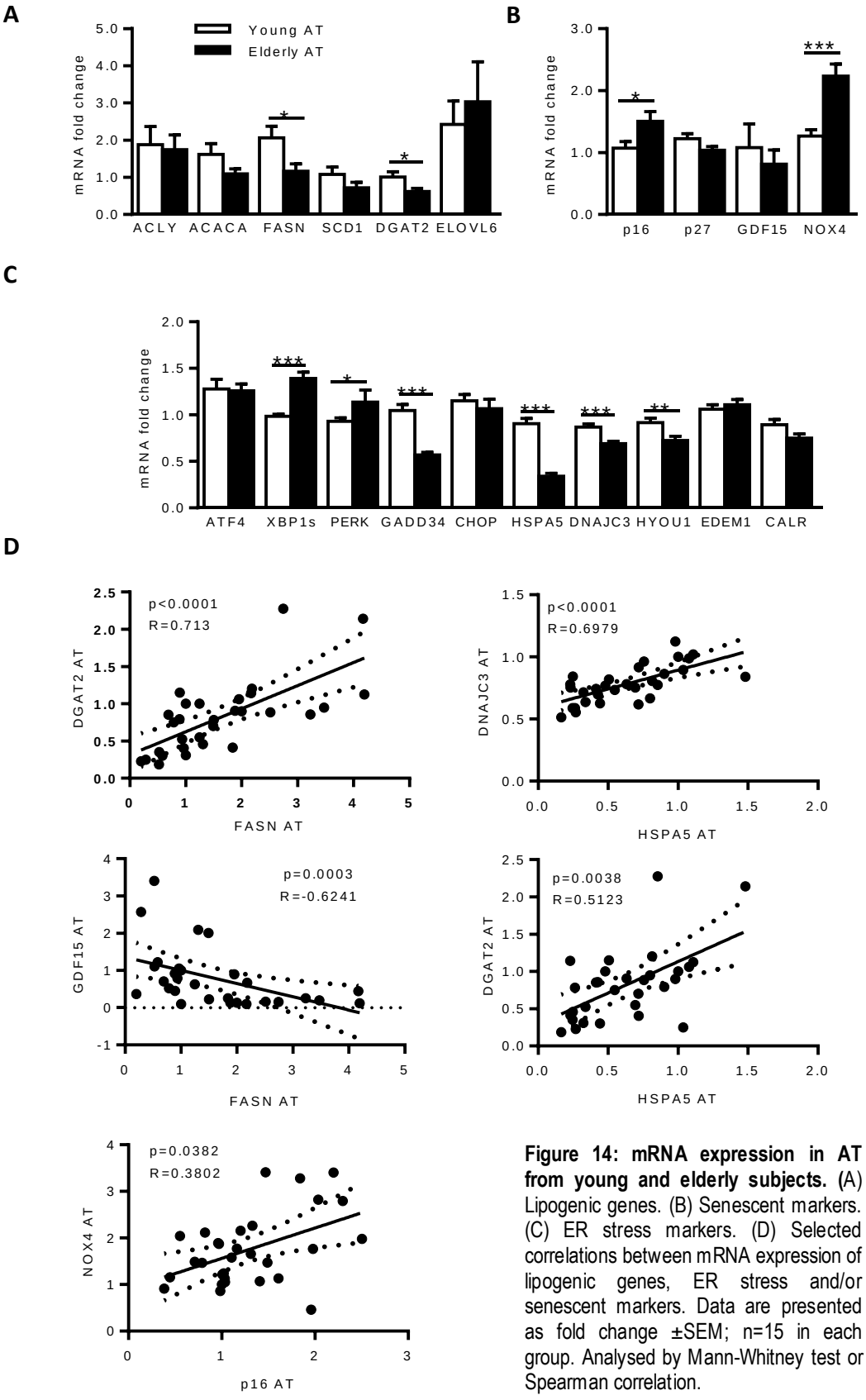


Figure 14: mRNA expression in AT from young and elderly subjects. (A) Lipogenic genes. (B) Senescent markers. (C) ER stress markers. (D) Selected correlations between mRNA expression of lipogenic genes, ER stress and/or senescent markers. Data are presented as fold change ±SEM; n=15 in each group. Analysed by Mann-Whitney test or Spearman correlation.

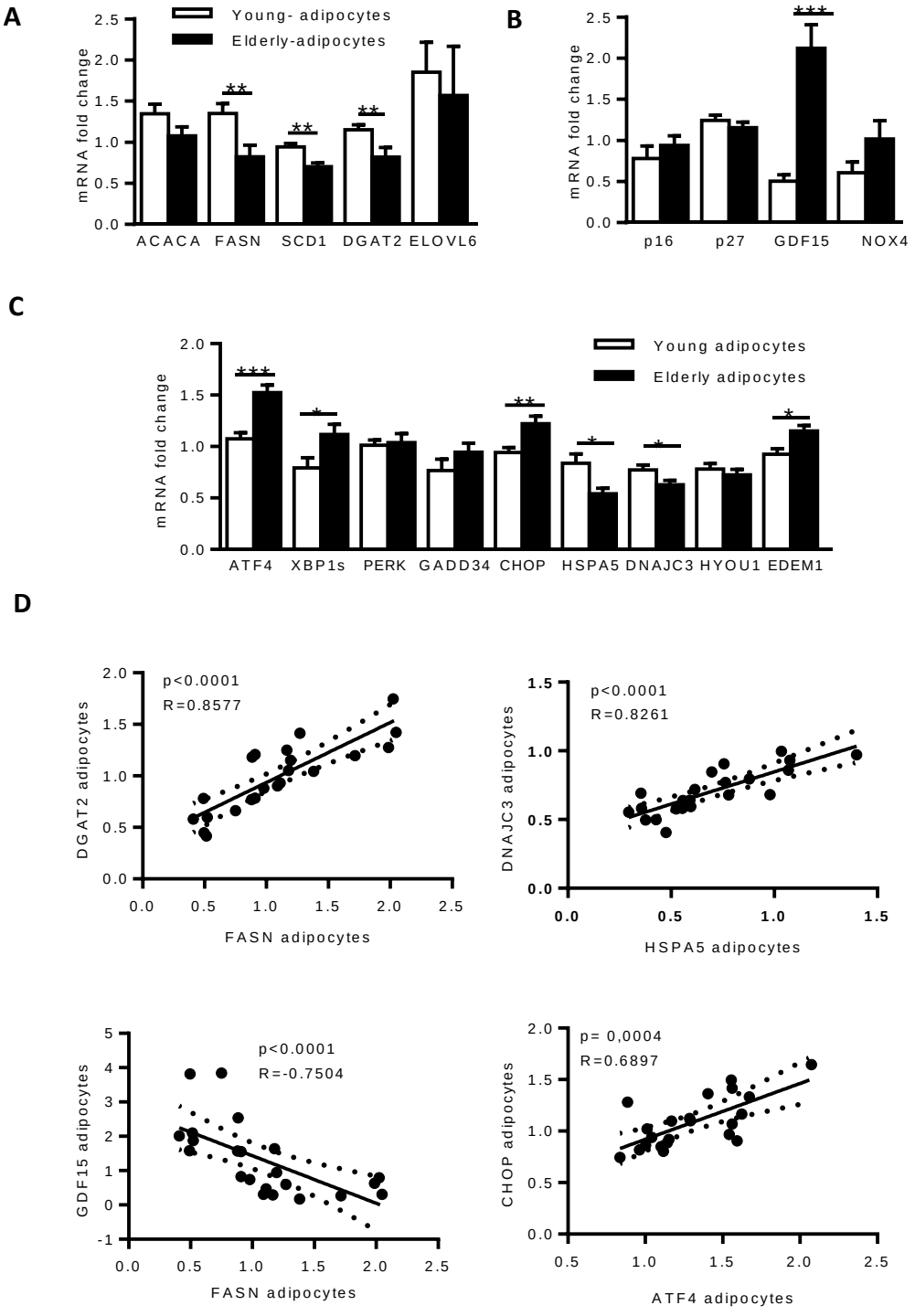


Figure 15: mRNA expression in adipocytes differentiated *in vitro* from young and elderly subjects. (A) Lipogenic genes. (B) Senescent markers. (C) ER stress markers. (D) Selected correlations between mRNA expression of lipogenic genes, ER stress and/or senescent markers. Data are presented as fold change ± SEM; n=11 in each group. Analysed by Mann-Whitney test or Spearman correlation.

4.3 Experimental part C

4.3.1 Introduction to experimental part C

VLCD are often prescribed in obesity treatment to achieve rapid weight loss. Generally, this type of dietary intervention consists of 500-800 kcal per day during 1-2 months, and leads to improvement in metabolic profile (such as plasma total cholesterol, triglycerides, HDL, insulin etc.) and insulin sensitivity [237]. A study that compared the effects of VLCD and bariatric surgery has shown that VLCD drives almost the same improvements of insulin sensitivity, β-cell function and lipid parameters as bariatric surgery when the same reduction of body weight and fat mass is achieved [284]. However, some of the positive effects of severe calorie restriction are observed already before the loss of fat mass is accomplished. Whole body/hepatic insulin resistance measured by *homeostasis model assessment for insulin resistance* (HOMA-IR) or *quantitative insulin sensitivity check index* QUICKI improved as soon as after two days of VLCD [285, 286]. Similarly the beneficial effects of bariatric surgery on carbohydrate metabolism were observed within several days after bariatric operation in type 2 diabetic patients, before significant weight loss has occurred [287]. Mechanisms of the beneficial metabolic effects of the calorie restriction *per se* are not well understood. It might be hypothesized that modifications of immune and metabolic characteristics of AT might occur and play a role in this process, in spite of the fact that there is no change in AT mass. While the response of inflammation-related cytokines during 1 month VLCD was investigated in a number of studies (reviewed in [237, 288]), the effects of a very short calorie restriction was studied rarely [285]. Similarly, it was shown that the expression of metabolism-related genes in AT was reduced after 1 month of VLCD [257], but the response to shorter calorie restriction (e.g. several days) was not thoroughly studied.

Therefore, in this longitudinal study we compared the effects of 2 days and 28 days of VLCD on metabolic and inflammation-related indices in subcutaneous abdominal AT (SAAT) and their possible relationship with systemic inflammatory and metabolic status in moderately obese women. We investigated expression of the respective genes in SAAT as well as secretion of cytokines in SAAT explants. The design of the study is on **Figure 16**.

According to recent studies, the diet- induced metabolic changes might be partially controlled by FGF21. FGF21 is released by liver and stimulates fatty acid oxidation and ketogenesis [289]. Recently, it was shown, in mice and in cell cultures that FGF21 may affect adipose tissue metabolic pathways (lipogenesis, lipolysis) [290, 291]. Thus, FGF21 levels and their association with changes in SAAT were also investigated.

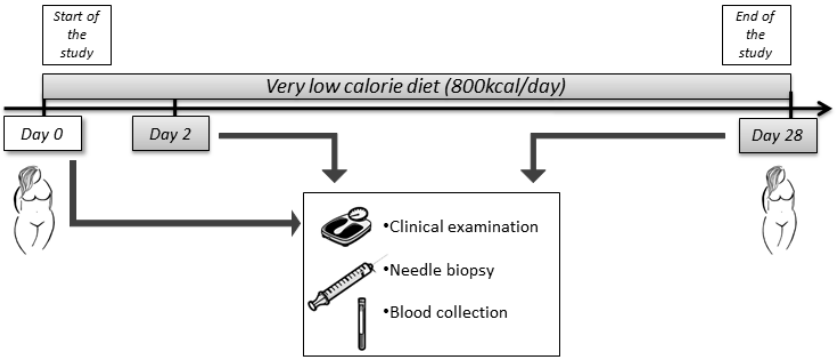


Figure 16: The design of the study. 17 obese pre-menopausal women were included in the study. Clinical examination, needle biopsy of SAAT and blood collection were performed at indicated days (day 0 - before the start of VLCD, day 2 – after 2days of VLCD, day 28 – at the end of one-month VLCD). Samples from needle biopsies and plasma were used for further analysis of inflammatory and metabolic characteristics during the intervention.

4.3.2 Results: experimental part C

Effect of dietary intervention on clinical and laboratory characteristics of obese women.

Anthropometric and biochemical parameters of subjects before and during two stages of the diet are presented in Table 1. At day 2 of VLCD the subjects´ body weight was reduced by 1.4%, while fat mass was not changed. After 28 days of VLCD a body weight loss of 9.2% was achieved, associated with a decrease of 16.5% of fat mass (kg).

Plasma glucose levels and TAG were not changed significantly during any phase of the intervention. FFA and β-hydroxybutyrate levels were elevated both after 2 days and 28 days of VLCD. Total cholesterol and HDL cholesterol levels decreased after 28 days of VLCD. Insulin and insulin resistance estimated by HOMA-IR decreased after 2 days of the diet by 13.7% and 16.4 %, while at the day 28 these variables decreased by 40 % and 44 %, respectively (**Table 6**).

Effect of dietary intervention on mRNA gene expression in SAAT.

Genes regulated after 2 days of VLCD

Among all the genes analyzed, those that were downregulated at day 2 were: three lipogenic genes (SCD1, FASN, and ELOVL6), lipogenic transcription factor SREBP1c, and fibrotic enzyme – lysyl oxidase (LOX).

Upregulated genes at day 2 were: lipases (ATGL, HSL), ATGL coactivator CGI58, transcription factor PPARγ and fatty acid translocase CD36. mRNA expression of glucose transporter GLUT1 had tendency to increase after 2 days of VLCD (p=0.09).

All other genes were not changed at day 2 of VLCD, explicitly I would mention the genes involved in β-oxidation (CPT1B, ACOX, ACADM, PPARα, PCG1) (**Figure 17C**), the genes involved in fibrosis (TLR4, collagens, TGFβ1, MMP9) (**Figure 17E**), and in inflammation (macrophage markers and cytokines) (**Figure 17F**), and several genes related to lipogenesis and lipolysis.

Genes regulated after 28 days of VLCD

Genes ***downregulated*** after 28 days of VLCD were: all lipogenic enzymes (SCD1, FAS, DGAT2, ACLY, ACACA, ELOVL6) and two lipogenic transcription factors (SREBP1c, ChREBP) (**Figure 17A**); lipolytic genes and regulators - MGL, G0S2 (an inhibitor of ATGL), PLIN1 (an inhibitor of HSL), and DGAT1 (an enzyme involved in the re-esterification of fatty acids and in lipogenesis) (**Figure 17B**); genes associated with β-oxidation of fatty acids – CPT1, ACOX1 and ACAD (**Figure 17C**); insulin stimulated glucose transporter (GLUT4) (Fig 2D); leptin (Fig 2D), and fibrotic enzyme - lysyl oxidase (**Figure 17E**).

Genes ***upregulated*** after 28 days of VLCD were some macrophage markers, namely CD163, MSR1, IRF5, and CCR2. The increase of mRNA expression of other markers (ACP5, FCGBP, ITGAX) and cytokines (IL8, MCP1, TNFα, IL6 and IL10) was observed but it did not reach statistical significance (**Figure 17F**)

Expression of all other genes was not significantly modified at the end of 28 days of the dietary intervention, specifically: genes involved in lipolysis: HSL, ATGL, CGI58, CD36 (**Figure 17B**), transcription factor PPARα, PPARγ and PPARγ coactivator PGC1α, IRS1, and genes involved in fibrosis (**Figure 17E**).

Correlations of the diet-induced changes in gene expression in SAAT and in metabolic parameters during VLCD intervention

2-days changes: Changes of circulating free fatty acids and glycerol after 2 days of VLCD correlated with changes in mRNA expression of CGI58 (Suppl. Fig 1.). The changes of glycerol after 2 days of VLCD correlated with expression changes of HSL and ATGL (**Suppl. Figure 1**). The changes of HOMA-IR after 2 days of VLCD tended to correlate with changes of HSL and ATGL expression (data not shown).

28-days changes: The changes in mRNA expression of leptin and LOX correlated positively with the changes of mRNA expression of lipolytic and lipogenic enzymes, β-oxidation, and IRS1 during 28 days of VLCD (**Suppl. Table 1**). The changes of HOMA-IR correlated with

changes of plasma levels and secretion of leptin (**Suppl. Figure 1, Suppl. Table 1**). Changes of cholesterol, insulin and TAG correlated with the changes of expression of several lipolytic and lipogenic genes (i.e. HSL, SCD1, FASN, DGAT2) (**Suppl. Figure 1**).
Changes of plasma FGF21 correlated positively with corresponding changes of β-hydroxybutyrate (r=0.537, p=0.048), and negatively with corresponding fold changes of ATGL, DGAT2, PPARγ and GLUT4 expression (**Suppl. Figure 1,** data not shown).

Secretion of cytokines/adipokines in SAAT during VLCD. *In vitro* secretion of cytokines IL6 and MCP1 from SAAT explants did not change after 2 days of VLCD but increased after 28 days of VLCD. Secretion of IL8 and TNFα was not significantly changed after 2 days, and tended to be increased after 28 days of VLCD (p=0.053; p=0.066; respectively). Secretion of IL10 was not significantly changed in either VLCD phase (**Figure 18**). Secretion of leptin was not changed after 2 days of VLCD but decreased significantly after 28 days of VLCD (**Figure 18**).

Plasma levels of cytokines, CRP, FGF21, and leptin during VLCD

Plasma concentration of cytokines IL6 and MCP1 increased after 2 days of VLCD, and returned to baseline after 28 days of VLCD (**Figure 19**). Similarly, CRP concentration had a tendency to increase after 2 days of VLCD (p=0.07) and decreased under the baseline values after 28 days of VLCD (Fig 4). IL8, IL10, TNFα and cortisol levels were not significantly changed either after 2 or 28 days of VLCD. The average plasma leptin levels did not change significantly after 2 days of VLCD (decrease by 21%, p=0.21), however, the response showed a high interindividual variability. After 28 days of VLCD the decrease of leptin was markedly pronounced (by 49%, p<0.001). FGF21 was not changed after 2 days of VLCD and was elevated after 28 days of VLCD (**Figure 19**).

	Before diet	2 days VLCD	28 days VLCD
Weight (kg)	93.5 ± 2.3	92.1 ± 2.3[a]	84.9 ± 2.3[a]
BMI (kg.m^{-2})	32.7 ± 0.9	32.2 ± 0.9[a]	29.7 ± 0.8[a]
Fat mass (kg)	38.9 ± 2.0	38.6 ± 1.9	32.3 ± 1.6[a]
Fat free mass (%)	59.6 ± 1.2	59.3 ± 1.2	63.5 ± 1.1[b]
Waist circumference (cm)	99.9 ± 1.7	98.6 ± 1.6[b]	92.6 ± 1.6[a]
Glucose (mmol.l^{-1})	5.0 ± 0.1	5.1 ± 0.1	4.9 ± 0.2
Insulin (mU.l^{-1})	10.2 ± 1.0	8.0 ± 0.6[c]	5.3 ± 0.6[a]
Free fatty acids (µmol.l^{-1})	820 ± 56	1156 ± 119[c]	1115 ± 70[b]
Glycerol (µmol.l^{-1})	124 ± 16	147 ± 14	113 ± 10
Triglycerides (mmol.l^{-1})	1.12 ± 0.12	1.04 ± 0.07	0.93 ± 0.10
HDL (mmol.l^{-1})	1.25 ± 0.05	1.21 ± 0.06	1.06 ± 0.04[a]
Total Cholesterol (mmol.l^{-1})	4.82 ± 0.20	4.86 ± 0.18	3.87 ± 0.13[a]
β-Hydroxybutyrate (mmol.l^{-1})	114 ± 19	379 ± 63[b]	603 ± 124[b]
HOMA -IR	2.3 ± 0.2	1.8 ± 0.1[c]	1.3 ± 0.2[a]
QUICKI	0.342 ± 0.005	0.354± 0.004[c]	0.386 ± 0.009[a]

Table 6: Clinical characteristics of 17 obese women before the diet, after 2 days and after 28 days of VLCD. Abbreviations: BMI, body mass index; HOMA-IR, homeostasis model assessment for insulin resistance; HDL, high-density cholesterol, QUICKI, Quantitative insulin sensitivity check index
Data are presented as mean ± SEM.
[a] $p < 0.001$ when compared with baseline (before the diet) values (one way ANOVA with repeated measures, followed by post hoc pairwise comparisons with Bonferroni adjustment for multiple testing);
[b] $p < 0.01$ when compared with baseline (before the diet) values (one way ANOVA with repeated measures, followed by post hoc pairwise comparisons with Bonferroni adjustment for multiple testing);
[c] $p < 0.05$ when compared with baseline (before the diet) values (one way ANOVA with repeated measures, followed by post hoc pairwise comparisons with Bonferroni adjustment for multiple testing);

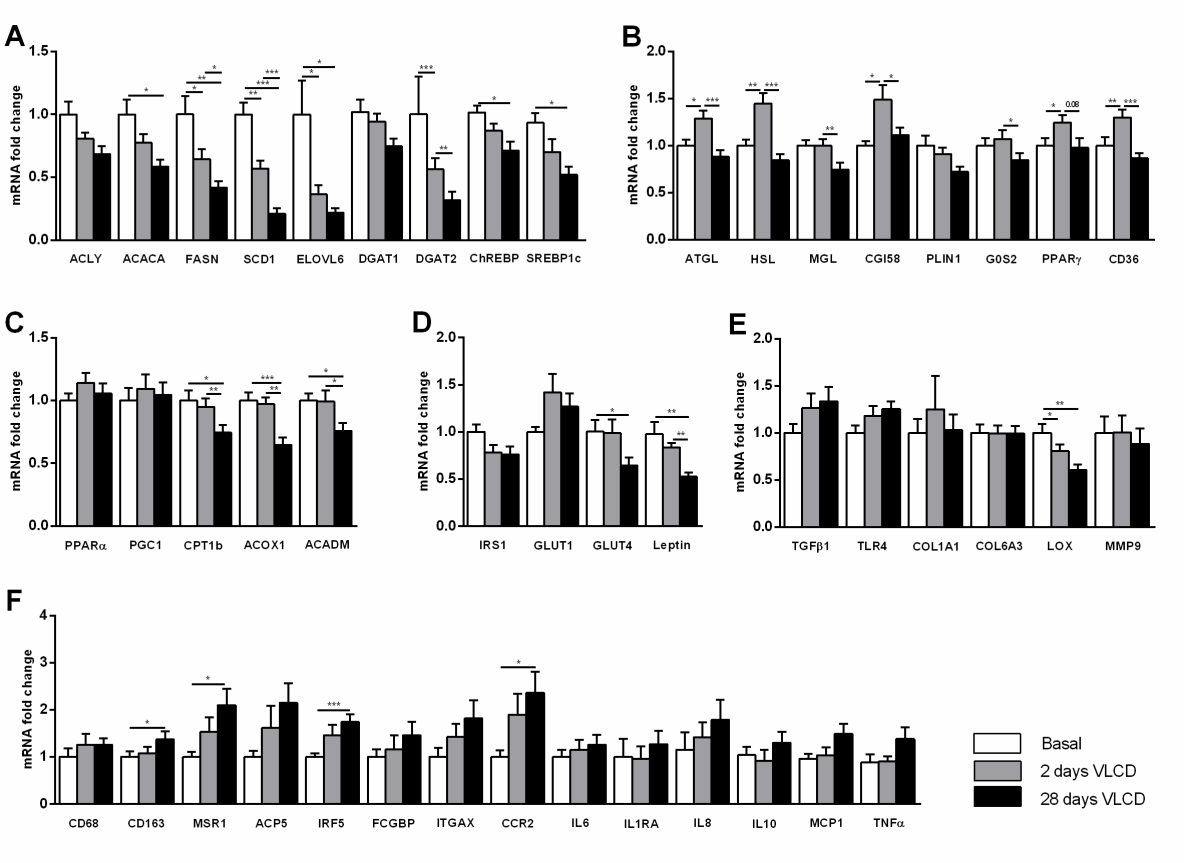


Figure 17: mRNA expression of genes in subcutaneous AT of obese women before the diet (white), 2 days after VLCD (grey) and 28 days after VLCD (black). (A) lipogenesis, (B) lipolysis, (C) β-oxidation, (D) insulin/glucose receptors and leptin, (E) fibrosis, (F) inflammation. Data are presented as fold change ± SEM, related to mean basal (before diet) gene expression; normalized to PPIA expression (n=16).
* p <0.05; ** p< 0.01; ***p< 0.001 compared to pre-diet levels or values at 2 days of VLCD (One-way ANOVA with repeated measures, followed by post-hoc pairwise comparisons with Bonferroni adjustment for multiple testing).

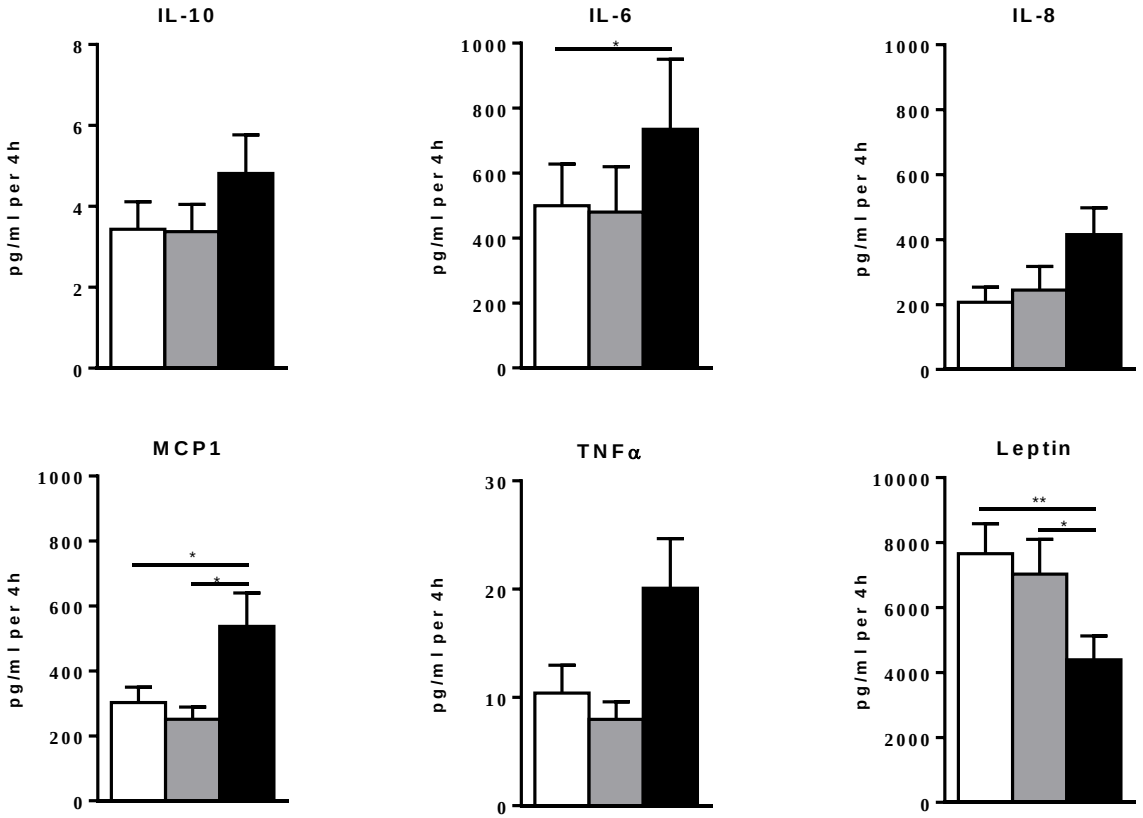


Figure 18: Secretion of cytokines / adipokines from subcutaneous adipose tissue of obese women before the diet (white), 2 days after VLCD (grey) and 28 days after VLCD (black). Data are presented as concentration of secreted protein (pg/ml per 4h) ± SEM (n=16); * p <0.05; ** p< 0.01; ***p< 0.001 compared to pre-diet levels or values at 2 days of VLCD (One-way ANOVA with repeated measures, followed by post-hoc pairwise comparisons with Bonferroni adjustment for multiple testing).

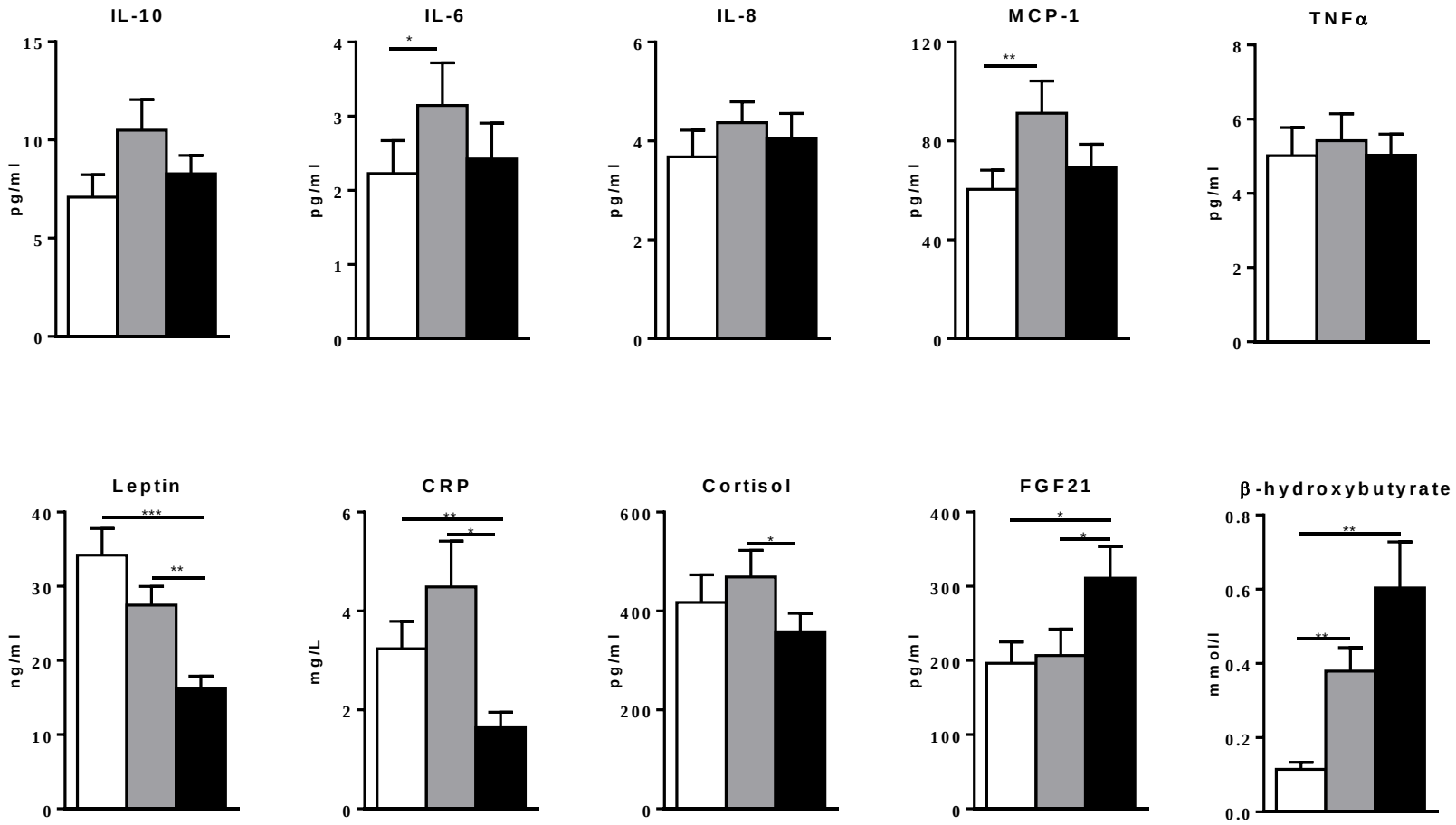
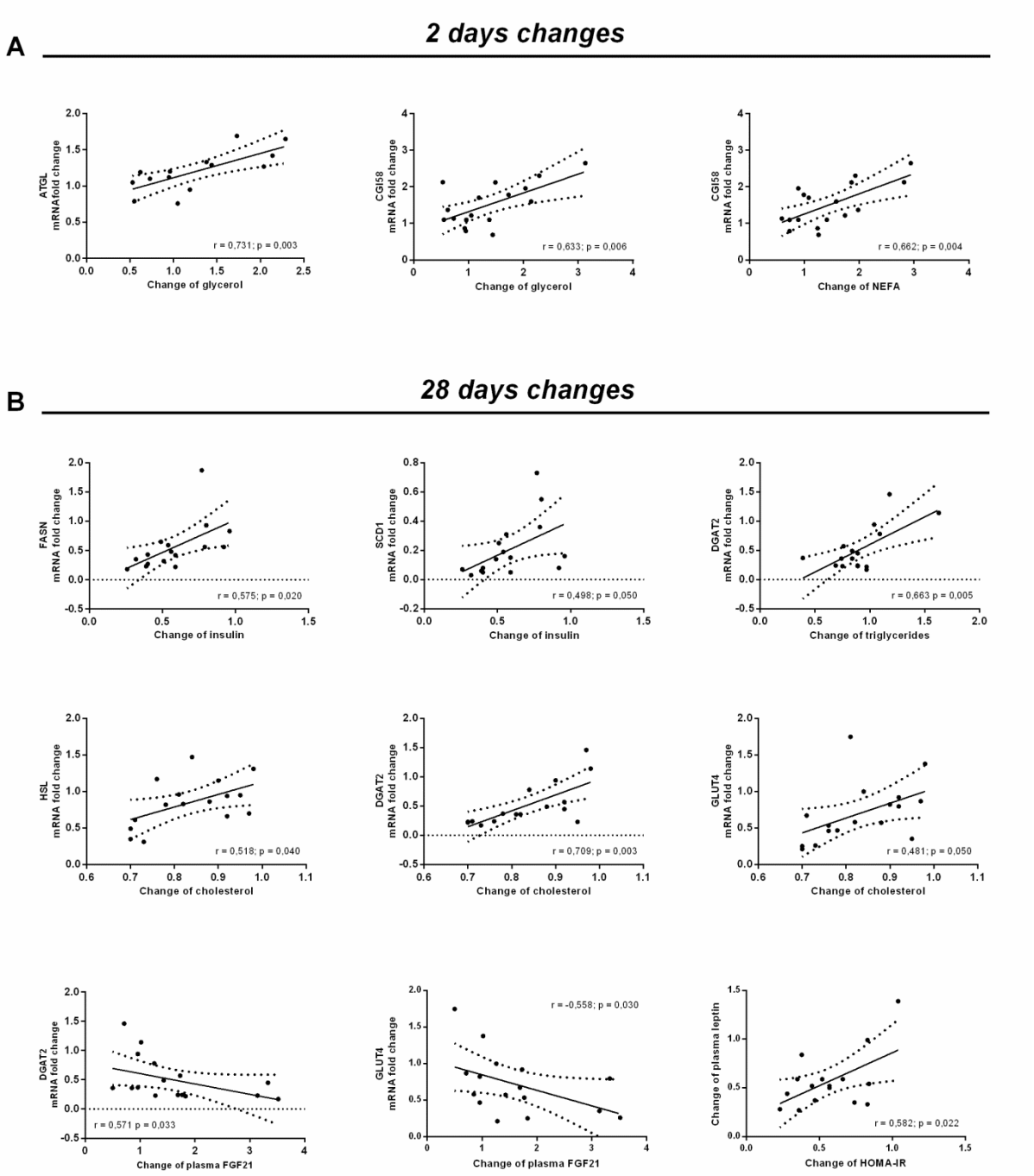


Figure 19: Plasma levels of cytokines and hormones in obese women before the diet (white), 2 days after VLCD (grey) and 28 days after VLCD (black). Data are presented as mean ± SEM, (n=17); * p <0.05; ** p< 0.01; ***p< 0.001 compared to pre-diet values or values at 2 days of VLCD (One-way ANOVA with repeated measures, followed by post-hoc pairwise comparisons with Bonferroni adjustment for multiple testing).



Supplementary figure 1: Correlations of selected mRNA gene expression in AT and fold changes of selected metabolic factors after A) 2 days and B) 28 days of VLCD. Analysed by linear regression, Pearson correlation coefficient is presented (n=16).

2 days of VLCD

mRNA (except of HOMA-IR)	Leptin (mRNA)	Leptin (secretion)	LOX (mRNA)	CD36 (mRNA)
ATGL	**0.662**[b]	0.276	0.284	**0.746**[c]
HSL	**0.647**[b]	0.135	0.469	**0.776**[c]
MGL	**0.636**[a]	-0.092	0.176	0.121
SCD1	**0.645**[c]	0.297	0.189	0.430
FASN	**0.668**[c]	0.250	0.513	**0.522**[a]
ACOX1	0.363	-0.194	0.207	0.051
ACADM	**0.551**[a]	0.335	0.254	0.256
PPARγ	**0.607**[b]	-0.104	**0.534**[a]	**0.841**[c]
PLIN	0.384	0.229	**0.593**[a]	0.239
GLUT4	0.386	0.408	**0.608**[a]	0.173
IRS1	0.300	0.300	**0.664**[b]	-0.248
SREBP	0.322	0.465	0.551	-0.047
ChREBP	**0.550**[a]	0.191	0.501	0.035
HOMA-IR	0.273	**0.782**[c]	-0.064	0.182

28 days of VLCD

mRNA (except of HOMA-IR)	Leptin (mRNA)	Leptin (secretion)	LOX (mRNA)	CD36 (mRNA)
ATGL	**0.706**[a]	**0.593**[a]	**0.658**[b]	**0.645**[b]
HSL	**0.712**[c]	**0.607**[a]	**0.641**[b]	**0.636**[b]
MGL	**0.709**[a]	**0.583**[a]	**0.756**[c]	0.248
SCD1	**0.702**[b]	**0.596**[a]	0.431	**0.507**[a]
FASN	**0.512**[a]	0.497	**0.589**[a]	0.488
ACOX1	0.414	0.332	**0.604**[a]	0.108
ACADM	0.372	0.347	**0.817**[c]	0.279
PPARγ	**0.641**[b]	**0.757**[a]	0.077	**0.819**[c]
PLIN	**0.594**[a]	0.423	**0.846**[c]	0.346
GLUT4	**0.593**[a]	**0.591**[a]	0.493	0.165
IRS1	**0.554**[a]	0.388	**0.668**[b]	0.280
SREBP	0.507	0.503	0.470	0.249
ChREBP	0.475	0.374	**0.689**[c]	0.242
HOMA-IR	0.445	**0.550**[a]	-0.426	0.439

Supplementary table 1: Correlations between diet-induced changes of selected genes/factors. Correlations between fold changes of metabolic factors during during 2 days and 28 days of VLCD ((value at 2 days/ baseline) or (value at 28 days/ baseline)). Pearson's correlation coefficient is presented (n=16). [a]$p<0.05$; [b]$p\leq0.01$; [c]$p\leq0.001$.

4.4 Experimental part D

4.4.1 Introduction to experimental part D

Obesity is one of the greatest challenge of the 21th century. As a serious medical complication, obesity impairs quality of life and constitutes one of a major economic burden worldwide. Another important contributor to the growing prevalence of the metabolic syndrome is the aging of the population [292, 293]. In fact, the median age of the population is increasing almost as dramatically as obesity. In 2015, the worldwide population of the elderly rose by 55 million thus reaching 8.5 % of the total population. As a result the median age of the total population is currently at the highest peak in human history (28 years in 2010) [294, 295].

Importantly, elderly population lives in the same obesogenic environment as other age groups and this poses a new health challenge: obesity in the elderly. Compared to obese children and adults in their productive age who can profit from obesity treatment, there is no evidence that weight loss has beneficial effects on AT of the elderly. Furthermore, appropriate treatment for obesity in older persons is controversial because weight loss in the elderly could have potential harmful effects, such as sarcopenia (the loss of skeletal muscle and function), thus exacerbating the age-related frailty.

Therefore, the aim of the project was to compare and define effects of moderate calorie restriction on *in vivo* and *in vitro* physiology of AT in two groups of obese men: juniors and seniors, with a special attention to biology of preadipocytes differentiated into adipocytes *in vitro* (**Figure 20**).

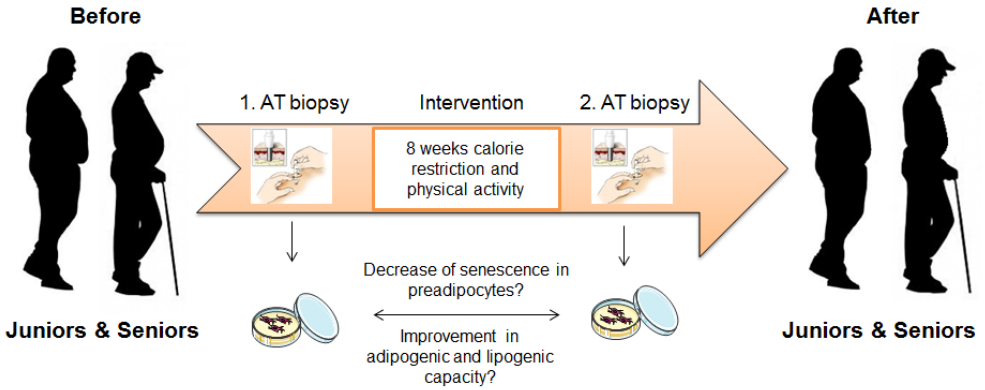


Figure 20: Design of the study. Obese juniors (age 30-40) and seniors (age 60-70) underwent eight weeks of calorie restriction (at least 20 % reduction of energetic need per day) in order to reach moderate weight loss. To prevent sarcopenia, subjects performed physical activity with professional sport trainer at least three times a week for 45-60 min. Needle biopsies of subcutaneous AT were taken before and after the intervention and used to isolate preadipocyte. To determine possible impact of the intervention, experiments/analysis were performed on preadipocytes and adipocytes differentiated *in vitro*.

4.4.2 Results : Experimental part D

Note: *Experiments performed in Project D vary with number of subjects. That is because cells from all subjects were not available for all the experiments. For comparison between juniors and seniors, only results from paired subjects are shown. In most cases, results from comparison within maximum number of subjects are not shown. These results did not differ statistically significantly from results analysed with only paired subjects.*

Clinical and laboratory characteristics of subjects

Anthropometric and biochemical parameters of subjects (juniors vs. seniors, before vs. after the intervention) are presented in **Table 7**. The two groups did not differ in any parameter except for age (35.82 ± 0.70 and 64.50 ± 1.10) and glucose disposal rate (GDR; 4.86 ± 0.38 and 3.79 ± 0.41).

The intervention induced weight loss in both groups. Compared with baseline values, the subject´s body weight decreased by 4.32 % in a group of juniors and 5.67 % in a group of seniors. BMI and fat mass (kg) decreased similarly in both groups. Lean body mass (%) increased significantly only in the group of juniors. The intervention decreased levels of cholesterol and LDL in both groups.

Preadipocyte proliferation

The rate of proliferation of preadipocytes assessed by Wst-1 assay did not differ between juniors and seniors (**Figure 21A**) and was not affected by the intervention in either group. (**Figure 21B, C**). Also, levels of another marker of proliferative status of preadipocytes, i.e. Akt phosphorylation at S473 in response to growth hormones, insulin, serum and other growth factors, were not different between the groups, either before and after the intervention (**Figure 22**).

Senescence status of preadipocytes

To determine quantitatively the level of senescence in preadipocytes in passage 3 or 4 (P3/4), activity of βgal at pH 6.0 and cellular size, two frequently used senescence markers, was analysed using flow cytometer. Representative images of the results and gating strategy are depicted on **Figure 23**. No difference in the percentage of βgal positive and large cells was between juniors and seniors, and before/after the intervention (**Figure 24**). Similarly, no

difference in mRNA expression of p16, a prototypical marker of senescence, was found between the groups and in response to the intervention (**Figure 25**).

Differentiation and metabolic capacity of adipocytes differentiated *in vitro*

As detected by ORO staining**,** no statistically significant difference in capacity to accumulate neutral lipids was found between juniors and seniors, even though there was a tendency to accumulate less lipids in the group of seniors (**Figure 26**). Similarly, the intervention did not increase significantly the capacity to accumulate the lipids in any of the groups. Metabolic assay using radioactive 2-deoxy glucose did not show any difference in the capacity of insulin stimulated glucose transport between juniors and seniors (**Figure 27**). However, the intervention improved the capacity of glucose uptake exclusively in the group of seniors. To confirm this result, Western blot was performed to quantify the phosphorylation of IRS1 and Akt upon insulin stimulation. Nevertheless, no difference was found between juniors and seniors, before or after the intervention (**Figure 28 and Figure 29**).

Gene expression of adipocytes differentiated *in vitro*

In total, mRNA gene expression of 91 genes involved in adipogenesis and lipogenesis (**Figure 30**), fatty acid oxidation and lipolysis (**Figure 31**), senescence and inflammation (**Figure 32**), ER stress, anti-oxidant reaction and insulin sensitivity (**Figure 33**), browning/whitening (**Figure 34**), fibrosis and other genes (**Figure 35**) was analysed. There was no statistically significant difference in the mRNA level encoding any of analysed genes between juniors and seniors prior the intervention. Interestingly, the intervention led to the increase of *PPARγ* and *ChREBP* mRNA levels in the group of seniors. Similarly, the expression of genes involved in fatty acid oxidation (Succinate Dehydrogenase Complex Flavoprotein Subunit A or *SDHA*, Ubiquinol-Cytochrome C Reductase Core Protein II or *UQCRC2*, *PPARα*), lipolysis (*ATGL, HSL, CD36*) and browning of AT (*GK, ELOVL3*) was increased after the intervention in the group of seniors. Interestingly, mRNA expression of *DPT* (marker of WAT) was decreased significantly when compared only the effect of intervention in seniors by Wilcoxon test. Similarly, mRNA expression of genes involved in *brightening/browning* (*CITED, TMEM26, PDK4, SIRT3*) was increased significantly in seniors when analysed by Wilcoxon test. mRNA expression of the other genes stayed unchanged and expression of *DLK1* and *MMP9* was not detected.

	JUNIORS			SENIORS			JUNIORS X SENIORS	JUNIORS X SENIORS
	Before	After	Before X after	Before	After	Before X after	Before	After
Age (year)	35.82 ± 0.70	35.13 ± 0.74	NS	64.50 ± 1.10	64.25 ± 1.21	NS	***	***
Weight (kg)	107.29 ± 3.42	102.96 ± 4.05	**	101.50± 3.41	96.59 ± 3.64	***	NS	NS
BMI	33.53 ± 0.53	32.41 ± 0.77	***	33.98 ± 0.50	32.22 ± 0.64	***	NS	NS
Fat mass (kg)	40.01 ± 2.18	36.65 ± 3.18	***	36.65 ± 2.02	33.46 ± 2.42	**	NS	NS
Muscular mass (kg)	35.06± 1.21	34.43 ± 1.54	NS	31.70 ± 0.61	30.95 ± 0.64	NS	NS	NS
Lean body mass (%)	59.46 ± 1.31	61.25 ±2.10	**	60.84 ± 0.81	62.22 ± 1.19	NS	NS	NS
TAG (nmol/l)	1.90 ± 0.23	1.41 ± 0.22	NS	1.84 ± 0.14	1.24 ± 0.21	NS	NS	NS
Cholesterol (mmol/l)	5.06 ± 0.24	4.51 ± 0.29	*	5.09 ± 0.32	4.39 ± 0.78	*	NS	NS
HDL (mmol/l)	1.03 ± 0.07	1.04 ±0.07	NS	1.12 ± 0.04	1.11 ± 0.05	NS	NS	NS
VLDL (nmol/l)	0.86 ± 0.10	0.64 ± 0.10	NS	0.84 ±0.06	0.57 ±0.10	NS	NS	NS
LDL (mmol/l)	3.17 ± 0.26	2.89 ± 0.24	*	3.13 ± 0.30	2.68 ± 0.52	*	NS	NS
GDR (mg/min/kg)	4.86 ± 0.38	5.63 ± 0.26	NS	3.79 ± 0.41	4.20 ± 0.37	NS	*	*

Table 7: Clinical characteristics of obese juniors (n=11) and seniors (n=10) before and after the intervention. Data are presented as mean ± SEM. The comparison was done in each group for 8 paired subjects which did not differ from the whole group of juniors or seniors, respectively. Analysed by two-way ANOVA. *p<0.05, **p<0.01.

Figure 1

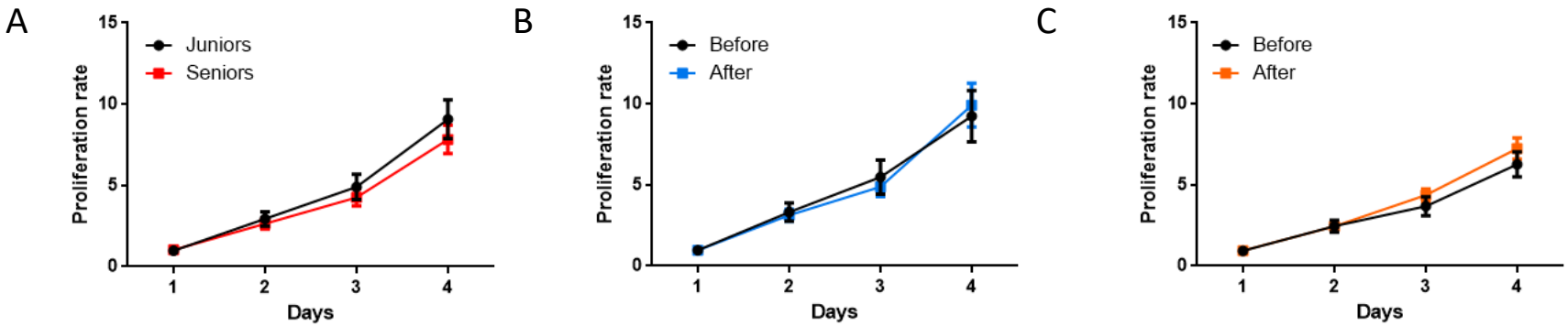


Figure 21: Proliferation rate of preadipocytes measured by Wst-1 assay. (A) Comparison of juniors (n=11) and seniors (n=10). Impact of the intervention is depicted in (B) for juniors (n=8) and in (C) for seniors (n=8). Expressed as a fold change (related to the absorbance in the day of seeding). Analysed by two-way ANOVA, ±SEM.

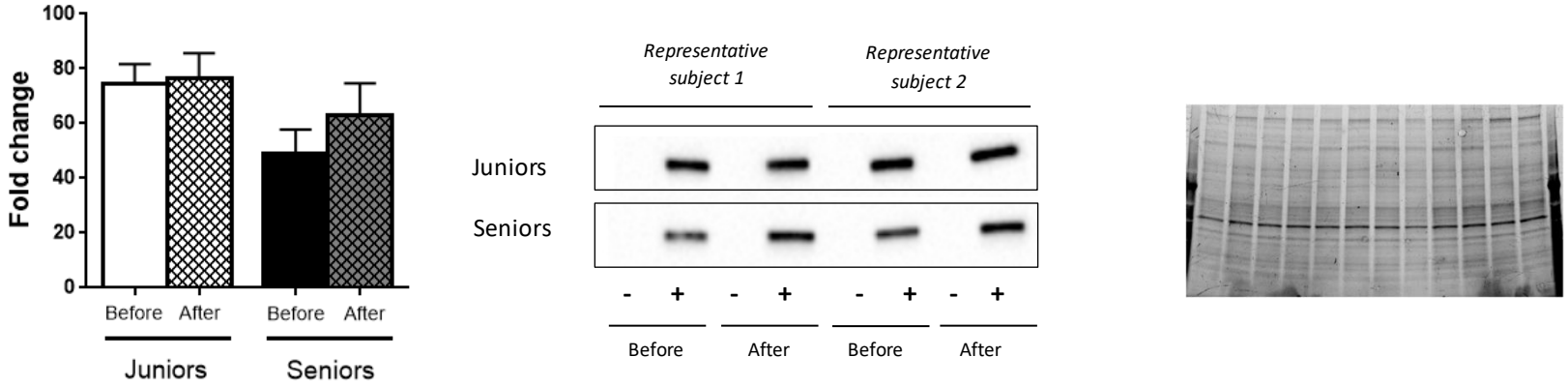


Figure 22: Sensitivity of preadipocytes to proliferative stimuli. (A) Quantitative bar graphs of S473-Akt phosphoryation in juniors (n=3) and seniors (n=7), before and after the intervention. Data are expressed as a fold change of induction. (B) Representative western blot. (C) Phosphorylation was related to total protein load. Analysed by two-way ANOVA, ±SEM.

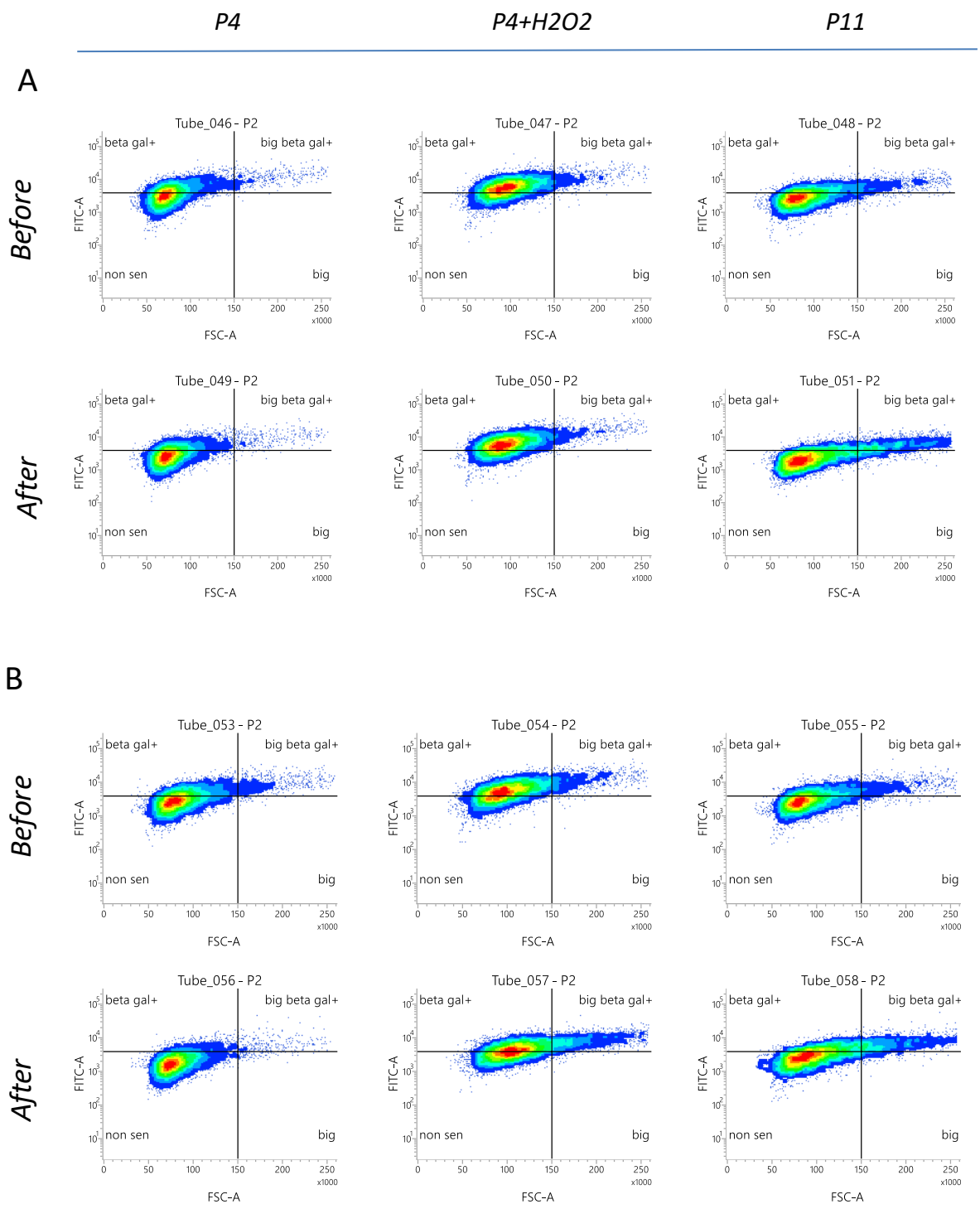


Figure 23: Gating strategy for the detection of senescence preadipocytes. Activity of βgal at pH 6.0 was determined on preadipocytes at P3/P4 using flow cytometer. Two parameters were taken into account during the analysis: % of β-galactosidase positive cells (determined on FITC channel) and cellular size (determined by Forward Scatter, FSC). Four populations were obtained: βgal negative cells, βgal positive cells, BIG βgal positive cells and BIG cells. Non-treated cells were used as a negative control and preadipocytes treated with H_2O_2 and preadipocytes at passage 11 served as positive controls. **A.** Representative images of junior before and after the intervention. **B.** Representative images of seniors before and after the intervention.

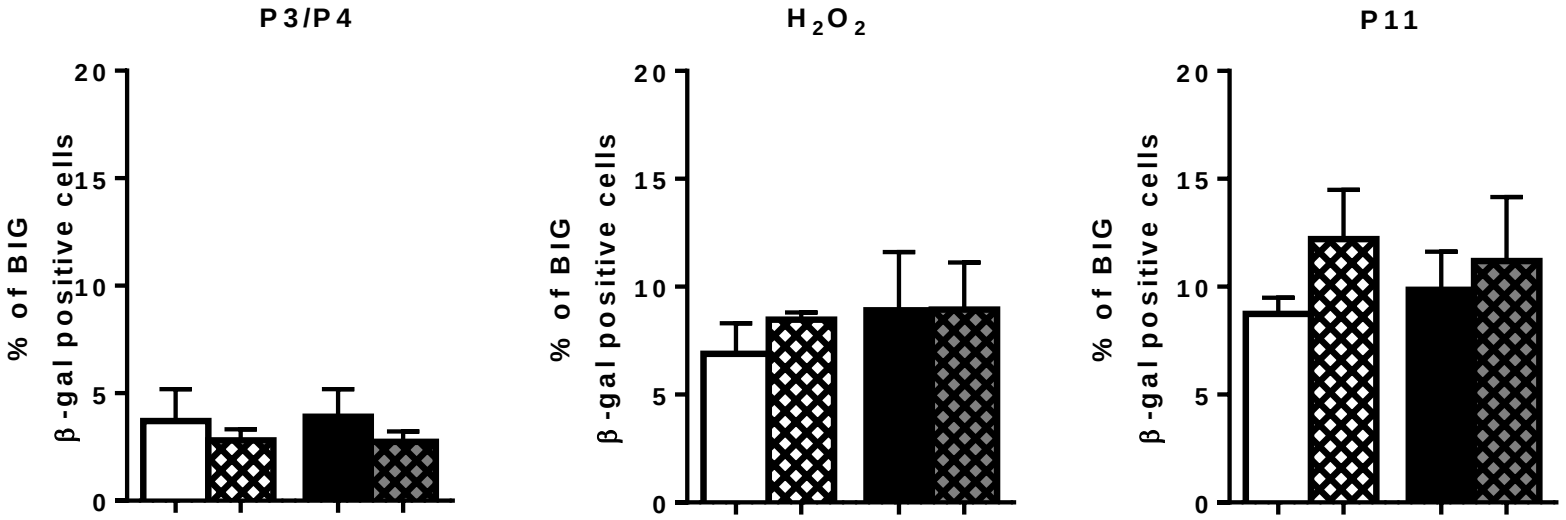


Figure 24: Senescence status of preadipocytes. Detected activity of βgal at pH 6.0 and cellular size were taken into account to determine percentage of senescent preadipocytes at P3/P4, P3/P4 treated with H_2O_2 and P11. Analysed by two-way ANOVA with Bonferroni adjustement for multiple testing; n=4-6 in each group;

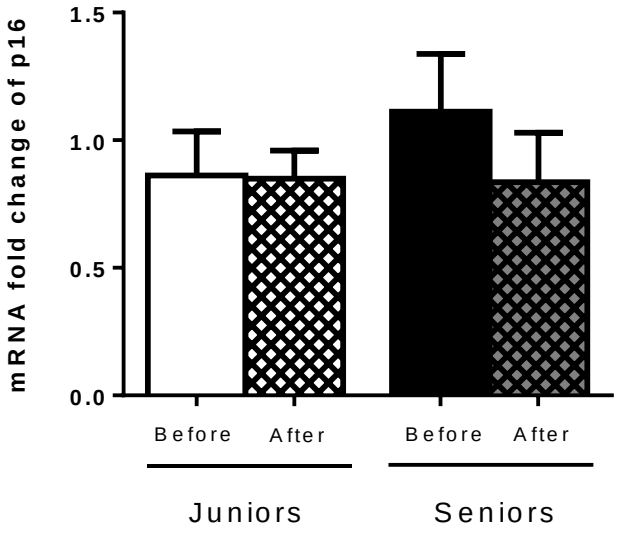


Figure 25: mRNA expression level of p16 in preadipocytes. Analysed by two-way ANOVA with Bonferroni adjustement for multiple testing; n=6 in each group;

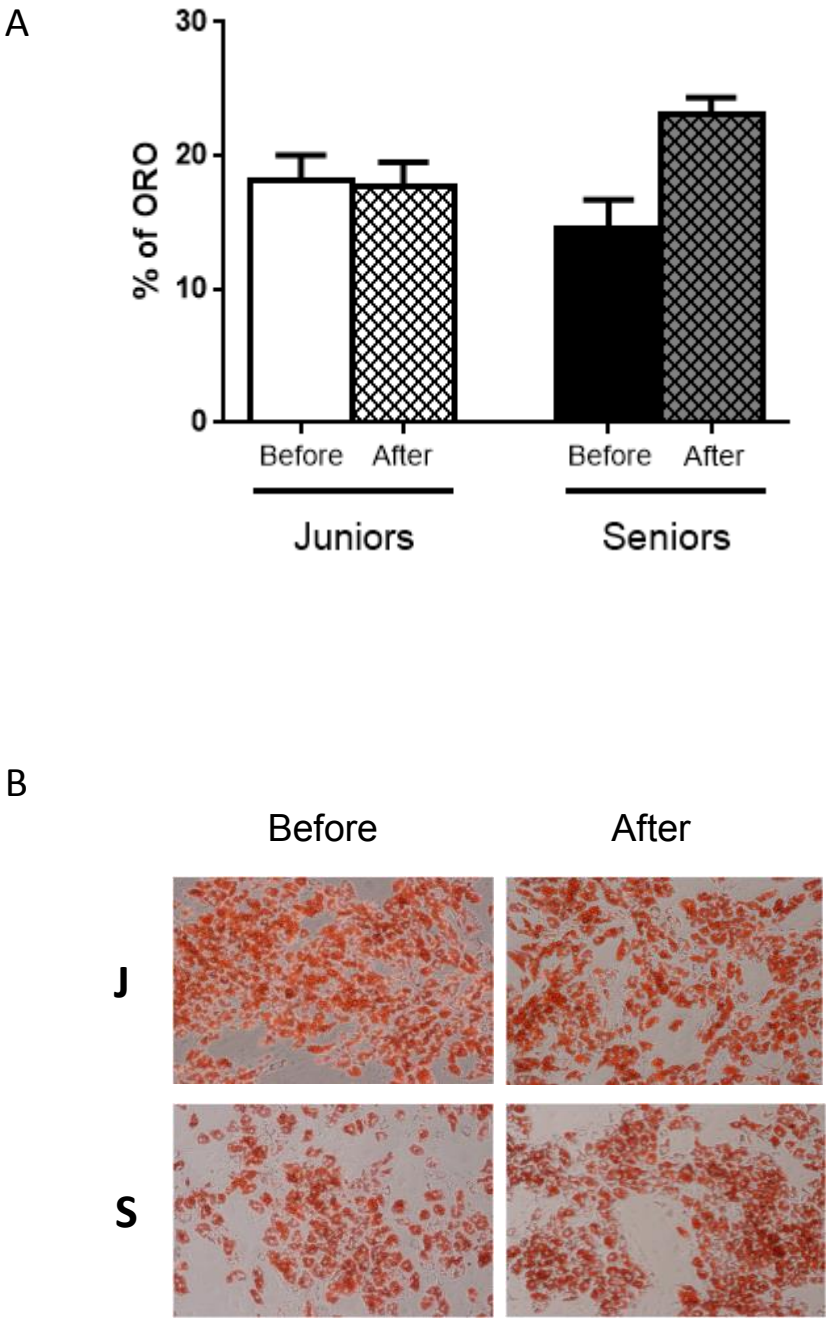


Figure 26: *In vitro* differentiation of preadipocytes. Cells were differentiated for 12 days and then the accumulation of lipids was analysed using Oil-Red O staining. (A) Comparison of level of differentiation between juniors and seniors (n=7 in each group) and the impact of the intervention. (B) Representative photographs of junior (J) and senior (S). Analysed by Mann-two-way ANOVA, ±SEM.

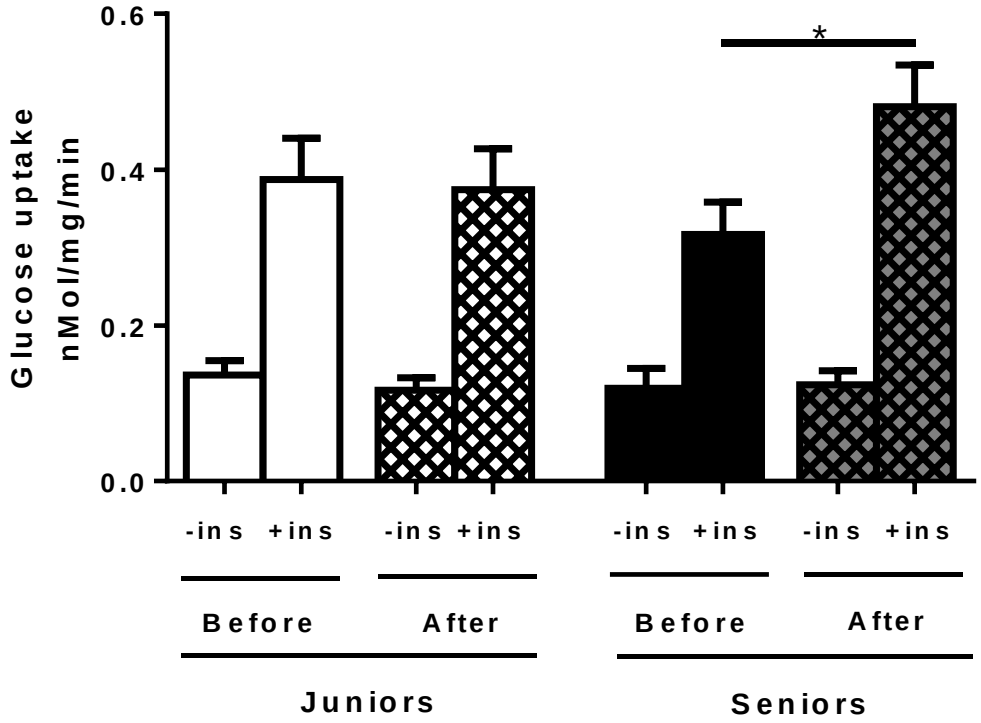


Figure 27: Glucose transport in adipocytes differentiated *in vitro*. Cells were differentiated for 12 days and deprived from insulin overnight. The next day, glucose transport in response to 100nM insulin was determined using radiolabelled 2-deoxyglucose. Comparison between juniors and seniors, before and after the intervention (n=7 in each group). Analysed by two-way ANOVA, ±SEM; *p<0.05.

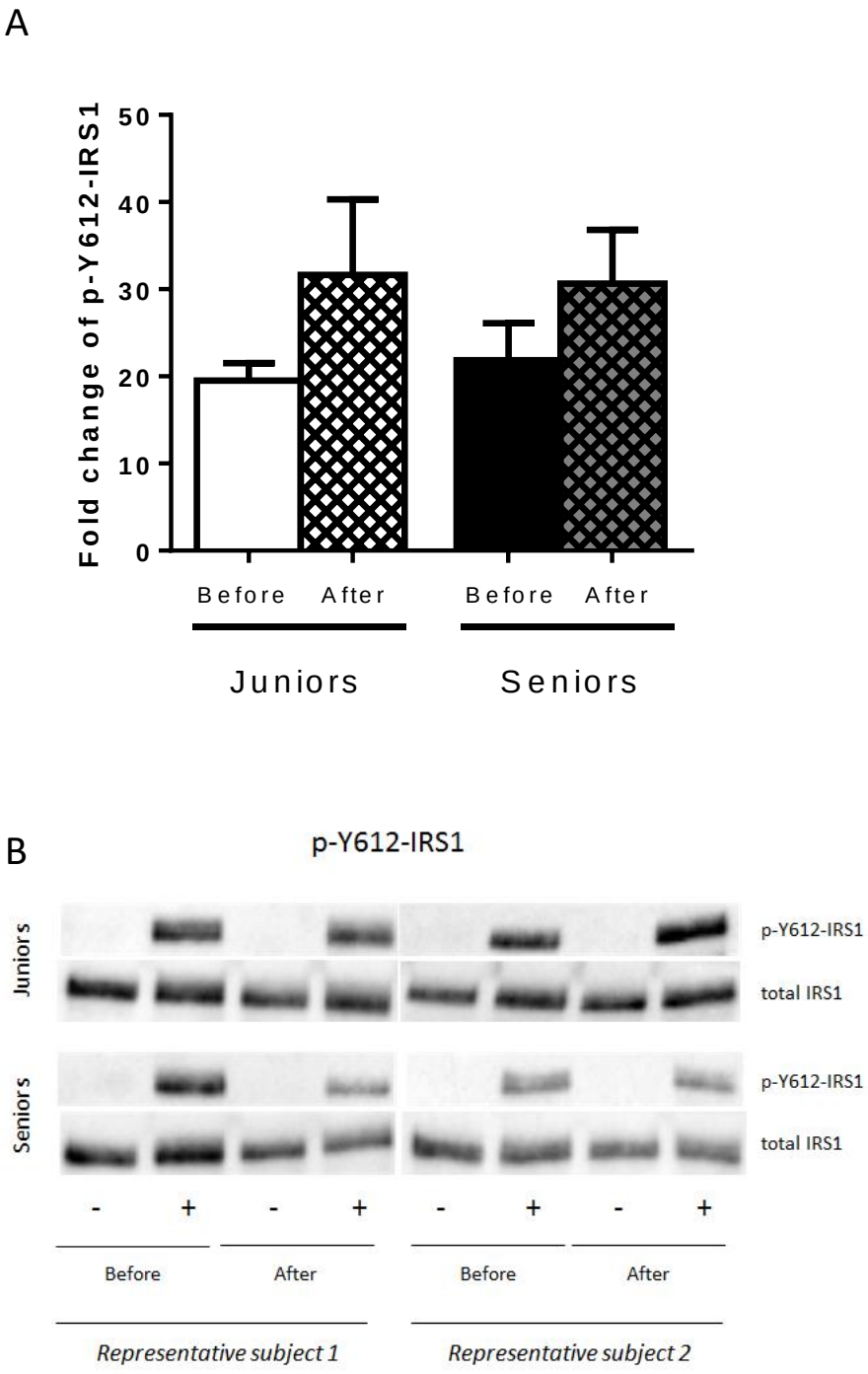
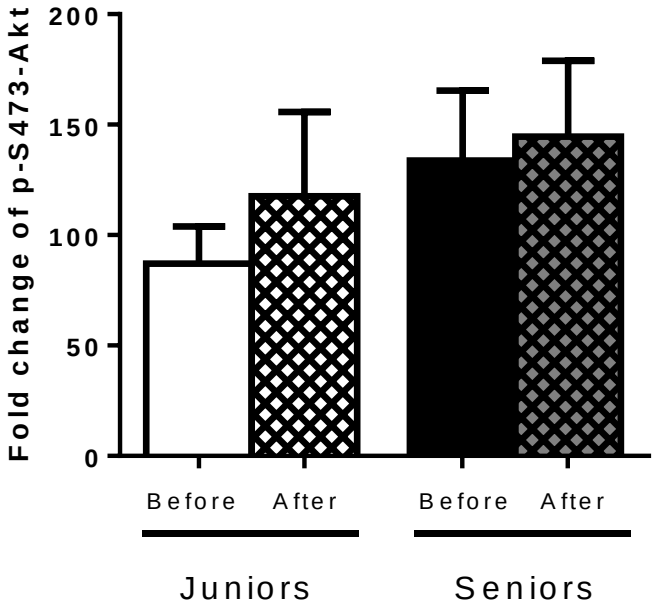


Figure 28: Insulin sensitivity determined by phosphorylation of IRS1. Cells were differentiated for 12 days and deprived of insulin overnight. The next day, 100 nM insulin was added for 10 min. Phosphorylation of Y612-IRS1 was determined using Western blot. (A) Bar graphs of comparison between juniors (n=7) and seniors (n=8), before and after the intervention. (B) Representative images from western blot. Analysed by two-way ANOVA, ±SEM.

A



B

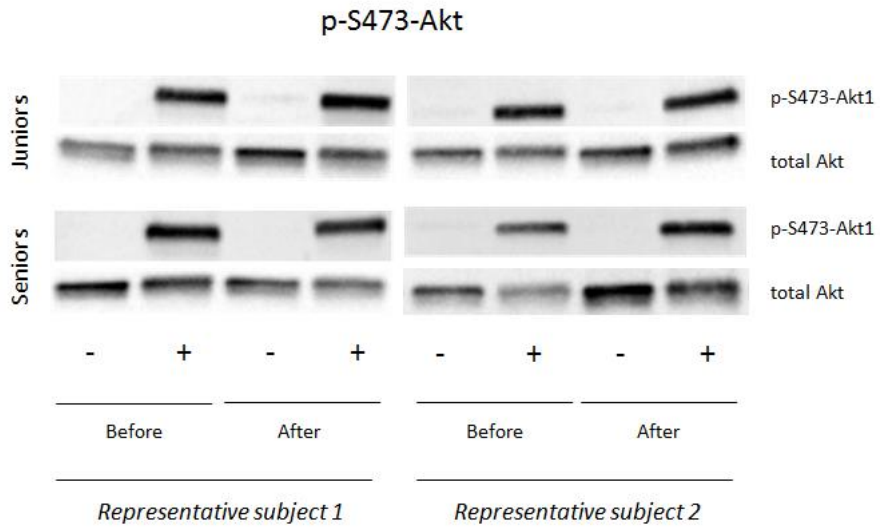


Figure 29: Insulin sensitivity determined by phosphorylation of Akt. Cells were differentiated for 12 days and deprived of insulin overnight. The next day, 100 nM insulin was added for 10 min. Phosphorylation of S473-Akt was determined using Western blot. (A) Bar graphs of comparison between juniors and seniors (n=8 in each group), before and after the intervention. (B) Representative images from western blot. Analysed by two-way ANOVA, ±SEM.

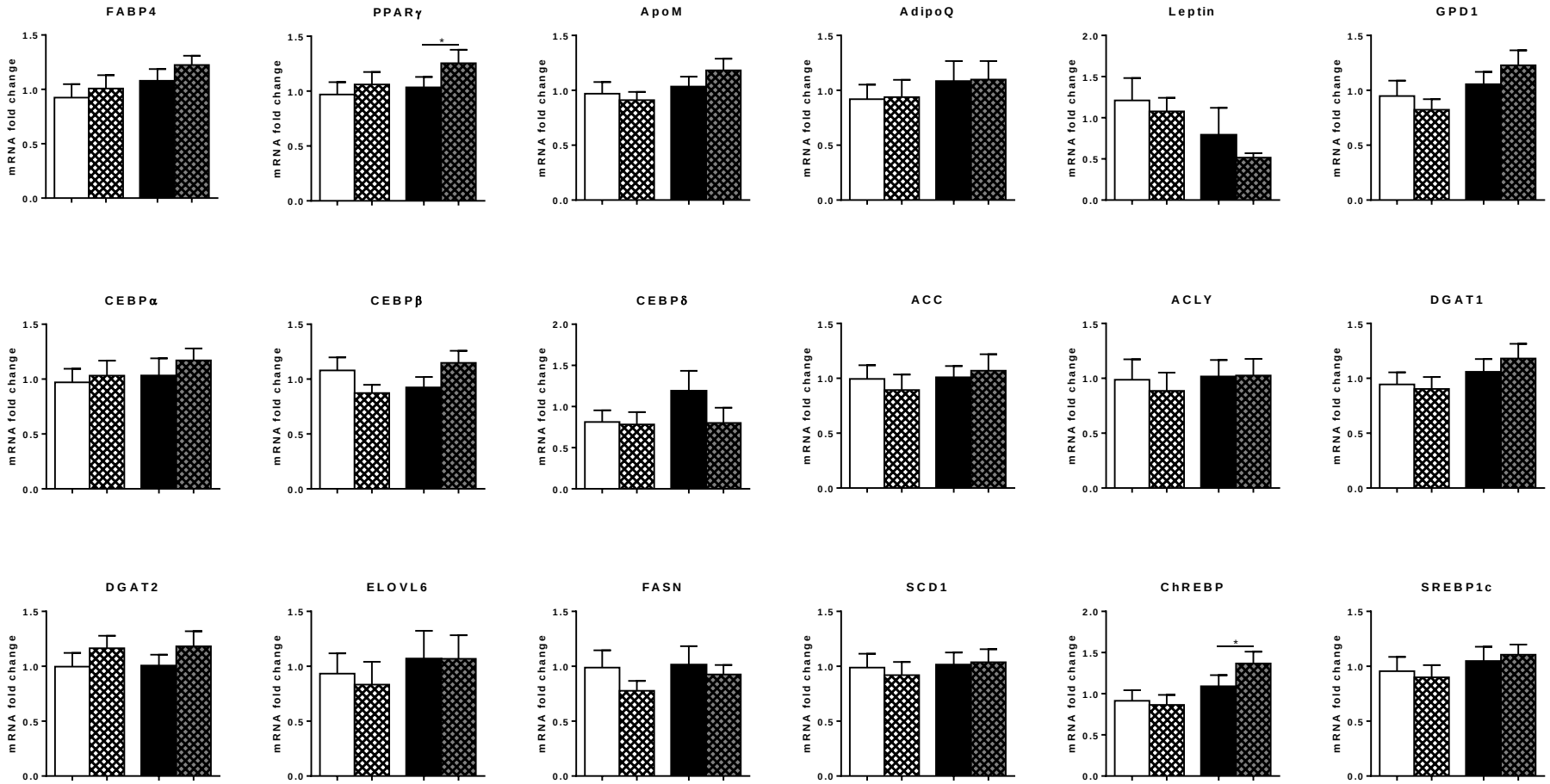


Figure 30: mRNA expression of genes involved in adipogenesis and lipogenesis from adipocytes differentiated *in vitro* of obese juniors and seniors before and after the intervention Data are presented as fold change ± SEM, normalized to PPIA expression; n=8 in each group. Analysed by two-way ANOVA.

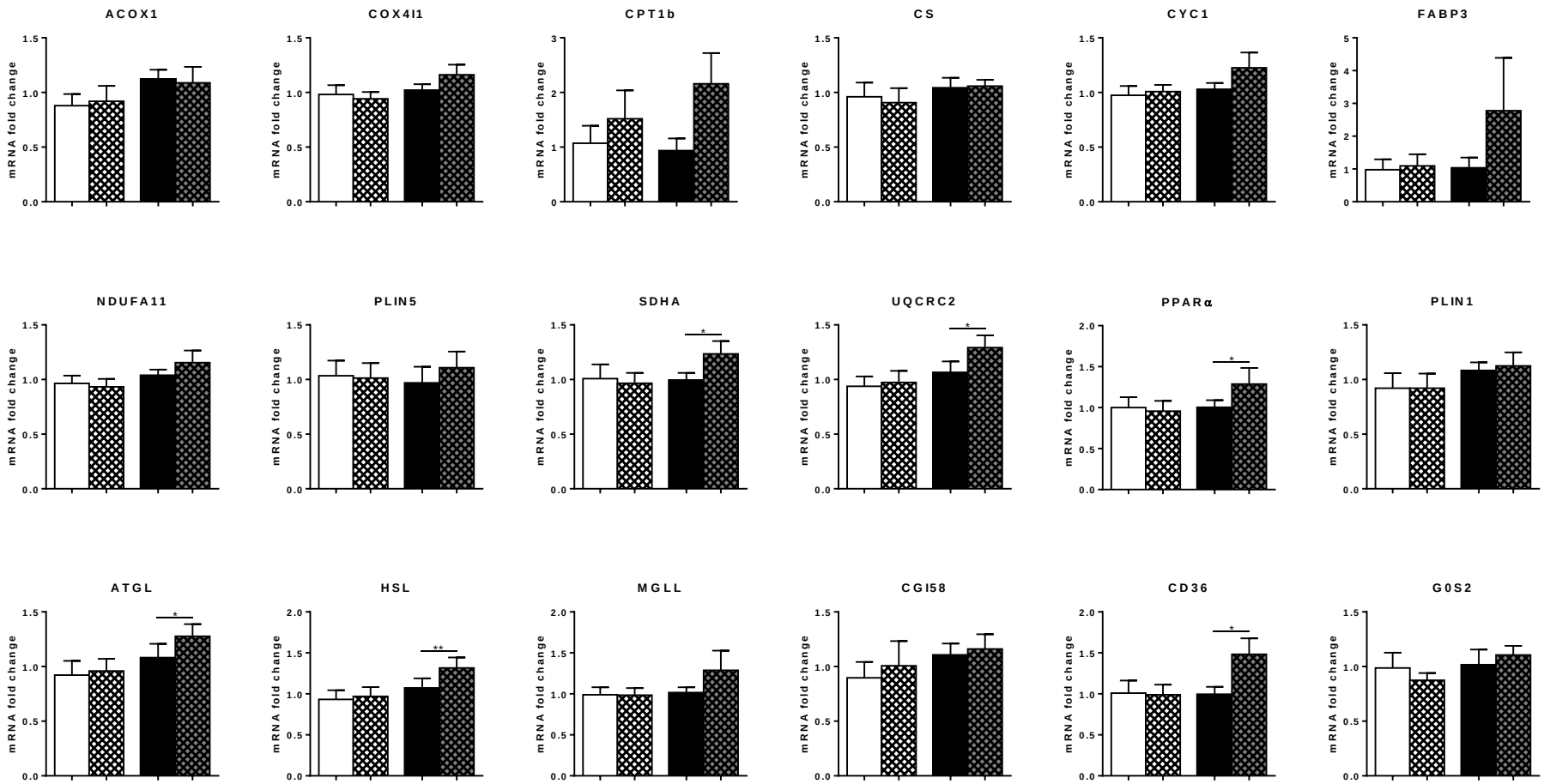


Figure 31: mRNA expression of genes involved in fatty acid oxidation and lipolysis from adipocytes differentiated *in vitro* of obese juniors and seniors before and after the intervention Data are presented as fold change ± SEM, normalized to PPIA expression; n=8 in each group. Analysed by two-way ANOVA. *p<0.05, **p<0.01.

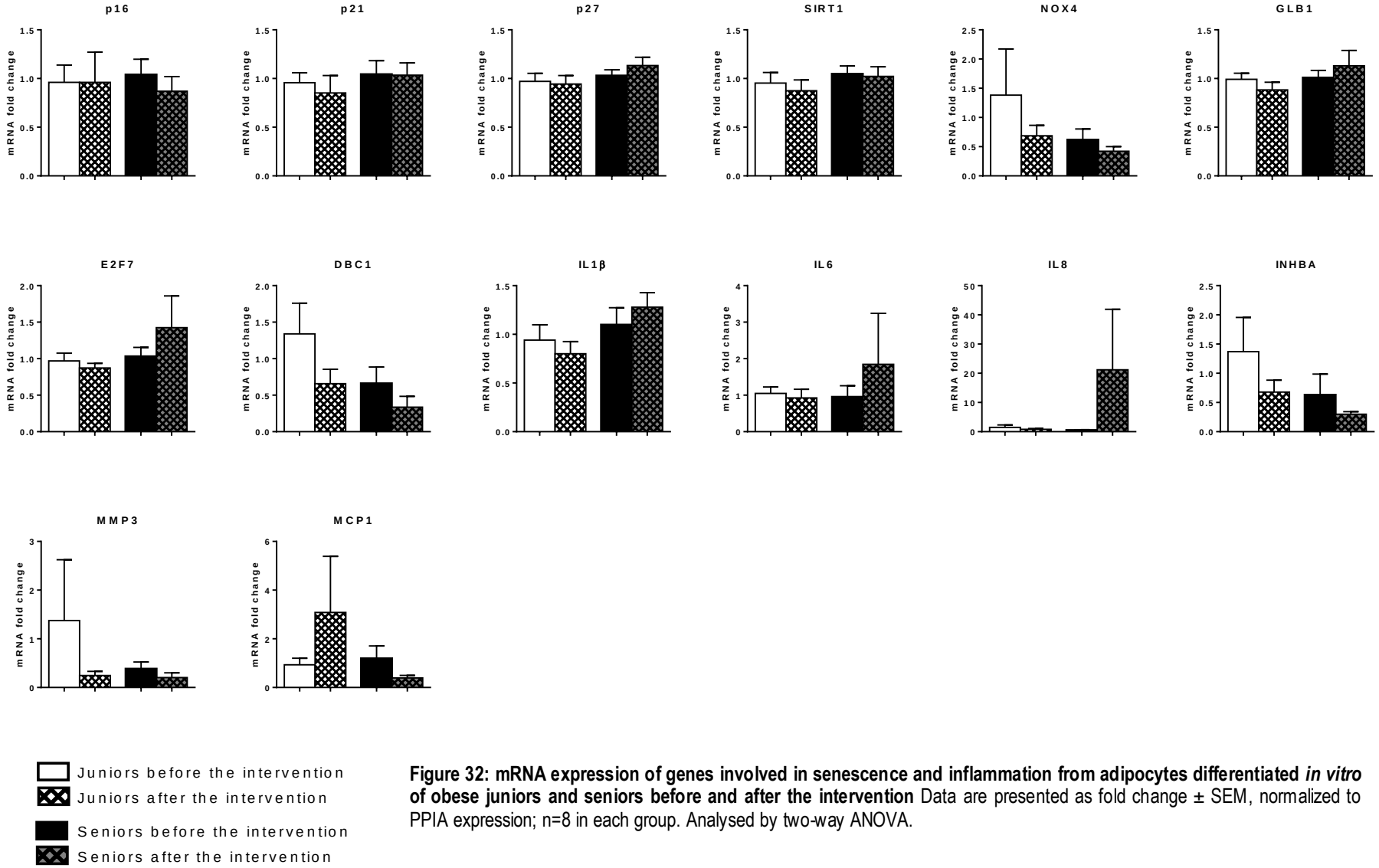


Figure 32: mRNA expression of genes involved in senescence and inflammation from adipocytes differentiated *in vitro* of obese juniors and seniors before and after the intervention Data are presented as fold change ± SEM, normalized to PPIA expression; n=8 in each group. Analysed by two-way ANOVA.

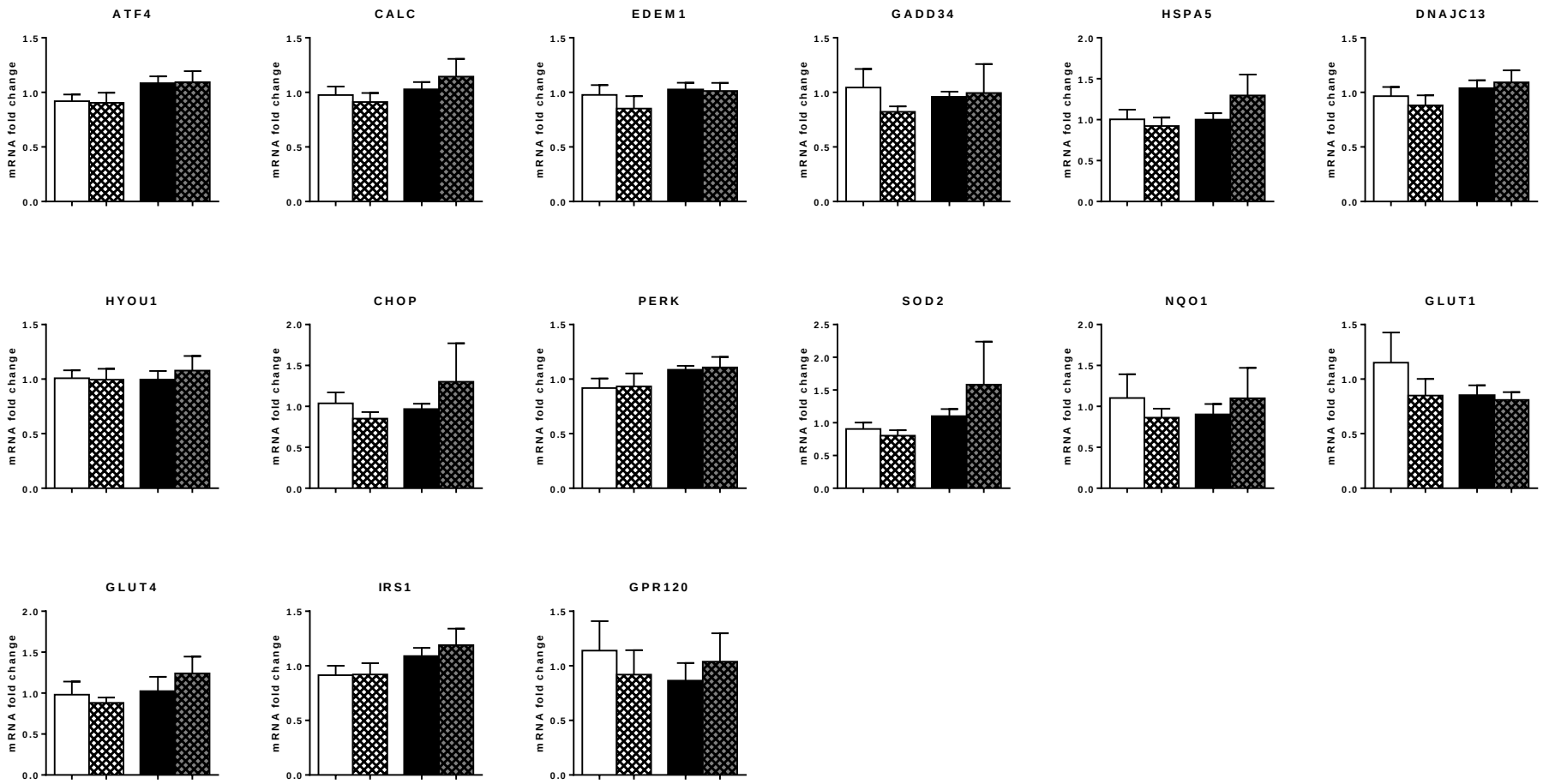


Figure 33: mRNA expression of genes involved in endoplasmic reticulum stress, anti-oxidant reactions and insulin sensitisation from adipocytes differentiated *in vitro* of obese juniors and seniors before and after the intervention Data are presented as fold change ± SEM, normalized to PPIA expression; n=8 in each group. Analysed by two-way ANOVA.

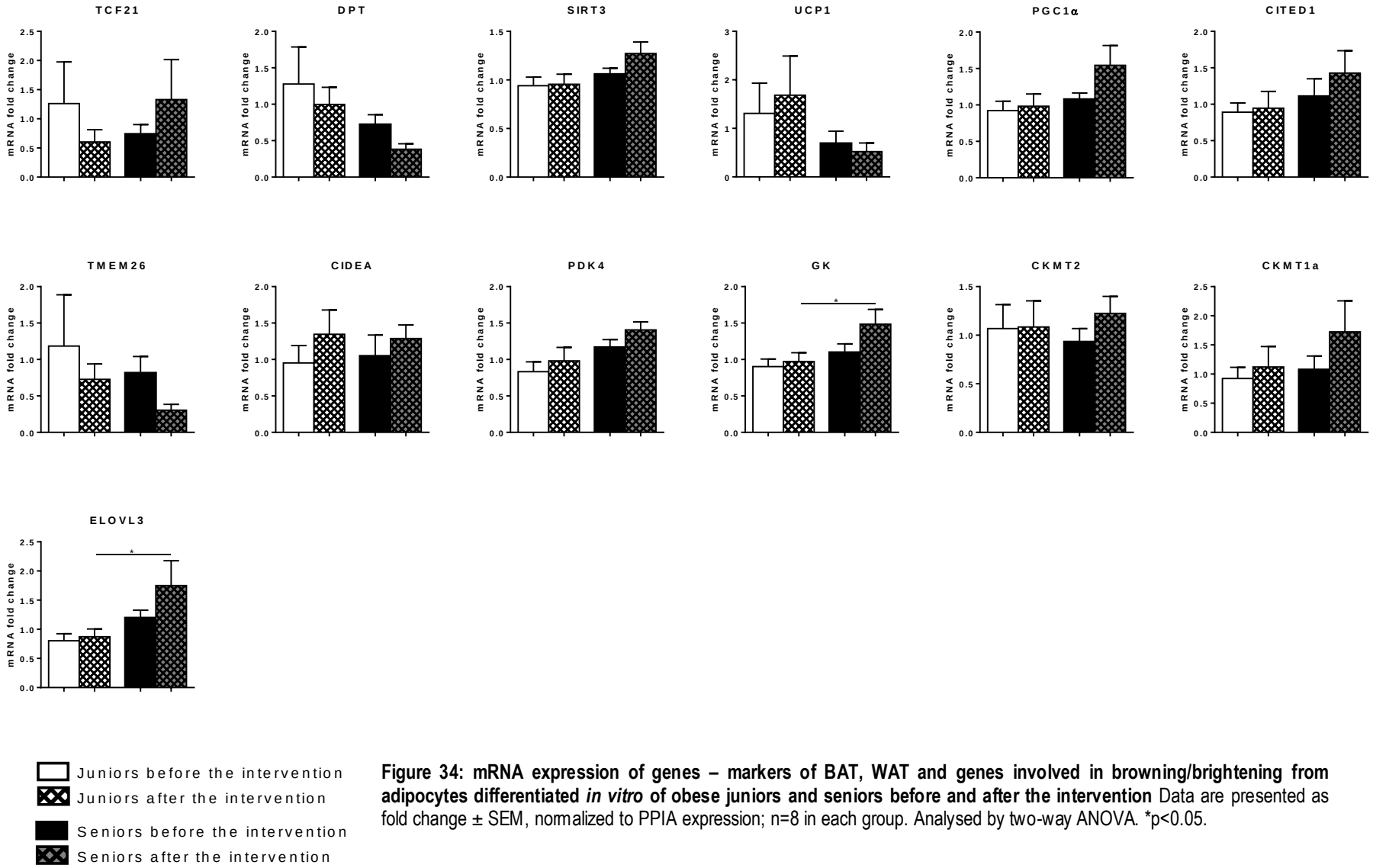


Figure 34: mRNA expression of genes – markers of BAT, WAT and genes involved in browning/brightening from adipocytes differentiated *in vitro* of obese juniors and seniors before and after the intervention Data are presented as fold change ± SEM, normalized to PPIA expression; n=8 in each group. Analysed by two-way ANOVA. *p<0.05.

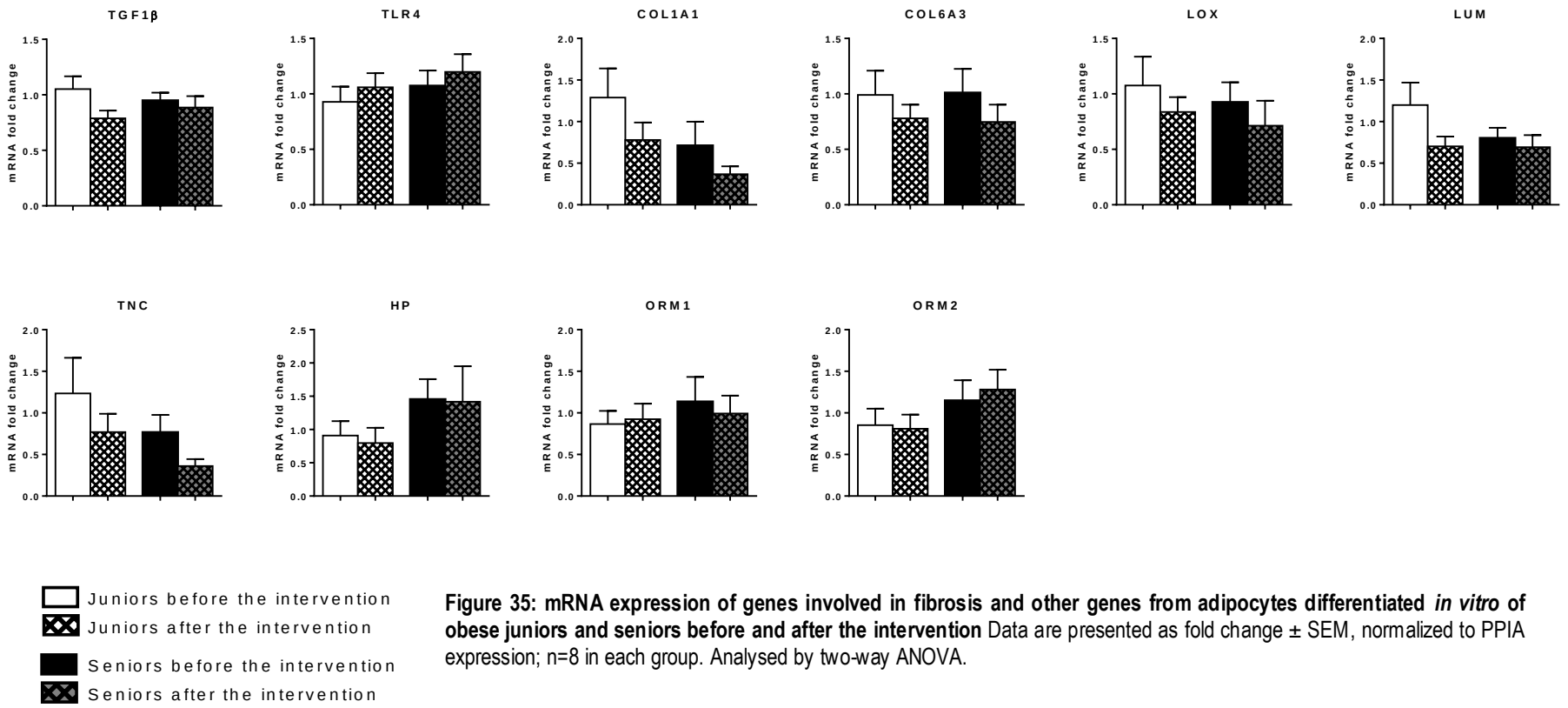


Figure 35: mRNA expression of genes involved in fibrosis and other genes from adipocytes differentiated *in vitro* of obese juniors and seniors before and after the intervention Data are presented as fold change ± SEM, normalized to PPIA expression; n=8 in each group. Analysed by two-way ANOVA.

5 Discussion

5.1 Discussion to experimental part A

Pathways activated by ER stress represent primarily an adaptive homeostatic process that aims at protecting cellular metabolism disturbed by various insults. Extreme severity or chronicity of ER stress is however linked with poor cellular survival and suboptimal metabolic performance [296]. This study brings evidence that severe ER stress substantially reduces lipogenic capacity of adipocytes while chronic low ER stress impairs lipogenesis through inhibition of adipogenic conversion of preadipocytes. As adipocytes are cells primarily dedicated to synthesis of lipids, the inhibition of lipogenesis represents a major disturbance of their metabolic function. Indeed, lipogenesis is repressed in obese AT [297], whose demands for the synthetic and secretory activity are enhanced and result in persistent deficiency of ER capacity. Notably, adipocytes used in this study were differentiated and exposed to ER stressors in fatty acid free medium, so the described effects of ER stress on lipogenesis actually represents effects on DNL. *In vivo* significance of DNL in AT was considered negligible until recent discovery that in lean subjects, up to 20 % of TAG were synthetized in AT *de novo* [61]. As we did not observe any effect of ER stress on mitochondria OXPHOS capacity (data not shown), the impact of ER stress on lipogenesis in adipocytes appears to be direct and not exerted through suppression of mitochondrial function.

Lipogenesis has been previously found to be regulated by activity of UPR components in liver [269, 298]. While UPR activation by glucose deprivation blocks lipogenesis in liver cells [299], under non-limiting glucose inflow *in vivo* ER stress induced formation of lipid droplets leading to hepatic steatosis [300, 301]. Thus, the negative effect of ER stress on lipogenesis in the presence of glucose - as observed in this study - seems to be specific for AT. Despite this tissue specific effect of ER stress, a pathophysiological outcome of unresolved ER stress is the same in both adipose and liver cells, i.e. a deterioration of their primary metabolic specialization. Thus, eliciting ER stress in both AT and liver at the same time may explain a vicious circle leading to profound disturbance of the whole body lipid metabolism in obese. Notably, opposing effects of ER stress on lipogenesis in adipocytes versus liver cells fits well with fact that DNL seems to be regulated in AT and liver in an opposing manner. This disparity is probably based on differential activation of two key transcription factors SREPB1c and ChREBP in both tissues, since expression of both of them

has been shown to be lower in AT but higher in liver from obese compared to lean subjects [261, 302]. Nevertheless, chronic ER stress of low intensity that more closely imitates *in vivo* conditions was inefficient to diminish lipogenesis in mature adipocytes. This suggests that chronic ER stress seen in obesity could have an impact rather on the newly recruited preadipocytes and thus could impair necessary renewal of AT. This hypothesis is supported by our observation that ER stress activating all arms of the UPR strongly inhibits adipogenic conversion of preadipocytes when present prior or in early stages of this process. Sensitivity of adipogenesis to low ER stress was reported also by Kim et al. [303]. Early effect of ER stress on adipogenesis suggests a regulation of a key adipogenic factor PPARγ or its upstream regulators. Indeed, cells pretreated with TM prior induction of adipogenesis were unable to enhance PPARγ expression as much as cells exposed to regular adipogenic medium for 3 days. Moreover, expression and transcriptional activity of PPARγ in 3T3-L1 cells was recently found to be inhibited by ATF3, a transcriptional inhibitor inducible by ER stress [304]. Nevertheless, it remains unclear whether ATF3 plays a role in adipogenesis also under conditions of low chronic ER stress. Interestingly, expression of ER stress marker HSPA5 was increased 24 hours after onset of differentiation by TG dose that does not have this effect in quiescent preadipocytes (compare Fig. 2C and Fig. 3A). These data suggest that hormonal stimulation to adipogenesis represents in fact additional ER stress above the one induced by low doses of TG.

In conclusions, we found that acute ER stress is a powerful inhibitor of lipogenesis in adipocytes, both at the level of mRNA expression and de novo TAG synthesis, while low intensity ER stress blocked lipogenesis through an impairment of adipogenesis. These effects of ER stress could therefore contribute to decreased lipogenic capacity of adipose tissue seen in obesity.

5.2 Discussion to experimental part B

Accumulation of TAG in subcutaneous adipocytes is regulated mainly by the balance between lipolytic and lipogenic activity of these cells. In aging, accumulation of TAG in subcutaneous AT is diminished, but the molecular basis of this phenomenon remains elusive. In fact, to our knowledge, no analysis of the activity of lipogenic or lipolytic genes in subcutaneous AT in respect to aging was ever done in humans. Because aging is in general characterized by the progressive functional decline of various cellular processes, we hypothesized that lower deposition of TAG in aged subcutaneous AT is driven mainly by decreased lipogenesis. To test our hypothesis, we analyzed gene expression in subcutaneous AT from two age differing groups of women. Since subcutaneous AT consists of a variety of cells including stem, endothelial and immune cells, we employed a model of *in vitro* differentiated adipocytes originating from the same donors to analyze aging-related changes in expression pattern specifically in adipocytes.

Our study brought the evidence of a prerequisite for lower capacity of aged subcutaneous AT to accumulate fat, i.e. lower mRNA expression of two major lipogenic genes (one of them linked to DNL) in both, whole tissue and adipocytes, from the elderly (**Figure 14A, 15A**). Indeed, lower activity of lipogenic enzymes and lower accumulation of TAG mirrors lower mRNA expression of lipogenic markers in adipocytes as previously documented by tracer studies performed in our and other laboratories [305, 306].

Since lipogenesis occurs in the ER and can be regulated by ER stress [264, 269, 305], a condition that was also proposed as one of the hallmarks of aging [307], we quantified mRNA expression of ER stress markers and correlated it with lipogenic gene expression. In contrast to aged murine AT [283], aged human subcutaneous AT exhibited lower expression of major ER chaperone HSPA5, together with lower expression of its co-chaperone DNAJC3 and ATP/ADP exchange factor HYOU1 (**Figure 14C,15C**) [308, 309]. It is possible that higher expression of HSPA5 and other markers in AT of aged mice reflected not only the aging process but also aging-related alterations of body composition, because the groups in the mice study were not matched for fat mass parameters that could substantially differ in young (4-6 months) vs. old animals (18-20 months). On the other hand, the results of our study are in accordance with several other studies performed on various (but not adipose) tissues showing aging-related reduction of the expression and activity of many ER chaperones and enzymes. It was suggested that their functional decline results in chronic ER stress [307, 310, 311]. Thus, it is possible that with aging, levels of ER chaperones become mildly reduced, which leads to

chronic but low intensity ER stress that cannot fully activate IRE1 branch of the UPR necessary to restore ER homeostasis. This hypothesis is supported by our observations that mRNA expression of HSPA5 and its co-chaperones was decreased despite higher levels of XBP1s, a powerful transcription factor controlling the expression of a cluster of genes related to folding (**Figure 14C, 15C**), while the ability of adipocytes from the elderly to upregulate HSPA5 expression in response to acute ER stress (triggered by high doses of thapsigargin or tunicamycin) remained fully preserved (data not shown) [312]. On the other hand, we found higher expression of both ATF4 and its target gene CHOP [313] in adipocytes from the elderly, and thus, low intensity ER stress in aging adipocytes appears to be sufficient to trigger PERK-ATF4 axis of the UPR.

Although the expression of major chaperone HSPA5 and its co-chaperones in subcutaneous AT correlated with expression of lipogenic markers, this relationship was not found in adipocytes, which implies an involvement of other AT resident cells in this relationship. Indeed, both immune and endothelial cells are sensitive to ER stress and upon activation of the UPR they can produce a number of proinflammatory cytokines [314, 315] , which were implicated in the worsened physiological functions of adipocytes [168]. Besides, we have previously observed that lipogenic capacity of mature adipocytes is not influenced by experimentally induced chronic low ER stress that could resemble the type of ER stress occurring in aging [305].

To address the impact of accumulation of senescent cells on AT lipogenesis, we analyzed several markers of senescent cells, namely CDK inhibitors and markers of oxidative stress and mitochondrial dysfunction that are considered also as markers of senescence [316-318]. Concomitantly increased levels of p16 mRNA and NOX4 are indeed suggestive of higher numbers of senescent cells in subcutaneous AT from the elderly. The lack of correlation between the expression of these two markers and lipogenic genes, however, does not corroborate the existence of cause-effect relationship between the accumulation of dysfunctional senescent cells and decreased lipogenesis in aged subcutaneous AT. Indeed, cells contributing to increased p16 and NOX4 expression in aged SAT were probably not adipocytes, i.e. the cells exerting strong downregulation of lipogenic gene expression. Nevertheless, GDF15 expression, one of the genes implicated in aging, was strongly correlated with lipogenic markers in both subcutaneous AT and adipocytes. GFD15 is a cytokine that might be induced by various cellular stresses, including mitochondrial dysfunction that may contribute to senescence and aging [318]. Indeed a down regulation of mitochondrial enzymes in adipose tissue with aging was shown in mice [319]. Thus, the

strong negative relationship between GDF15 expression and lipogenesis could suggest that the aging-related decline of lipogenesis in subcutaneous AT is related to mitochondrial dysfunction in adipocytes. Indeed, lipogenesis is an ATP-demanding process and thus it could be limited by any disturbance of oxidative phosphorylation [320].

Major limitation of this study is the fact that it is based only on the analysis of mRNA levels of marker genes and as such it cannot provide strong conclusions on subcutaneous AT expression and activity of certain UPR proteins that are regulated mostly post-transcriptionally. Nevertheless, mRNA levels of lipogenic, ER chaperones and p16/NOX4 are quite strong determinants of actual protein levels as discussed above [317, 318].

Another limitation of the presented study is the discordance of BMI and weight between the groups, which appears to be driven by difference in fat free mass. This discordance is rather impossible to overcome when comparing the young and the elderly, because both factors (lean and fat mass) are changing with age. Thus, we believe that the matching for fat mass instead of weight and BMI is more suitable to analyze aging-dependent changes in adipose tissue.

In conclusions, decreased capacity of subcutaneous AT adipocytes to accumulate TAG appears to be linked to diminished expression of lipogenic enzymes in the elderly that is probably driven by mitochondrial dysfunction and not by enhanced ER stress and accumulation of senescent cells.

5.3 Discussion to experimental part C

The aim of this study was to elucidate metabolic and immune effects of the calorie restriction per se, when compared with the restriction accompanied with the body fat mass loss. Therefore, in moderately obese women, whole body and AT characteristics were compared between the early (2 days) and later phase (28 days) of VLCD. The main finding is that the responses of metabolic and inflammation-related characteristics in SAAT and in plasma differed markedly between the two phases of the diet.

The 2 days of calorie restriction modified genes involved in lipolysis and lipogenesis in SAAT, while the inflammatory status of AT was not changed. The increased levels of free fatty acids and β-hydroxybutyrate in plasma reflect enhanced AT lipolysis and hepatic ketogenesis, similarly as seen in fasting [321]. Increased expression of major AT lipases in SAAT - HSL, ATGL, with its cofactor CGI58 could contribute to higher release of free fatty acids from AT to plasma, as reviewed by Nielsen et al. [322]. Indeed, in this study the increase in plasma free fatty acids and glycerol after 2 days of VLCD correlated with the changes in expression of CGI58, and the changes in plasma glycerol correlated with SAAT lipases –ATGL and HSL. In addition, increased mRNA expression of CD36 - fatty acid transporter in SAAT was found at the early phase of VLCD and this expression correlated with increased free fatty acid plasma levels. This goes in line with published studies showing that the expression of CD36 in AT increases in response to the acute increase of free fatty acid plasma levels induced by lipid infusion [323] and also in response to chronic elevation of free fatty acid plasma levels in obese subjects with metabolic syndrome [324]. On the other hand, in the study of Hames et al. [325], CD36 was shown to facilitate the free fatty acids uptake by AT when the levels of free fatty acids in plasma are low, i.e. after consumption of carbohydrate-containing meal. Thus, the higher expression of CD36 observed after 2 days of calorie restriction could provide AT better free fatty acid absorption capacities for the anticipated refeeding phase. Also, it was suggested that free fatty acids released from adipocyte by lipolysis are immediately taken back by CD36 to secure cycling of free fatty acids, and this mechanism may prevent excessive release of free fatty acids under condition of stimulated lipolysis [326]. The up-regulation of CD36 in our study is likely driven by PPARγ activation. Indeed, PPARγ mRNA expression was elevated during the early VLCD phase and this change correlated with the changes of mRNA expression of CD36 and with changes of lipolytic genes (HSL, ATGL) in this study. Up-regulated PPARγ expression and activity

might be associated by enhanced availability of lipolysis-derived fatty acids (PPARγ ligands), as suggested by Haemmerle et al [327, 328].

In contrast to the effect of 2 days of calorie restriction on lipolytic genes, expression of lipogenic enzymes in SAAT was reduced. This reduction was likely linked to a downregulation of SREBP1c - one of the lipogenic transcription factors, which is regulated by insulin [329]. The improvement in insulin sensitivity during the early phase of VLCD was not associated with the metabolic gene expression in SAAT, except borderline correlation with several lipolytic genes (i.e. HSL, ATGL). Thus, changes occurring in AT in response to short calorie restriction do not appear to play major role in the metabolic improvement induced by 2 days of VLCD. Therefore, the potential role of other insulin sensitive organs in the diet- induced metabolic changes must be taken into account. Lara-Castro et al. [330] demonstrated that the decrease of the intramyocellular lipids after 6 days of VLCD was closely related to insulin resistance. In contrast to that, Jazet et al. [331] found no changes in markers of insulin signaling and glucose transport in skeletal muscle after 2 days of VLCD but they found a diet-induced decrease of endogenous glucose production. The latter points to the role of the liver in the very short VLCD-induced metabolic changes.

As no significant change in gene expression and secretion of pro-inflammatory (IL6, IL8, TNFα, MCP1) and anti-inflammatory (IL10, IL1Ra, TGFβ1) cytokines, and macrophage markers (CD68, CD163, IRF5, MSR1, ACP5, CCR2, FCGBP, ITGAX) in SAAT were observed after two days of VLCD, it may be concluded that the early improvement of insulin sensitivity (metabolic changes) was not related to changes of immune status of AT. Indeed, several studies using opposite dietary intervention, i.e. short overfeeding in healthy men, showed similar dichotomy between AT inflammation and insulin sensitivity under conditions of mild weight gain when insulin sensitivity was impaired despite no induction of inflammatory cytokines and macrophage activation in SAAT [332, 333].

In contrast to the changes induced by 2 days of VLCD, after 28 days of severe calorie restriction mRNA expression of lipolytic genes (ATGL, HSL, MGL, PLIN1, CGI58) returned to the pre-diet levels. The no change in ATGL or HSL mRNA expression after 1 month of VLCD– when compared to the pre-diet condition - is in agreement with some of the previous studies of our and other teams [237, 334]. However, the decrease in expression of lipolytic genes in day 28 compared to day 2 does not essentially mean that lipolysis is attenuated. Free fatty acid levels in plasma were elevated at 28 day similarly to day 2 indicating maintenance of higher lipolytic rate. The decrease to baseline in expression of lipases at late phase of VLCD was accompanied by a decrease of G0S2, which has been shown as dominant inhibitor

of ATGL in adipocytes [335], and by reduction in DGAT1, which is responsible for free fatty acid re-esterification. Reduced inhibition of ATGL together with reduced re-esterification can thus still ensure the free fatty acid release needful to cover the energy demand of the organism. Thus, our data supports the hypothesis that G0S2 acts predominantly as a long-term regulator of ATGL, while CGI58 is more important for the regulation of acute lipolytic response [322]. Furthermore, at day 28 a marked decrease of lipogenic genes, genes of β-oxidation, and insulin stimulated glucose transport (GLUT4) in SAAT was observed. These processes probably prevent excessive breakdown as well as storage of free fatty acids in adipocytes during longer-term shortage and secure its adequate release from AT to serve as substrates for other organs.

The increase in SAAT inflammatory state after 28 days of VLCD is in agreement with published results of our and other groups [285, 336-338]. Previously we have shown that 28 days VLCD was not associated with an increase in macrophage content in SAAT [339]. Thus it might be assumed that the observed increase of macrophage marker expression in response to strong calorie restriction reflects stimulation of macrophage activation rather than their accumulation. The increase of pro-inflammatory (IL6, IL8, MCP1, TNFα) but also anti-inflammatory (IL10, IL1Ra) cytokines together with M1 (IGTAX, CCR) and M2 (CD163) macrophage markers is in accordance with the published findings that macrophages in SAAT are of mixed phenotype [340]. The role of the increase in inflammatory state in SAAT after 28 days of severe calorie restriction is still unclear.

Interestingly, the only gene identified to be significantly downregulated in both phases of VLCD was lysyl oxidase (LOX), one of the genes involved in fibrosis and ECM remodeling. LOX catalyzes the cross-linking of collagens in AT and, thus, it is one of a key factors contributing to the fibrosis of AT observed in obesity [341]. Inhibition of LOX also resulted in the improvement of several metabolic parameters – ameliorated glucose and insulin levels, decreased HOMA index and reduced plasma TAG level in obese rats [341]. Indeed, the decrease of LOX expression found in our study was associated with changes in SAAT metabolism genes (**Suppl. Table 1**). Importantly, the reduction of lipogenesis and fibrosis observed during weight loss in our study represent the opposite processes to those found in the overfeeding studies [332, 333].

Leptin appeared as the only adipokine for which a quantitative relationship with the diet-induced changes of HOMA-IR was found. In addition, leptin changes were correlated also with a number of metabolic-related genes in adipose tissue and with plasma FGF21 levels. Leptin was shown to act on peripheral tissues by regulation of fatty acid oxidation and

energy expenditure through activation of AMPK, induction of free fatty acid oxidation genes and increased transport of free fatty acids to mitochondria [342]. It might be suggested that this adipokine is a sensor of metabolic changes in SAAT and the signal which mediates the metabolic interplay with other organs during calorie restriction. Further studies in this issue in obese humans should be warranted.

After 28 days of VLCD, the diet induced improvement of metabolic indices (TAG, insulin, cholesterol) was correlated with changes in lipogenic genes (e.g., SCD1, FASN, DGAT2). It was shown in mice that decrease of SCD1 expression (in liver or systemic level) was correlated with improvement of metabolic profile and insulin sensitivity [343, 344]. Thus, we may hypothesize that the decrease of lipogenic genes in AT is paralleled with the decrease of lipogenesis in liver, which is probably one of important contributors to the improvement of metabolic profile and IS during VLCD.

FGF21 was shown to act as a metabolic regulator during fasting through stimulating ketogenesis and fatty acid oxidation in liver in mice [289]. In addition, in mice and 3T3-L1 adipocytes the role of FGF21 in down-regulation of lipogenesis and lipolysis in SAAT was shown [289-291]. In line with this, we observed negative correlation between the increase of plasma FGF21 during the 28 days VLCD and the changes in mRNA expression of genes involved in SAAT lipogenesis (FASN, DGAT2) and glucose uptake (GLUT4). As FGF21 plasma levels are increased only in later phase of the diet [345] it might be suggested that FGF21 plays a role in the regulation of a switch from a short-term to a longer-term calorie restriction. The down-regulation of lipogenesis in association with insulin-stimulated glucose transport into AT supports the role of FGF21 in the saving of substrates for other organs during famine.

In conclusion, our findings show that the early (2 days) and later (28 days) phases of VLCD differ in respect to metabolic and inflammatory response in SAAT. Although in both phases the effects of severe calorie restriction represent the reaction to shortage of calories/nutrients (i.e. induced lipolysis, reduced storage of lipids), the expression of regulatory cofactors involved in these processes is different in the early and later phase of VLCD. The diet-induced modifications in metabolic and inflammation-related functions of AT did not appear to play a pivotal role in the improvement of insulin sensitivity at early phase of VLCD. The processes observed after 28 days of VLCD probably contribute to adaptation of SAAT to prolonged calorie restriction through saving of substrates. Moreover, the correlation of the changes in metabolic genes in SAAT with metabolic indices and FGF21 suggest the possible cross-talk of SAAT with liver function during VLCD.

5.4 Discussion to experimental part D

Little is known about the calorie restriction in the elderly due to its potential harmful effects, e.g. sarcopenia. Therefore, in this unique study we compared the effect of mild eight week calorie restriction accompanied with exercise to maintain the muscle mass on certain aspects of AT biology in two groups of obese men differing in age (juniors vs. seniors).

Aging is associated with AT redistribution and with a relative loss of subcutaneous AT. Nevertheless, the mechanism of subcutaneous fat loss within aging is not completely understood. Observations in rodents suggest that this decline in peripheral body fat is associated with a deteriorated ability of preadipocytes to differentiate [215, 216]. Since new fat cells are continuously produced from preadipocytes [61], age-related alterations in the number of preadipocytes may also contribute to the loss of fat. In the study of Van Harmelen et al., a negative correlation between donor age and proliferation of subcutaneous cells was found [346]. Our results, however, do not prove that the capacity of preadipocytes to replicate or the sensitivity to proliferative stimuli is decreased in the group of seniors. This could be given by the fact that in the study of Van Harmelen, the age of subjects range was from 17-61 and the study included 29 subjects. Nevertheless, in our results we observed a trend showing the handicap for the cells from the group of seniors. Therefore, a group including more subjects could give more evidence to our results.

Calorie restriction may reverse typical cellular features of aging, e.g. cellular senescence, telomere erosion, epigenetic alterations, mitochondrial dysfunction, genomic instability and impaired nutrient sensing among others [347]. Considering that these changes apply also on preadipocytes, one would predict faster proliferation after calorie restriction. However, our results showed that a moderate calorie restriction does not influence preadipocyte capacity to proliferate. This is in agreement with the results of study performed by Tomlinson et al., on 12 obese subjects undergoing VLCD for ten weeks, when no improvement in proliferation or in the number of cells division over the period of seven days was observed [348]. Similarly, previous data from our laboratory showed that three months dietary intervention including 23 obese women did not change the number of days for which the preadipocytes were in culture (from the establishment of culture up to the first passage), neither the total yield of cells in passage 3 [349]. *In vitro* preadipocytes are exposed to overly optimal conditions which do not enable to see the possible impact of the intervention. A different methodological approach is required. For instance, immunohistochemical detection of proliferative markers in SVF could give us better answer. However, it would therefore be

difficult to distinguish preadipocytes from other SVF-cells. Another explanation may be related to the type and duration of calorie restriction. Here, we used moderate calorie restriction combined to physical exercise follow up of the patients in order to prevent loss of skeletal muscle mass in elderly obese patients.

There is mounting evidence that senescent cells contribute to ageing and age-related pathologies. Therefore, it is surprising that we did not observe a difference in numbers of senescent cells or p16 expression in preadipocytes between juniors and seniors. The relatively low age and relatively healthy status of the seniors group could be the reason of this discrepancy. Another reason for this could be that the original precursors which were already senescent did not multiply in the culture, making impossible to detect them at confluence state. On the other hand, senescent cells arise in culture by repeated passaging (*replicative senescence*). This prerequisite is correct as we obtained more βgal positive and big cells at P11 compared to P3/P4.

Obesity is also associated with accumulation of senescent cells [199]. Therefore, it was logical to assume that weight loss could lead to the opposite process: elimination of senescent cells. However, there has not been much evidence for support of this hypothesis up to now. Recently, it was shown that exercise prevents the accumulation of senescent cells in mice fed on high fed diet and consequently nullifies the damaging effects of fast-food diet on health [350]. This effect accompanied by a decrease of expression of inflammatory cytokines was observed, except of other organs, in visceral AT. It is possible that the changes within subcutaneous AT are less pronounced. Of course, a final conclusion cannot be drawn without deeper analysis of SASP of preadipocytes. Therefore, gene expression analysis using microarrays is warranted.

It has been hypothesized that metabolic complications in obesity are related to the dysfunction of hypertrophic adipocytes or to the inability of AT to meet increased energetic surplus by hyperplastic growth, i.e. by recruitment and differentiation of new adipocytes. Evidence for the latter came from the study in which insulin-resistance type 2 diabetic subjects exhibited decreased expression of genes involved in differentiation when compared with healthy controls [351]. In addition, it was shown that insulin-sensitizing drug rosiglitazone, a member of the thiazolidinedione class of glucose-lowering medicines, markedly increases the differentiation of adipose progenitors into adipocytes [352]. Surprisingly, we did not confirm statistically significant difference in differentiation capacity of preadipocytes between juniors and seniors. However, the trend for worsened adipogenesis in seniors was obvious. Interestingly, higher intrinsic ability of preadipocytes to differentiate

after weight loss which was found in our previous clinical study performed on obese women [349] was not achieved in this study. The reason for this discrepancy could be the degree of weight loss and overall length of the intervention. In the previous study, the subjects underwent calorie restriction for three months that was further followed by 3 month weight maintenance phase and the decrease of weight was 9.7 %, whereas in this study weight loss did reach only 4.3 % in a group of juniors and 5.7 % in a group of seniors. The study of Magkos et al. clearly show that moderate 5 % weight loss of the previous body weight influences positively insulin sensitivity in AT [353], but the effect of short dietary intervention resulting in lower weight loss on AT has not been much studied. Therefore, it is possible that the level of weight loss was not sufficient to lead to any detectable effects, which would explain most of the results in a group of juniors (no impact of the intervention on any of the measured parameters). On the other hand, observed changes were seen exclusively in a group of seniors. It is not clear why the intervention impacted the adipocyte biology in the group of seniors. The decrease of fat mass in % was the same in both groups (data not shown). Physical exercise was led by a professional trainer 3 times a week. All participants were encouraged to engage in physical activity (walking) at least 5 days a week in bouts lasting at least 45 min in duration. It can be hypothesized that the group of seniors exercised more. No matter the reason for the improvement in adipocyte metabolism in seniors, it is clear that this group was positively impacted by the intervention. Therefore, the following discussion will take into account only this group. An obvious trend showing higher level of differentiation in a group of seniors after the intervention is in compliance with higher degree of weight loss. When using qPCR for gene expression analyses, a method more precise than ORO, the mRNA expression of PPARγ, an essential adipogenic regulator, was increased as well.

One of the critical characteristics of mature adipocyte phenotype is the expression of GLUT4 and its translocation to the membrane upon insulin stimulation [354]. The effect of insulin, either *in vivo* or *in vitro*, on glucose transport requires proper post-receptor signalling. In our study, we observed only a trend toward lower IRS1 phosphorylation at Y612 (that activates insulin signalling) in seniors compared to juniors, but no impact of the intervention on the level of IRS1 or Akt phosphorylation in either group. However, we observed increased insulin-stimulated glucose uptake measured by 2-deoxy glucose (without increased *Glut4* mRNA expression). It was shown that insulin treatment can accelerate the rate of GLUT4 exocytosis [355]. Recently, TUSC5 was identified as an AT-specific protein that is involved in proper protein recycling, including insulin-mediated glucose uptake [356]. Furthermore,

TUSC5 was shown to be a PPARγ target. It would be intriguing to measure TUSC5 mRNA expression. Surely, we saw an increase in mRNA levels of PPARγ in seniors after the intervention. Thus, our data suggest that the increase in glucose uptake is due to accelerated rate of GLUT4 trafficking in response to insulin treatment.

We observed increased expression of genes involved in fatty acid oxidation (SDHA, UQCRC2, PPARα) and lipolysis (CD36, ATGL, HSL). This suggests an increased fatty acid handling: increased cycling and utilization reaction to negative energy balance. The last includes increased ability to oxidize substrates and increased lipolysis. Studies in which the subjects underwent weight loss (by VLCD or gastric bypass) showed also upregulation of ATGL [357] or increased mitochondrial respiratory capacity [358]. Another action that enhances oxygen consumption in mitochondria and is therefore associated with enhanced fatty acid metabolism is *browning/brightening* of AT. Recently, the role of BAT in lipid metabolism was explored in humans and it was shown that cold-induced BAT activation is associated with increased whole-body lipolysis, TAG-free fatty acid cycling, free fatty acid oxidation and AT insulin sensitivity. Cold also upregulated the expression of genes involved in lipid metabolism specifically in BAT [359]. Barquissau et al. showed that conversion of human white fat cells into brite adipocytes resulted in a major metabolic reprogramming inducing fatty acid anabolic and catabolic pathways [360]. Our results suggest that increased fatty acid metabolism could be caused by "briter" phenotype of cells. In brown/brite adipocytes, glucose oxidation is diminished and glucose is redirected towards glyceroneogenesis and favours TAG synthesis. Obviously, we observed increased level of GK mRNA. Notably, the mRNA level of ELOVL3 which was shown to have a role during increased fatty acid oxidation and is under the control of PPARα [361], was increased in a group of seniors after the intervention when compared to juniors. Nevertheless, the UCP1 is a protein necessary to sustain thermogenesis via high mitochondrial oxidative capacities. In our study, we did not observe a marked increase of UCP1. However, it was reported that human mature white adipocytes can acquire brown-like fat cell properties upon PPARγ and PPARα activation, both of them increased in our study. Therefore, it is possible that the intervention was too short or *light* or both to see UCP1 mRNA upregulation at the end of the intervention and without weight maintenance period. Other *brite* markers showed a trend to be increased. All data together would suggest that the intervention and moderate weight loss can lead to higher fatty acid oxidation capacity due to gradual starting of AT britening.

In conclusion, we show that if weight loss of at least 5.7 % is achieved by a moderate calorie restriction combined with exercise in seniors, it can have beneficial effects on metabolism in adipocytes, possibly because fat cells acquire more oxidative features.

6 Conclusions and future perspectives

This thesis focused on the study of biology of human AT, preadipocytes and adipocytes. The special attention was dedicated to regulation lipogenesis by ER stress, calorie restriction and aging.

First **Project A** assessed the impact of ER stress on differentiation and lipogenic capacity of human adipocytes. We showed that acute but not chronic low ER stress weakens lipogenic capacity of adipocytes. However, chronic exposure of preadipocytes to ER stress impaired both, lipogenesis and adipogenesis. These effects of ER stress could therefore contribute to the worsening of AT function seen in obesity.

Second **Project B** investigated the lipogenic capacity of human subcutaneous AT in relation to aging. By analysis of subcutaneous AT and adipocytes from two groups of women, the young and the elderly, we found that decreased capacity of subcutaneous AT adipocytes to accumulate triglycerides appears to be linked to diminished expression of lipogenic enzymes in the elderly that is probably not driven by the ER stress in adipocytes or accumulation of senescent cells. On the other hand, a strong relationship between the expression of lipogenic markers and GDF15 suggests that lipogenesis in the elderly might be reduced as a consequence of mitochondrial dysfunction.

Third **Project C** aimed at comparing metabolic and inflammation-related characteristics of subcutaneous AT in the early (2 days) and later (28 days) phase of a VLCD. Our results showed that the early and later phases of a VLCD differ with respect to metabolic and inflammatory responses in subcutaneous AT. The expression changes in subcutaneous AT in the early phase of the VLCD could not explain the effect of short calorie restriction on the improvement of insulin sensitivity.

The last **Project D** aimed to compare the effects of moderate calorie restriction on preadipocytes and adipocytes differentiated *in vitro* from obese juniors and seniors. Although wwe did not observe any effect of the intervention on metabolism of preadipocytes in either group, we observed an intervention-driven improvement in adipocyte metabolism selectively in the group of seniors. Therefore, our data suggest that moderate calorie restriction could initiate positive changes in metabolism of adipocytes in seniors.

In conclusion, this thesis brought evidence that lipogenesis in human AT can be inhibited by ER stress, severe caloric restriction and aging.

The findings of the thesis bring new questions and hypotheses. Even though calorie restriction has a clearly beneficial effects on AT, it seems that it cannot relieve ER stress in AT. This potential was, however, attributed to exercise, as a study of Khadir et al. showed that exercise itself (without calorie restriction and weight loss) is able to alleviate ER stress in obese individuals [362]. ER stress is also induced in aging in response to decline of ER chaperone levels, as suggested by our results and other studies. Intriguingly, exercise can normalize also this age-driven ER chaperone decline as shown in rats [363]. Furthermore, recent research proved that exercise prevents the accumulation of senescent cells and the expression of the SASP while nullifying the damaging effects of the fast food diets on parameters of health [350]. This study also demonstrated that exercise initiated after long-term fast food diet feeding reduces senescent phenotype markers in visceral AT while attenuating physical impairments. Thus, exercise may provide restorative benefit by mitigating accrued senescent burden. However, these studies were performed in animals, not in humans. Exercise could also mitigate mitochondrial dysfunction in AT that, based on our results, appears to be strongly linked to decreased lipogenesis in AT. Therefore, it can be suggested that long-time exercise could restore chaperones induction, alleviate ER stress, reduce numbers of senescent cells, relieve mitochondrial dysfunction and thus improve AT lipogenesis in obese individuals, as well as in the elderly. The clinical study dedicated to the investigation of long-time exercise on AT in the elderly women is currently under way in our laboratory. Thus, this longitudinal study employing advanced analyses of cellular composition and transcriptome, lipidome of AT, combined with the *in vitro* experiments on adipocytes could shed more light on the effects and functional mechanisms of exercise on AT in the elderly.

7 Bibliography

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Annexe 1

Stress of endoplasmic reticulum modulates differentiation and lipogenesis of human adipocytes

Michal Koc, **Veronika Mayerová**, Jana Kračmerová, Aline Mairal, Lucia Mališová, Vladimír Štich, Dominique Langin, Lenka Rossmeislová

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Stress of endoplasmic reticulum modulates differentiation and lipogenesis of human adipocytes

Michal Koc [a, b], Veronika Mayerová [a, b], Jana Kračmerová [a, b], Aline Mairal [a, c], Lucia Mališová [a, b], Vladimír Štich [a, b], Dominique Langin [a, c, d, e], Lenka Rossmeislová [a, b, *]

[a] Franco-Czech Laboratory for Clinical Research on Obesity, Third Faculty of Medicine, Prague, Czech Republic
[b] Department of Sport Medicine, Third Faculty of Medicine, Charles University in Prague, CZ-100 00, Czech Republic
[c] Inserm, UMR1048, Obesity Research Laboratory, Institute of Metabolic and Cardiovascular Diseases, 31432 Toulouse, Cedex 4, France
[d] University of Toulouse, UMR1048, Paul Sabatier University, 31432 Toulouse, Cedex 4, France
[e] Toulouse University Hospitals, Department of Clinical Biochemistry, 31059 Toulouse, Cedex 9, France

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ABSTRACT

Background: Adipocytes are cells specialized for storage of neutral lipids. This storage capacity is dependent on lipogenesis and is diminished in obesity. The reason for the decline in lipogenic activity of adipocytes in obesity remains unknown. Recent data show that lipogenesis in liver is regulated by pathways initiated by endoplasmic reticulum stress (ERS). Thus, we aimed at investigating the effect of ERS on lipogenesis in adipose cells.
Methods: Preadipocytes were isolated from subcutaneous abdominal adipose tissue from obese volunteers and *in vitro* differentiated into adipocytes. ERS was induced pharmacologically by thapsigargin (TG) or tunicamycin (TM). Activation of Unfolded Protein Response pathway (UPR) was monitored on the level of eIF2α phosphorylation and mRNA expression of downstream targets of UPR sensors. Adipogenic and lipogenic capacity was evaluated by Oil Red O staining, measurement of incorporation of radio-labelled glucose or acetic acid into lipids and mRNA analysis of adipogenic/lipogenic markers.
Results: Exposition of adipocytes to high doses of TG (100 nM) and TM (1 μg/ml) for 1–24 h enhanced expression of several UPR markers (HSPA5, EDEM1, ATF4, XBP1s) and phosphorylation of eIF2α. This acute ERS substantially inhibited expression of lipogenic genes (DGAT2, FASN, SCD1) and glucose incorporation into lipids. Moreover, chronic exposure of preadipocytes to low dose of TG (2.5 nM) during the early phases of adipogenic conversion of preadipocytes impaired both, lipogenesis and adipogenesis. On the other hand, chronic low ERS had no apparent effect on lipogenesis in mature adipocytes.
Conclusions: Acute ERS weakened a capacity of mature adipocytes to store lipids and chronic ERS diminished adipogenic potential of preadipocytes.

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1. Introduction

Adipocytes are cells highly specialized for storage of neutral lipids. They are equipped with dedicated receptors and transporters necessary for an uptake and transport of nonesterified fatty acids (NEFA) and with enzymatic cascade enabling NEFA incorporation into triglycerides. Moreover, adipocytes are able to synthetize lipids de novo, from glucose [1]. Glucose is necessary also for the synthesis of glycerol phosphate, the backbone of triglycerides. Thus lipogenic activity of adipocytes directly influence fatty acid and glucose plasma levels and this homeostatic effect is regulated by many factors [2,3]. Paradoxically, obesity impairs

Abbreviations: ER, endoplasmic reticulum; ERS, endoplasmic reticulum stress; NEFA, nonesterified fatty acids; TG, thapsigargin; TM, tunicamycin; UPR, unfolded protein response.

* Corresponding author. Department of Sport Medicine, Third Faculty of Medicine, Charles University in Prague, Ruská 87, 100 00 Prague, Czech Republic. Fax: +420 267 102 263.
E-mail address: Lenka.Rossmeislova@lf3.cuni.cz (L. Rossmeislová).

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capacity of adipocytes to synthetize and store lipids [4,5], which further contributes to high plasma NEFA levels, a putative cause of obesity-related hepatic and muscle insulin resistance [6]. The reason for the deterioration of lipogenic activity of adipocytes remains unclear. Notably, several enzymatic steps of lipogenesis and the formation of lipid droplets take place in the endoplasmic reticulum (ER), an organelle also essential for calcium homeostasis and protein folding [7,8]. The situation when the folding and other metabolic capacities of ER are overwhelmed is referred to as endoplasmic reticulum stress (ERS). ERS activates a defense mechanism called unfolded protein response (UPR) in order to enhance ER capacity and restore ER homeostasis [9]. The signs of chronic ERS have recently been found in obese and insulin resistant subjects [10,11]. The significance of ERS for metabolic health was confirmed by experiments on rodents corroborating ERS as a trigger of insulin resistance and other metabolic disturbances caused by obesity [12]. Importantly, ERS and consequently UPR were found to be important regulators of lipogenesis in liver [13]. But there is a lack of comprehensive studies that would investigate whether metabolic stress sensed through ER controls lipogenesis also in human adipose tissue. Thus, we aimed at investigating the effect of ERS on lipogenesis in human adipose cells.

2. Materials and methods

2.1. Cells and chemicals

Cells were derived from needle biopsies of subcutaneous adipose tissue from obese volunteers that were recruited at the Third Faculty of Medicine of Charles University, University Hospital Kralovske Vinohrady, Czech Republic, and Toulouse University Hospitals, France. Isolation, expansion and differentiation of cells was described previously [14]. The study was performed according to the Declaration of Helsinki and was approved by the respective Ethical Committees. Volunteers were informed on the study, and written informed consent was obtained before participation in the study.

Thapsigargin was supplied by Alexis (Lauzen, Switzerland), tunicamycin by LKT Laboratories, Inc. (St. Paul, Maine, USA) and Rosiglitazone by Cayman (Tallin, Estonia). Culture media were from Lonza Std. (Basel, Switzerland). FBS (qualified for MSC) was from ThermoFisher (Carlsbad, California, USA), FGFβ and EGF from Immunotools (Friesoythe, Germany). Other chemicals were from Sigma Aldrich (St. Louis, Missouri, USA).

2.2. Gene expression analysis

Isolation of RNA, cDNA synthesis and qRT-PCR was described previously [14]. TaqMan Gene expression assay for PPARγ, SCD1, FASN, DGAT2, SREBP1C, HSPA5, ATF4, EDEM1, PLIN1 were from Applied Biosystems (Carlsbad, California, USA). aP2 and XBP1 full-length and spliced were detected by specific primers (aP2-forward 5′-GCATGGCCAAACCTAACATGA-3′, aP2-reverse 5′ CCTGG CCCAGTATGAAGGAAA-3′, XBP1-total-forward 5′- CGCTGAGGAG-GAAACTGAA-3′, XBP1-total-reverse 5′- CACTTGCTGTTCCAGCT-CACTCAT/3′, XBP1-spliced-forward 5′- GAGTCCGCAGCAGGTGCA-3′, XBP1-spliced reverse 5′- ACTGGGTCCAAGTTGTCCAG-3′) by Sybr Green technology (Power Sybr Green Master Mix). Gene expression of target genes was normalized to expression of GUSB or RPS13 and fold change of expression was calculated using ΔΔ Ct method.

For western blotting, cells were harvested and lysates processed as described previously [15]. Antibodies against total and phosphorylated eIF2α were from Cell Signaling (Danvers, MA, USA).

2.3. Apoptosis assay

Cells were exposed to 0, 2.5 and 100 nM TG for 24 h. Then they were trypsinized and fixed in 70% ethanol at 4 °C overnight. After two washes with PBS, cells were stained with 50 μg/ml Propidium Iodide and treated with 0.1 mg/ml RNAse I diluted in PBS for 30 min at 37 °C. DNA content analysis was performed on FACSCalibur (BD Biosciences, Franklin Lakes, NJ, USA) and analyzed with FlowJo 8.2 (Tree Star Inc, Ashland, OR, USA).

2.4. Oil red O (ORO) staining

12 days differentiated cells were fixed and stained as described previously [14].

2.5. Glucose and acetic acid incorporation into lipids

Cells were incubated for 3 h in Krebs Ringer buffer or DMEM-no glucose medium supplemented with 2% BSA, 10 mM HEPES, 66 nM insulin, 2 mM glucose and 2 μCi D-[^{14}C(U)]glucose (PerkinElmer) or 5 mM acetic acid and 2 μCi [1−^{14}C]-Acetic Acid (PerkinElmer). Neutral lipids were extracted and analyzed as described [16]. Distribution of de novo incorporated ^{14}C among major lipid species was analyzed after lipid separation by thin-layer chromatography on Silica Gel plates developed in Heptane:Isopropylether:Acetic acid mixture (60:40:4) for 1 h, visualized by iodine vapor, quantitatively scraped from plate and analyzed by liquid scintillation counting. Results from metabolic measurements were normalized to total protein content of cell extracts.

2.6. Analysis of mitochondrial respiration

Oxygen consumption rates (OCR) was measured using an extracellular flux analyzer XF24 (Seahorse Bioscience, Copenhagen, Denmark). Preadipocytes were seeded at a density of 6000 cells per well (XF24 Cell Culture Microplate) and allowed to reach confluence when the differentiation was started. At day 11 cells were treated with ER stressors for 24 h. The culture medium was replaced with the XF Assay medium supplemented with 4 mM L-glutamine, 1 mM pyruvate and 5.5 mM glucose 1 h prior to measurement. OCR measurements were obtained before and after sequential additions of 1 μM oligomycin, 0.5 μM FCCP and 2 μM rotenone/antimycin A to the medium.

2.7. Statistical analysis

Data were analyzed using GraphPad Prism 6.0 software using Wilcoxon matched-pair signed rank or Mann Whitney test, as appropriate. The level of significance was set at $p < 0.05$.

3. Results

3.1. Acute high intensity ERS reduces adipogenesis and lipogenesis of human preadipocytes and adipocytes

To evaluate the effect of acute ERS on lipogenic capacity of adipocytes, we exposed *in vitro* differentiated adipocytes from 15 donors to two commonly used ER stressors, thapsigargin (TG) and tunicamycin (TM), for 24 h. Both, 100 nM TG and 1 μg/ml TM [17], dramatically enhanced expression of major ER chaperone HSPA5 (heat shock 70 kDa protein 5), a marker of ERS (Fig. 1A). The same treatment decreased mRNA levels of genes involved in lipogenesis, i.e. fatty acid synthase (FASN), stearoyl desaturase (SCD1), and diacylglycerol O-acyltransferase 2 (DGAT2) (Fig. 1A). The suppressive effect of ERS on lipogenesis was confirmed by a decreased

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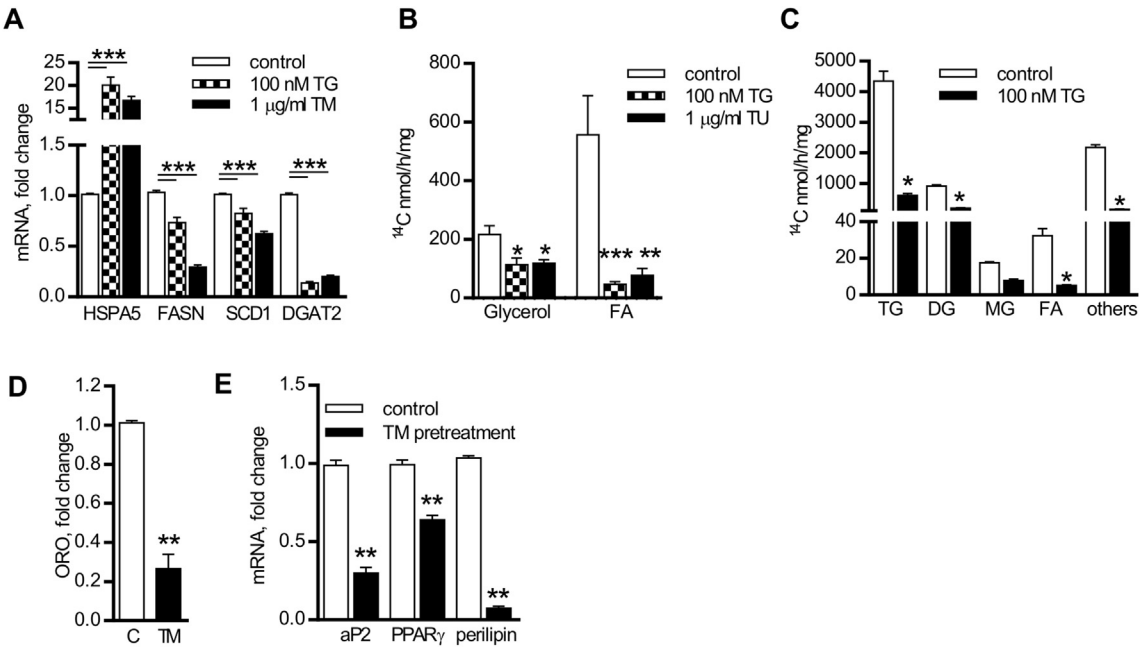

Fig. 1. Acute ERS lowers lipogenesis in human adipocytes and adipogenesis of preadipocytes. Preadipocytes were differentiated for 12 days and then incubated for 24 h with DMSO or 100 nM TG or 1 μg/ml TM. **A**. mRNA expression of HSPA5, FASN, SCD1 and DGAT2 was measured by qRT-PCR and normalized to GUSB expression (n = 15). **B**. Glucose carbon (^{14}C) incorporation into lipids (hydrolyzed into fatty acids (FA) and glycerol) during 3 h incubation was determined by liquid scintillation and normalized to protein content (n = 3). **C**. Distribution of de novo incorporated ^{14}C in lipid species was analyzed after TLC separation of extracted lipids (n = 2). **D**. Preadipocytes were exposed to 1 μg/ml TM for 4 h and then differentiated in the absence of TM for 12 days. Quantification of neutral lipids accumulation is expressed as a relative fold change to control (n = 5). **E**. Preadipocytes were exposed to 1 μg/ml TM for 4 h and then differentiated in the absence of TM for 3 days. mRNA expression of adipogenic markers was analyzed by qRT-PCR and normalized to RPS13 (n = 5). Data are means ± SE, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

capacity of adipocytes treated with TG to incorporate glucose carbon into lipids (Fig. 1B, C). Thus, in adipocytes, acute high intensity ERS lowers lipogenic capacity of adipocytes on both transcriptional and enzymatic level.

As lipogenesis in adipocytes was found to be dependent on mitochondrial activity [18,19] that could be impaired by calcium accumulation induced by ER stressors, we analyzed respiration capacity of adipocytes treated with 100 nM TG or 1 μg/ml TM for 24 h. Acute ERS had no impact on the proton leak by mitochondria nor the spare oxidative capacity (proton leak: control-116.4 ± 11.16, TG-106.6 ± 10.89, TM-94.94 ± 10.18 pmol/min; spare oxidative capacity: control- 631.1 ± 39.11, TG-555.1 ± 54.85, TM-677 ± 54.01 pmol/min).

In addition, we tested an effect of acute ER stress on adipogenic capacity of preadipocytes. In order to limit ERS only to preadipocytes we employed reversibly acting TM in high-dose (1 μg/ml). Confluent preadipocytes were treated with TM for 4 h, then washed by PBS several times and subjected to standard 12 day adipogenic procedure using media free of ERS inducer. This treatment resulted in approximately 60% reduction of neutral lipid content compared to control conditions (Fig. 1D) without apparent effect on the viability of cells. Moreover, the effect of TM-pretreatment of preadipocytes on adipogenesis was detectable already after 3 days of differentiation when mRNA levels of aP2, PPARγ and perilipin were reduced compared to control conditions (Fig. 1E).

3.2. Chronic low ERS impairs adipogenesis and associated lipogenesis

Obesity leads to chronic low intensity rather than acute high intensity ERS [10,20]. Therefore, we aimed at imitating chronic ERS in adipose cells by the use of TG dose capable of activating UPR without acute induction of downstream effectors [21]. To determine such a dose, we exposed both preadipocytes and mature adipocytes to 1, 2.5, 5 and 100 nM TG for 1, 4 and 24 h and then analyzed expression of genes representing early and late markers of unfolded protein response (UPR). Neither dose of TG caused appearance of hypodiploid apoptotic preadipocytes within 24 h (not shown). Early marker of UPR activation, i.e. phosphorylated eIF2α (PERK arm of UPR), was induced already by 2.5 nM TG (Fig. 2A) within 1 h, while an induction of expression of downstream ERS effectors (ATF6 arm- HSPA5 [22], PERK arm- ATF4 [23], IRE1 arm-EDEM1, XBP1 splicing [24]) within 4 and 24 h required higher TG concentrations (5 or 100 nM TG) (Fig. 2B–D). Therefore, 2.5 nM TG was selected for chronic treatments of cells.

We investigated whether low intensity but chronic ERS reduces adipogenic conversion of preadipocytes similarly as acute high intensity ERS. Preadipocytes were differentiated in the absence or presence of 2.5 nM TG. Chronic treatment of cells with TG led to a mild increase of mRNA levels of HSPA5, ATF4 and EDEM1 during the whole time course of differentiation (Fig. 3A). Capacity to accumulate neutral lipids was lowered by more than 50% in TG-treated adipocytes as detected by Oil Red O staining (Fig. 3B, C). This was accompanied by diminished mRNA levels of differentiation markers (i.e. a key adipogenic factor, PPARγ, transcription factor SREBP-1c and late adipogenic markers, aP2 and perilipin) (Fig. 3D, E). mRNA expression of the lipogenic genes SCD1, DGAT2 and FASN was also lowered (Fig. 3F).

To determine a critical period of time for ERS to exert inhibitory effect on adipogenesis, preadipocytes were differentiated in the presence of 2.5 nM TG for various days (0–6, 1–12, 3–12, 6–12, 9–12). Capacity to store neutral lipids evaluated by ORO staining was strongly impaired in adipocytes exposed to TG between days 0–6 and 1–12, mildly between days 3–12 and not between days 6–12 and 9–12 of differentiation (Fig. 3G, H). Lipogenesis measured

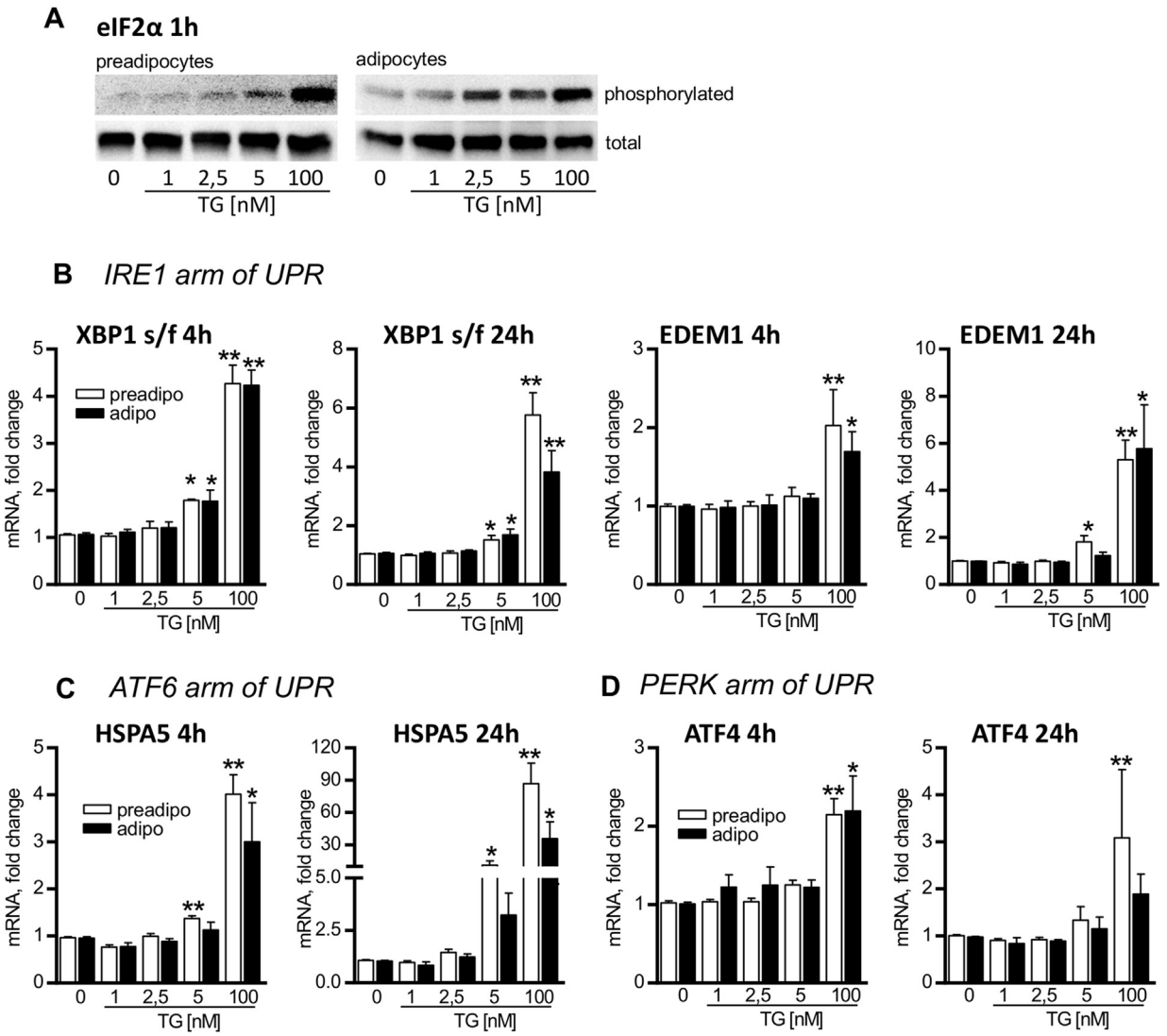

Fig. 2. Determination of TG dose appropriate for experiments with chronic low ERS. Cells were incubated with DMSO or 1, 2.5, 5, 100 nM TG for indicated time. **A**. Western blotting analysis of eIF2α activation (n = 3, the representative image is shown). **B**–**D**. mRNA expression of HSPA5, EDEM1 and ATF4 was measured by qRT-PCR and normalized to GUSB expression. mRNA expression of XBP1-spliced was normalized to XBP1-total expression (n = 4). Data are means ± SE, *p < 0.05, **p < 0.01.

as ^{14}C-glucose carbon incorporation into lipids was also not altered when cells were exposed to 2.5 nM TG at day 6–12 of differentiation (not shown).

3.3. Lipogenic capacity of mature adipocytes is not influenced by chronic low ERS

Next, we analyzed the effect of chronic (6 days) low ERS on adipocytes differentiated for 12 days. Accumulation of neutral lipids (^{14}C-glucose carbon incorporation) was not affected by 2.5 nM TG (Fig. 4A), similarly as seen when TG was applied between day 6 and 12 of adipogenesis (not shown). Only expression of perilipin was decreased while other lipogenic genes remained unaffected by this treatment (Fig. 4B).

4. Discussion

Pathways activated by ERS represent primarily an adaptive homeostatic process that aims at protecting cellular metabolism disturbed by various insults. Extreme severity or chronicity of ERS is however linked with poor cellular survival and suboptimal metabolic performance [25]. This study brings evidence that severe ERS substantially reduces lipogenic capacity of adipocytes while chronic low ERS impairs lipogenesis through inhibition of adipogenic conversion of preadipocytes. As adipocytes are cells primarily dedicated to synthesis of lipids, the inhibition of lipogenesis represents a major disturbance of their metabolic function. Indeed, lipogenesis is repressed in obese adipose tissue [26], whose demands for the synthetic and secretory activity are enhanced and result in persistent deficiency of ER capacity. Notably, adipocytes used in this study were differentiated and exposed to ER stressors in fatty acid free medium, so the described effects of ERS on lipogenesis actually represents effects on lipogenesis de novo (DNL). In vivo significance of DNL in adipose tissue was considered negligible until recent discovery that in lean subjects, 20% of triglycerides were synthetized in adipose tissue de novo [1]. As we did not observe any effect of ERS on mitochondria OXPHOS capacity, the impact of ERS on lipogenesis in adipocytes appears to be direct and not exerted through suppression of mitochondrial function.

Lipogenesis has been previously found to be regulated by activity of UPR components in liver [13,27]. While UPR activation by

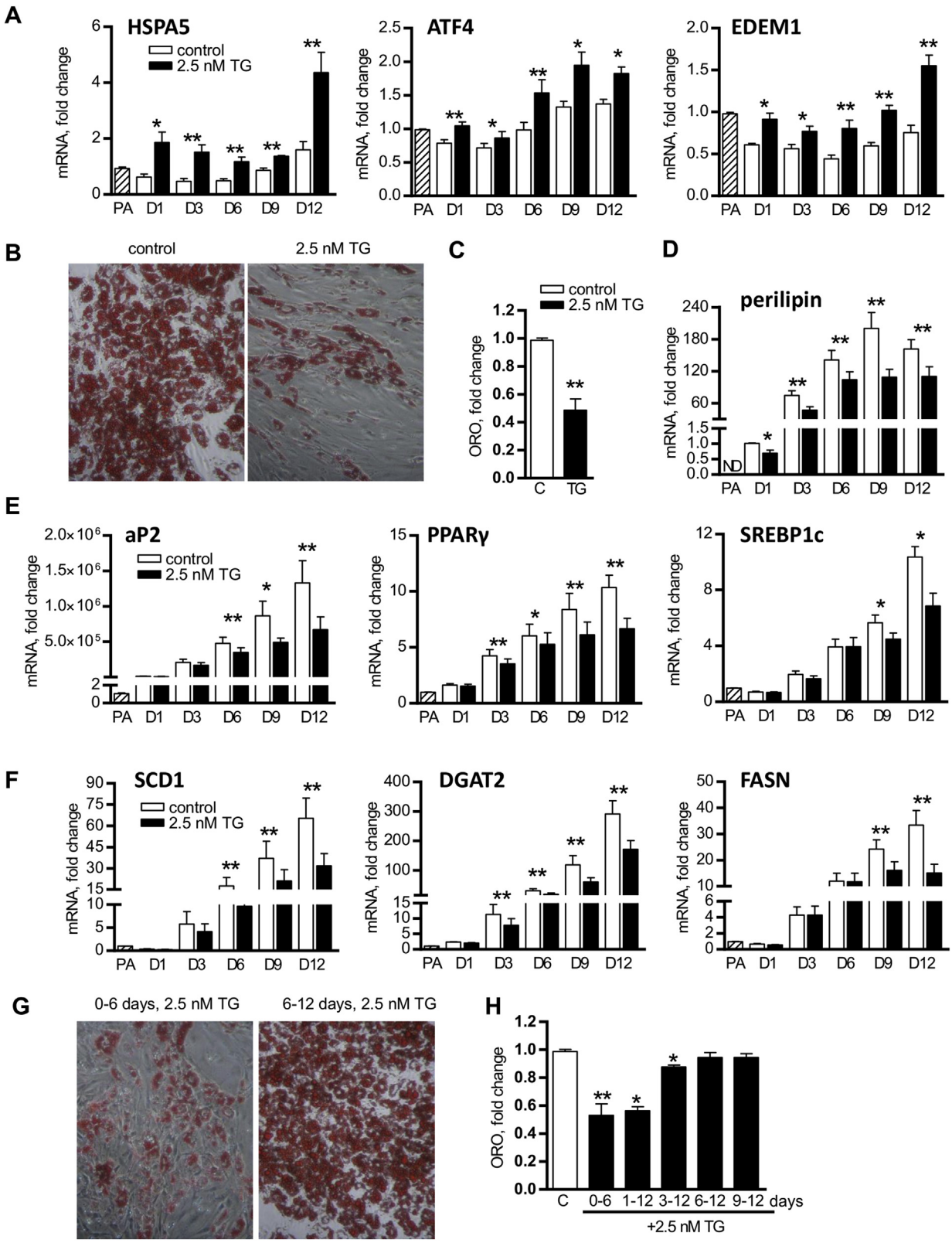

Fig. 3. Chronic low ERS impairs adipogenesis and associated lipogenesis. Preadipocytes (PA) were differentiated for 12 days in the absence or presence of 2.5 nM TG. Cells were harvested upon indicated days for mRNA analysis or at day 12 for ORO staining. mRNA expression of UPR effectors (**A**), adipogenic (**D, E**) and lipogenic markers (**F**) was measured by qRT-PCR and normalized to RPS13 expression (n = 4, ND-not detectable). **B**. Representative image of cells staining with ORO at day 12. **C**. Quantification of neutral lipids accumulation is expressed as a relative fold change to control (n = 5). **G**. Preadipocytes were differentiated and 2.5 nM TG was added to the differentiation media for indicated days. Representative image of cells staining with ORO at day 12. **H**. Preadipocytes were differentiated and 2.5 nM TG was added to the differentiation media for indicated days. Quantification of neutral lipids accumulation is expressed as a relative fold change to control (n = 5). Data are means ± SE, *p < 0.05, **p < 0.01.

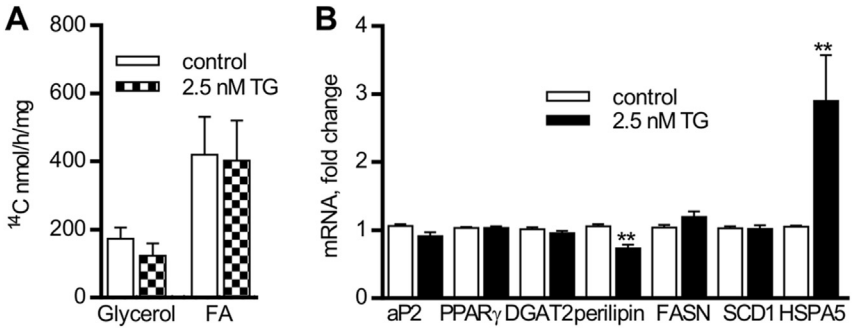

Fig. 4. Effect of low ERS stress on mature adipocytes. Mature adipocytes (12 days after onset of differentiation) were exposed for next 6 days to 2.5 nM TG. **A**. Glucose (^{14}C) incorporation into lipids (hydrolyzed into fatty acids FA and glycerol) during 3 h incubation of adipocytes treated as in Fig. 1B was determined by liquid scintillation and normalized to protein content (n = 3). **B**. mRNA expression of HSPA5, adipogenic and lipogenic markers was measured by qRT-PCR and normalized to RPS13 expression (n = 5). Data are means ± SE, $^{*}p < 0.05$, $^{**}p < 0.01$.

glucose deprivation blocks lipogenesis in liver cells [28], under non-limiting glucose inflow in vivo ERS induced formation of lipid droplets leading to hepatic steatosis [29,30]. Thus, the negative effect of ERS on lipogenesis in the presence of glucose – as observed in this study – seems to be specific for adipose tissue. Despite this tissue specific effect of ERS, a pathophysiological outcome of unresolved ERS is the same in both adipose and liver cells, i.e. a deterioration of their primary metabolic specialization. Thus, eliciting ERS in both adipose tissue and liver at the same time may explain a vicious circle leading to profound disturbance of the whole body lipid metabolism in obese. Notably, opposing effects of ERS on lipogenesis in adipocytes versus liver cells fits well with fact that DNL seems to be regulated in AT and liver in an opposing manner. This disparity is probably based on differential activation of two key transcription factors SREPB-1c and ChREBP in both tissues, since expression of both of them has been shown to be lower in AT but higher in liver from obese compared to lean subjects [4,31]. Nevertheless, chronic ERS of low intensity that more closely imitates in vivo conditions was inefficient to diminish lipogenesis in mature adipocytes. This suggests that chronic ERS seen in obesity could have an impact rather on the newly recruited preadipocytes and thus could impair necessary renewal of adipose tissue. This hypothesis is supported by our observation that ERS activating all arms of UPR strongly inhibits adipogenic conversion of preadipocytes when present prior or in early stages of this process. Sensitivity of adipogenesis to low ERS was reported also by Kim et al. [32]. Early effect of ERS on adipogenesis suggests a regulation of a key adipogenic factor PPARγ or its upstream regulators. Indeed, cells pretreated with TM prior induction of adipogenesis were unable to enhance PPARγ expression as much as cells exposed to regular adipogenic medium for 3 days. Moreover, expression and transcriptional activity of PPARγ in 3T3-L1 cells was recently found to be inhibited by ATF3, a transcriptional inhibitor inducible by ERS [33]. Nevertheless, it remains unclear whether ATF3 plays a role in adipogenesis also under conditions of low chronic ERS. Interestingly, expression of ERS marker HSPA5 was increased 24 h after onset of differentiation by TG dose that does not have this effect in quiescent preadipocytes (compare Figs. 2C and 3A). These data suggest that hormonal stimulation to adipogenesis represents in fact additional ERS above the one induced by low doses of TG.

In conclusions, we found that acute ERS is a powerful inhibitor of lipogenesis in adipocytes, both at the level of mRNA expression and de novo triglyceride synthesis, while low intensity ERS blocked lipogenesis through an impairment of adipogenesis. These effects of ERS could therefore contribute to decreased lipogenic capacity of adipose tissue seen in obesity.

Author contributions

M.K. performed experiments and data analysis and contributed to the writing of the manuscript, V.M, L.M, A.M. and J.K. performed experiments, V.S. performed adipose tissue biopsies and contributed to discussion and writing of the manuscript, D.L. contributed to discussion and writing of the manuscript. L.R. designed the study, performed experiments and data analysis and wrote the manuscript. L.R. is a guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Conflicts of interest

The authors declare no conflict of interest

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Transparency document

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Annexe 2

Comparison of Early (2 Days) and Later (28 Days) Response of Adipose Tissue to Very Low-Calorie Diet in Obese Women

Veronika Šrámková, Lenka Rossmeislová, Eva Krauzová, Jana Kračmerová, Michal Koc, Dominique Langin, Vladimír Štich, and Michaela Šiklová

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Comparison of Early (2 Days) and Later (28 Days) Response of Adipose Tissue to Very Low-Calorie Diet in Obese Women

Veronika Šrámková, Lenka Rossmeislová, Eva Krauzová, Jana Kračmerová, Michal Koc, Dominique Langin, Vladimír Štich, and Michaela Šiklová

Department of Sport Medicine (V.Š., L.R., E.K., J.K., M.K., V.Š., M.Š.), Third Faculty of Medicine, Charles University, 100 00 Prague 10, Czech Republic; Franco-Czech Laboratory for Clinical Research on Obesity (V.Š., L.R., E.K., J.K., M.K., D.L., V.Š., M.Š.), Third Faculty of Medicine, Prague, and Institut des Maladies Métaboliques et Cardiovasculaires, Université Toulouse III Paul Sabatier, Unité Mixte de Recherche, F-31432 Toulouse, France; Second Department of Internal Medicine (E.K., V.Š.), University Hospital Kralovske Vinohrady, 100 34 Prague, Czech Republic; and INSERM (D.L.), Unité Mixte de Recherche 1048, Institute of Metabolic and Cardiovascular Diseases, and University of Toulouse, Paul Sabatier University, and Department of Clinical Biochemistry (D.L.), Toulouse University Hospitals, F-31432 Toulouse, France

Context: Beneficial metabolic effects of calorie restriction found in the early stage of hypocalorie diets may be caused by the modulation of metabolic and endocrine function of adipose tissue.

Objective: The objective of the study was to compare metabolic and inflammation-related characteristics of sc adipose tissue (SAAT) in the early (2 d) and later (28 d) phase of a very low calorie diet (VLCD).

Design, Setting, Intervention, and Patients: Seventeen moderately obese premenopausal women followed an 800 kcal/d VLCD for 28 days. Anthropometric measurements, blood sampling, and a biopsy of SAAT were performed before the diet and after 2 and 28 days of the VLCD.

Main Outcome Measure(s): mRNA expression of 50 genes related to lipid metabolism, inflammation, and fibrosis were analyzed in SAAT. Secretion of adipokines was determined in SAAT explants and adipokines, fibroblast growth factor 21 (FGF21) and C-reactive protein were measured in plasma.

Results: In the early phase of the VLCD, the expression of lipolytic genes was increased, whereas the expression of lipogenic genes was significantly suppressed. The inflammatory markers in SAAT remained unchanged. At the later phase, expression of genes involved in lipogenesis and β-oxidation was markedly suppressed, whereas the expression of inflammatory markers was increased. The changes of lipogenic genes after 28 days of the VLCD correlated with FGF21 changes.

Conclusion: The early and later phases of a VLCD differ with respect to metabolic and inflammatory responses in SAAT. The expression changes in SAAT in the early phase of the VLCD could not explain the effect of short calorie restriction on the improvement of insulin sensitivity. An interplay of SAAT with liver function during VLCD mediated by FGF21 might be suggested. **(*J Clin Endocrinol Metab* 101: 5021–5029, 2016)**

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Abbreviations: AT, adipose tissue; ATGL, adipose triglyceride lipase; CRP, C-reactive protein; DGAT2, diacylglycerol acyltransferase 2; FFA, free fatty acid; FGF21, fibroblast growth factor 21; GLUT4, glucose transporter 4; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment for insulin resistance; HSL, hormone-sensitive lipase; IS, insulin sensitivity; LOX, lysyl oxidase; MCP-1, monocyte chemoattractant protein-1; PPAR, peroxisome proliferator-activated receptor; qPCR, quantitative PCR; SAAT, sc AT; SREBP1c, sterol regulatory element-binding protein-1c; VLCD, very low-calorie diet.

Very low-calorie diets (VLCDs) are often prescribed in obesity treatment to achieve rapid weight loss. Generally, this type of dietary intervention consists of 500–800 kcal/d during 1–2 months and leads to an improvement in metabolic profile (such as plasma total cholesterol, triglycerides, high-density lipoprotein [HDL]cholesterol, insulin, etc) and insulin sensitivity (IS) (1). A study that compared the effects of VLCD and bariatric surgery has shown that VLCD drives almost the same improvements of IS, β-cell function, and lipid parameters as bariatric surgery when the same reduction of body weight and fat mass is achieved (2). However, some of the positive effects of severe calorie restriction are observed already before the loss of fat mass is accomplished. Whole-body/hepatic insulin resistance measured by the homeostasis model assessment for insulin resistance (HOMA-IR) or quantitative insulin sensitivity check index improved as soon as after 2 days of a VLCD (3, 4). Similarly the beneficial effects of bariatric surgery on carbohydrate metabolism were observed within several days after bariatric operation in type 2 diabetic patients, before significant weight loss has occurred (5). Mechanism of the beneficial metabolic effects of the calorie restriction per se are not well understood. It might be hypothesized that modifications of immune and metabolic characteristics of adipose tissue (AT) might occur and play a role in this process despite the fact that there is no change in AT mass. Whereas the response of inflammation-related cytokines during a 1-month VLCD was investigated in a number of studies (reviewed in references 1 and 6), the effects of a very short calorie restriction was studied rarely (3). Similarly, it was shown that the expression of metabolism-related genes in AT was reduced after 1 month of a VLCD (7), but the response to a shorter calorie restriction (eg, several days) was not thoroughly studied.

Therefore, in this longitudinal study, we compared the effects of 2 days and 28 days of a VLCD on metabolic and inflammation-related indices in sc adipose tissue (SAAT) and their possible relationship with systemic inflammatory and metabolic status in moderately obese women. We investigated the expression of the respective genes in SAAT as well as the secretion of cytokines in SAAT explants.

According to recent studies, the diet-induced metabolic changes might be partially controlled by fibroblast growth factor 21 (FGF21). FGF21 is released by the liver and stimulates fatty acid oxidation and ketogenesis (8). Recently it was shown, in mice and in cell cultures, that FGF21 may affect adipose tissue metabolic pathways (lipogenesis, lipolysis) (9, 10). Thus, FGF21 levels and their association with changes in SAAT were also investigated.

Subjects and Methods

Subjects, dietary protocol, and clinical examination

Seventeen metabolically healthy obese women (aged 35 ± 7 y, mean body mass index 32.6 ± 3.6 kg/m^{-2}) were recruited for the study. All subjects were drug free and healthy, as determined by medical history and laboratory findings. All patients had a stable weight for at least 3 months prior to inclusion. All subjects underwent a VLCD intervention program, during which they received 800 kcal/d (liquid formula diet; Redita; representing an intake of 52 g of protein, 118 g of carbohydrates, and 12.9 g of fat per day). Patients consulted a dietitian once a week. The evaluation of physical activity was performed before the start of the study by the International Physical Activity Questionnaire, and the subjects were recommended not to change their habitual activity during the study. The design of the study is shown in Figure 1. Clinical investigation was carried out at day 0 (baseline), day 2 (2 d of the VLCD), and day 28 of the VLCD. During these investigations the subjects were examined at 8:00 AM after overnight fasting. Body weight and waist and hip circumferences were measured, and body composition was assessed by bioimpedance (QuadScan 4000; Bodystat). The needle biopsy of SAAT and the samples of peripheral blood were taken. The study was approved by Ethical Committee of the Third Faculty of Medicine, Charles University in Prague, and all subjects gave their informed consent before the start of the study.

Secretion of cytokines from SAAT explants

AT samples obtained by biopsy were processed as previously described (11). Briefly, AT was washed in saline and separated to several aliquots. Two aliquots (200–500 mg) were snap frozen in liquid nitrogen for subsequent gene expression analysis. Another aliquot of approximately 400 mg was cut into small pieces and the explants were incubated in 4 mL of Krebs/Ringer phosphate buffer (pH 7.4) supplemented with 20 g/L of BSA and 1 g/L of glucose at 37°C in a shaking water bath with air as the gas phase. After 4 hours of incubation, the conditioned medium was collected, the cellular debris was removed by centrifugation, and the cell-free supernatant was stored at −80°C until analysis.

Analysis of plasma and SAAT conditioned media

Plasma samples were prepared from uncoagulated peripheral blood by centrifugation. Plasma glucose was determined with a glucose oxidase technique (Beckman Instruments). Plasma insulin was measured using an Immunotech insulin immunoradiometric assay kit (Immunotech).

β-Hydroxybutyrate, glycerol, and free fatty acids (FFAs) were analyzed by enzymatic colorimetric assays (Randox Laboratories Ltd). A multiplex immunoassay at the MagPIX or Luminex 200 was used to analyze the following: 1) plasma cytokines IL-6, IL-8, IL-10, and TNF-α (high sensitivity human cytokine Milliplex panel; Merck-Millipore); 2) cytokines in conditioned media, IL-6, IL-8, IL-10, monocyte chemoattractant protein-1 (MCP-1), TNF-α, and leptin (human adipocyte kit; Merck-Millipore). The circulating levels of MCP-1, leptin, FGF21, and C-reactive protein (CRP) were quantified by ELISA kits (eBioscience and R&D Systems).

Gene expression analysis

Total RNA was isolated from 200- to 500-mg aliquots of AT using an RNeasy lipid tissue RNA minikit (QIAGEN). The RNA

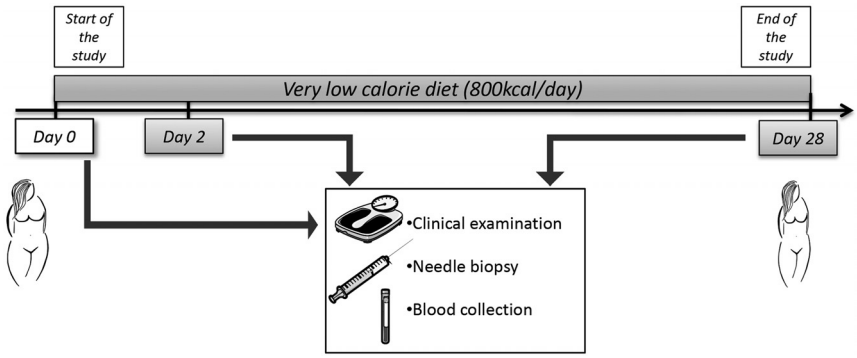

Figure 1. The design of the study. Seventeen obese premenopausal women were included in the study. Clinical examination, needle biopsy of SAAT, and blood collection were performed at indicated days (d 0, before the start of VLCD; d 2, after 2 d of VLCD; d 28, at the end of 1 mo of VLCD). Samples from needle biopsies and plasma were used for further analysis of inflammatory and metabolic characteristics during the intervention.

concentration was measured using Nanodrop1000 (Thermo Fisher Scientific). To remove genomic DNA, deoxyribonuclease I (Invitrogen) treatment was applied. Six hundred nanograms of total RNA were reverse transcribed using a high-capacity cDNA reverse transcription kit (Applied Biosystems). For microfluidics, 4 ng of cDNA was preamplified within 16 cycles to improve detection of target genes during subsequent real-time quantitative PCR (qPCR; TaqMan Pre Amp master mix kit; Applied Biosystems). For the preamplification, 20× TaqMan gene expression assays of all target genes (the list of genes in Supplemental Table 1) were pooled together and diluted with water to the final concentration 0.2× for each probe. The real-time qPCR was performed in duplicates on Biomark real-time qPCR system using 96 × 96 array (Fluidigm). In addition, the mRNA expression of *CD36*, peroxisome proliferator-activated receptor (*PPAR*)-γ, adipose triglyceride lipase (*ATGL*), hormone-sensitive lipase (*HSL*), diacylglycerol acyltransferase (*DGAT*)-2, *IL-6*, *IL-8*, *IL-10*, *MCP-1*, *TNF-α*, and *leptin* was quantified by qPCR without preamplification on an ABI PRISM 7500 (Applied Biosystems). Data were normalized to reference gene *PPIA*, which proved to be superior over two other measured reference genes, *PUM1* and *GUSB* (not shown). The method of $2^{(-\delta Ct)}$ was calculated for statistical analysis, and the final values for the figures were expressed as fold change related to mean basal value.

Statistical analysis

Data are presented as means ± SEM. Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software, Inc). The comparison of the anthropometric, biochemical, and other variables before the diet, at day 2, and at day 28 of the VLCD was done using a one-way ANOVA with repeated measures, followed by post hoc pairwise comparisons with Bonferroni adjustment for multiple testing. Correlations of fold changes of all parameters during 2 days and 28 days of the VLCD (value at d 2/baseline) or (value at d 28/baseline) were assessed by Pearson's correlation. The difference of $P < .05$ was considered as statistically significant.

Results

Effect of dietary intervention on clinical and laboratory characteristics of obese women

Anthropometric and biochemical parameters of subjects before and during two stages of the diet are presented in Table 1. At day 2 of the VLCD, the subjects´ body weight was reduced by 1.4%, whereas fat mass was not changed. After 28 days of the VLCD, a body weight loss of 9.2% was achieved, associated with a decrease of 16.5% of fat mass (kilograms).

Plasma glucose levels and triglycerides were not changed significantly during any phase of the intervention. FFA and β-hydroxybutyrate levels were elevated after both 2 days and 28 days of the VLCD. Total cholesterol and HDL cholesterol levels decreased after 28 days of the VLCD. Insulin and insulin resistance estimated by HOMA-IR decreased after 2 days of the diet by 13.7% and 16.4%, whereas at day 28 these variables decreased by 40% and 44%, respectively (Table 1).

Effect of dietary intervention on mRNA gene expression in sc abdominal adipose

Genes regulated after 2 days of VLCD

Among all the genes analyzed, those that were down-regulated at day 2 were as follows: three lipogenic genes (*SCD1*, *FASN*, and *ELOVL6*), the lipogenic transcription factor sterol regulatory element-binding protein-1c (*SREBP1c*), and fibrotic enzyme-lysyl oxidase (*LOX*).

Up-regulated genes at day 2 were as follows: lipases (*ATGL*, *HSL*), ATGL coactivator *CGI58*, transcription factor *PPARγ*, and fatty acid translocase *CD36*. mRNA expression of glucose transporter *GLUT1* had a tendency to increase after 2 days of the VLCD ($P = .09$).

All other genes were not changed at day 2 of the VLCD; explicitly we would mention the genes involved in β-oxidation (*CPT1β*, *ACOX*, *ACADM*, *PPARα*, *PCG1*) (Figure 2C), the genes involved in fibrosis (*TLR4*, collagens, *TGFβ1*, *MMP9*) (Figure 2E) and in inflammation (macrophage markers and cytokines) (Figure 2F), and several genes related to lipogenesis and lipolysis.

Genes regulated after 28 days of VLCD

Genes down-regulated after 28 days of VLCD were as follows: all lipogenic enzymes (*SCD1*, *FAS*, *DGAT2*, *ACLY*, *ACACA*, *ELOVL6*) and two lipogenic transcription factors (*SREBP1c*, carbohydrate-responsive element binding protein) (Figure 2A); lipolytic genes and regulators, *MGL*, *G0S2* (an inhibitor of ATGL), *PLIN1* (an inhibitor of HSL), and *DGAT1* (an enzyme involved in the reesterification of fatty acids and in lipogenesis) (Figure 2B); genes associated with β-oxidation of fatty acids, *CPT1*, *ACOX1*, and *ACAD* (Figure 2C); insulin-stimu-

Table 1. Clinical Characteristics of 17 Obese Women Before the Diet and After 2 Days and After 28 Days of VLCD

	Before Diet	2 Days of VLCD	28 Days of VLCD
Weight, kg	93.5 ± 2.3	92.1 ± 2.3[a]	84.9 ± 2.3[a]
BMI, kg/m^{-2}	32.7 ± 0.9	32.2 ± 0.9[a]	29.7 ± 0.8[a]
Fat mass, kg	38.9 ± 2.0	38.6 ± 1.9	32.3 ± 1.6[a]
Fat-free mass, %	59.6 ± 1.2	59.3 ± 1.2	63.5 ± 1.1[b]
Waist circumference, cm	99.9 ± 1.7	98.6 ± 1.6[b]	92.6 ± 1.6[a]
Glucose, mmol/L^{-1}	5.0 ± 0.1	5.1 ± 0.1	4.9 ± 0.2
Insulin, mU/L^{-1}	10.2 ± 1.0	8.0 ± 0.6[c]	5.3 ± 0.6[a]
FFAs, μmol/L^{-1}	820 ± 56	1156 ± 119[c]	1115 ± 70[b]
Glycerol, μmol/L^{-1}	124 ± 16	147 ± 14	113 ± 10
Triglycerides, mmol/L^{-1}	1.12 ± 0.12	1.04 ± 0.07	0.93 ± 0.10
HDL, mmol/L^{-1}	1.25 ± 0.05	1.21 ± 0.06	1.06 ± 0.04[a]
Total cholesterol, mmol/L^{-1}	4.82 ± 0.20	4.86 ± 0.18	3.87 ± 0.13[a]
β-Hydroxybutyrate, mmol/L^{-1}	114 ± 19	379 ± 63[b]	603 ± 124[b]
HOMA-IR	2.3 ± 0.2	1.8 ± 0.1[c]	1.3 ± 0.2[a]
QUICKI	0.342 ± 0.005	0.354 ± 0.004[c]	0.386 ± 0.009[a]

Abbreviations: BMI, body mass index; QUICKI, quantitative insulin sensitivity check index. Data are presented as mean ± SEM.

[a] $P < .001$ when compared with baseline (before the diet) values (one way ANOVA with repeated measures, followed by post hoc pairwise comparisons with Bonferroni adjustment for multiple testing)

[b] $P < .01$ when compared with baseline (before the diet) values (one way ANOVA with repeated measures, followed by post hoc pairwise comparisons with Bonferroni adjustment for multiple testing).

[c] $P < .05$ when compared with baseline (before the diet) values (one way ANOVA with repeated measures, followed by post hoc pairwise comparisons with Bonferroni adjustment for multiple testing).

lated glucose transporter 4 (*GLUT4*) (Figure 2D); leptin (Figure 2D); and fibrotic enzyme *LOX* (Figure 2E).

Genes up-regulated after 28 days of VLCD were some macrophage markers, namely *CD163*, *MSR1*, *IRF5*, and *CCR2*. The increase of mRNA expression of other markers (*ACP5*, *FCGBP*, *ITGAX*) and cytokines (*IL-8*, *MCP-1*, *TNFα*, *IL-6*, and *IL-10*) was observed, but it did not reach statistical significance (Figure 2F).

Expression of all other genes was not significantly modified at the end of 28 days of the dietary intervention, specifically the following: genes involved in lipolysis (*HSL*, *ATGL*, *CGI58*, *CD36*) (Figure 2B), transcription factors *PPAR*. *PPARγ*, and *PPARγ* coactivactor 1α, insulin receptor substrate 1, and genes involved in fibrosis (Figure 2E).

Correlations of the diet-induced changes in gene expression in SAAT and in metabolic parameters during VLCD intervention

Two-day changes

Changes of circulating FFAs and glycerol after 2 days of the VLCD correlated with changes in mRNA expression of *CGI58* (Supplemental Figure 1). The changes of glycerol after 2 days of the VLCD correlated with expression changes of *HSL* and *ATGL* (Supplemental Figure 1, data not shown). The changes of the HOMA-IR after 2 days of the VLCD tended to correlate with changes of *HSL* and *ATGL* expression (data not shown).

Twenty-eight-day changes

The changes in mRNA expression of *leptin* and *LOX* correlated positively with the changes of mRNA expression of lipolytic and lipogenic enzymes, β-oxidation, and insulin receptor substrate 1 during 28 days of the VLCD (Supplemental Table 2). The changes of the HOMA-IR correlated with changes of plasma levels and the secretion of leptin (Supplemental Figure 1 and Supplemental Table 2). Changes of cholesterol, insulin, and triglycerides correlated with the changes of expression of several lipolytic and lipogenic genes (ie, *HSL*, *SCD1*, *FASN*, *DGAT2*) (Supplemental Figure 1).

Changes of plasma FGF21 correlated positively with corresponding changes of β-hydroxybutyrate ($r = 0.537$, $P = .048$) and negatively with corresponding fold changes of *ATGL*, *DGAT2*, *PPAR*, and *GLUT4* expression (Supplemental Figure 1, data not shown).

Secretion of cytokines/adipokines in sc abdominal adipose tissue during VLCD

In vitro secretion of cytokines IL-6 and MCP-1 from SAAT explants did not change after 2 days of VLCD but increased after 28 days of VLCD. Secretion of IL-8 and TNFα was not significantly changed after 2 days and tended to be increased after 28 days of the VLCD ($P = .053$ and $P = .066$, respectively). Secretion of IL-10 was not significantly changed in either VLCD phase (Figure 3). Secretion of leptin was not changed after 2 days of the

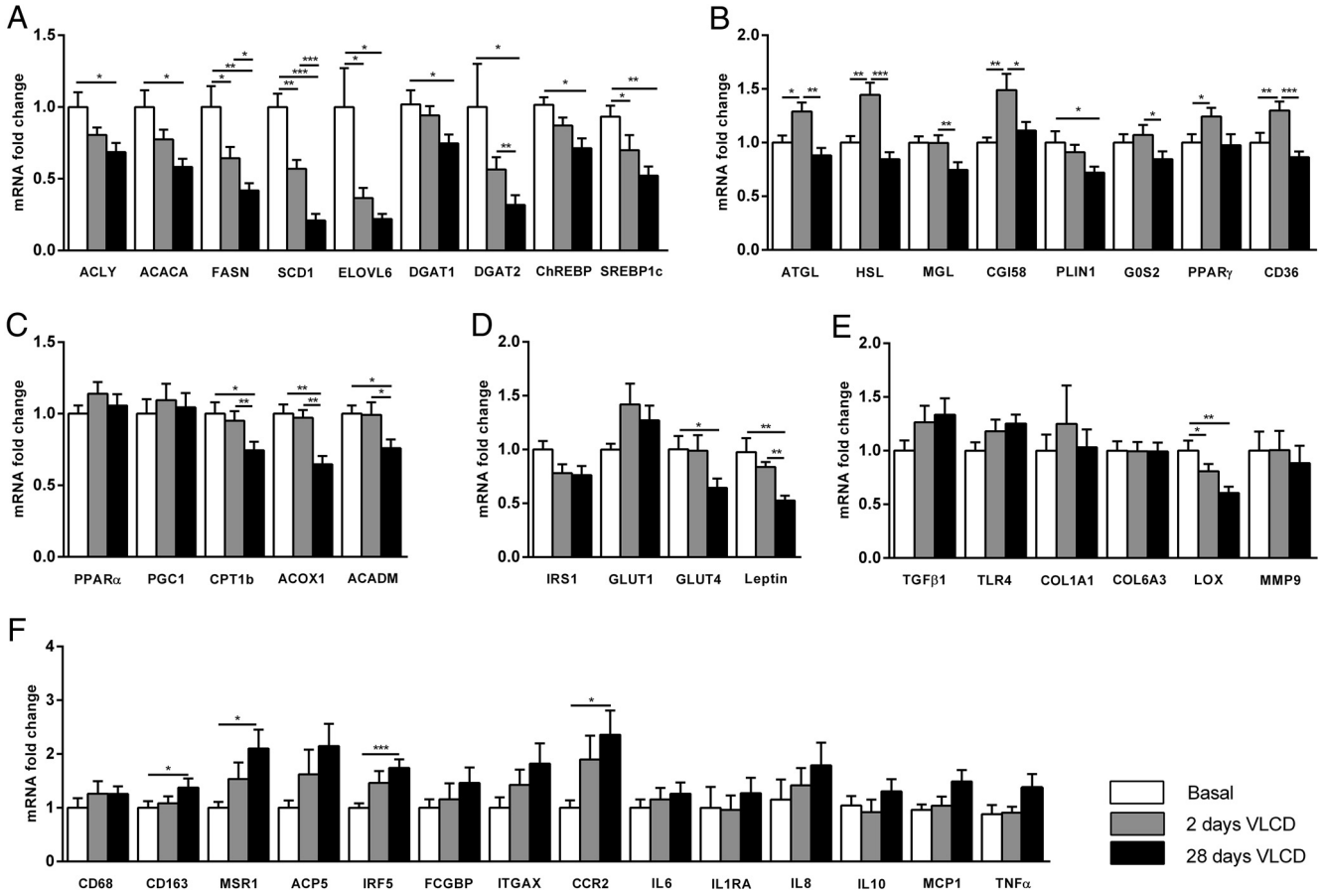

Figure 2. mRNA expression of genes in SAAT of obese women before the diet (white), 2 days after the VLCD (gray), and 28 days after the VLCD (black). Lipogenesis (A), lipolysis (B), β-oxidation (C), insulin/glucose receptors and leptin (D), fibrosis (E), and inflammation (F) are shown. Data are presented as fold change ± SEM, related to mean basal (before diet) gene expression, normalized to *PPIA* expression (n = 16). *, $P < .05$, **, $P < .01$, ***, $P < .001$, compared with prediet levels or values at 2 days of the VLCD (one way ANOVA with repeated measures, followed by post hoc pairwise comparisons with Bonferroni adjustment for multiple testing).

VLCD but decreased significantly after 28 days of the VLCD (Figure 3).

Plasma levels of cytokines, CRP, FGF21, and leptin during VLCD

Plasma concentration of cytokines IL-6, and MCP1 increased after 2 days of the VLCD and returned to baseline after 28 days of the VLCD (Figure 4). Similarly, CRP concentration had a tendency to increase after 2 days of the VLCD ($P = .07$) and decreased under the baseline values after 28 days of the VLCD (Figure 4). IL-8, IL-10, TNFα, and cortisol levels were not significantly changed after either 2 or 28 days of the VLCD. The average plasma leptin levels did not change significantly after 2 days' VLCD (decrease by 21%, $P = .21$); however, the response showed a high interindividual variability. After 28 days of the VLCD, the decrease of leptin was markedly pronounced (by 49%, $P < .001$). FGF21 was not changed

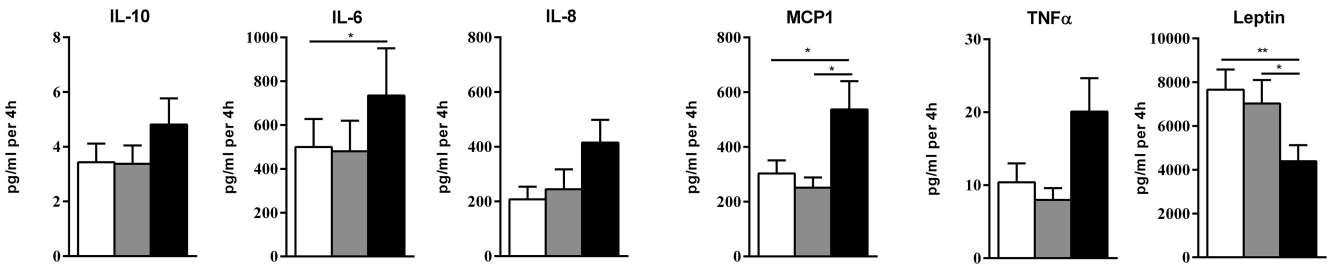

Figure 3. Secretion of cytokines/adipokines from SAAT of obese women before the diet (white), 2 days after the VLCD (gray), and 28 days after the VLCD (black). Data are presented as a concentration of secreted protein (picograms per milliliter per 4 h) ± SEM (n = 16). *, $P < .05$, **, $P < .01$, ***, $P < .001$, compared with prediet levels or values at 2 days of the VLCD (one way ANOVA with repeated measures, followed by post hoc pairwise comparisons with Bonferroni adjustment for multiple testing).

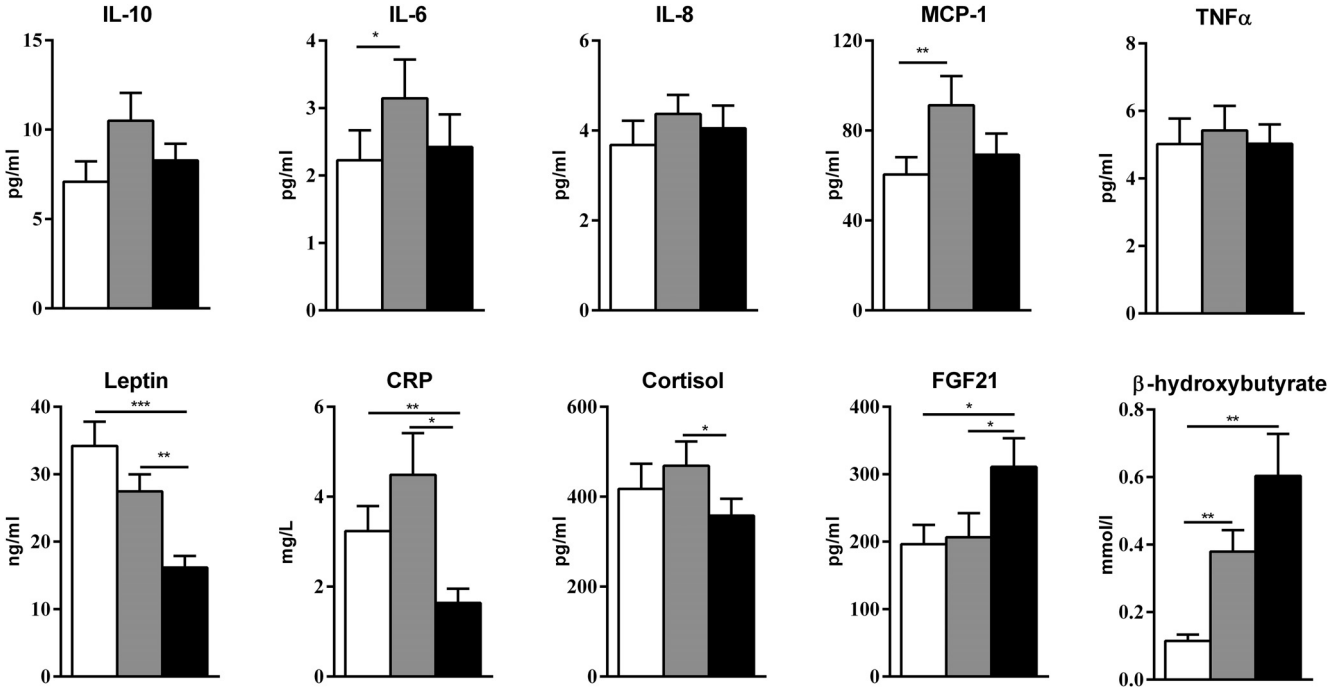

Figure 4. Plasma levels of cytokines and hormones in obese women before the diet (white), 2 days after the VLCD (gray), and 28 days after the VLCD (black). Data are presented as mean ± SEM (n = 17). *, $P < .05$, **, $P < .01$, ***, $P < .001$, compared with prediet values or values at 2 days of the VLCD (one way ANOVA with repeated measures, followed by post hoc pairwise comparisons with Bonferroni adjustment for multiple testing).

after 2 days of the VLCD and was elevated after 28 days of the VLCD (Figure 4).

Discussion

The aim of this study was to elucidate metabolic and immune effects of the calorie restriction per se, when compared with the restriction accompanied with the body fat mass loss. Therefore, in moderately obese women, whole-body and AT characteristics were compared between the early (2 d) and later phase (28 d) of VLCD. The main finding is that the responses of metabolic and inflammation-related characteristics in SAAT and in plasma differed markedly between the two phases of the diet.

The 2 days of calorie restriction modified genes involved in lipolysis and lipogenesis in SAAT, whereas the inflammatory status of AT was not changed. The increased levels of FFAs and β-hydroxybutyrate in plasma reflect enhanced AT lipolysis and hepatic ketogenesis, similarly as seen in fasting (12). Increased expression of major AT lipases in SAAT, *HSL*, *ATGL*, with its cofactor *CGI58*, could contribute to a higher release of FFAs from AT to plasma, as reviewed by Nielsen and Moller (13). Indeed, in this study the increase in plasma FFAs and glycerol after 2 days of the VLCD correlated with the changes in expression of *CGI58*, and the changes in plasma glycerol correlated with SAAT lipases, *ATGL* and *HSL*. In addition, increased mRNA expression of *CD36*, a fatty acid transporter in SAAT, was found at the early phase of the VLCD, and this expression correlated with increased FFA plasma levels. This is in line with published studies showing that the expression of *CD36* in AT increased in response to the acute increase of FFA plasma levels induced by lipid infusion (14) and also in response to chronic elevation of FFA plasma levels in obese subjects with metabolic syndrome (15). On the other hand, in the study of Hames et al (16), CD36 was shown to facilitate the FFA uptake by AT when the levels of FFAs in plasma are low, ie, after consumption of a carbohydrate-containing meal. Thus, the higher expression of *CD36* observed after 2 days of calorie restriction could provide AT better FFA absorption capacities for the anticipated refeeding phase. Also, it was suggested that FFAs released from adipocyte by lipolysis are immediately taken back by CD36 to secure cycling of FFA, and this mechanism may prevent excessive release of FFA under condition of stimulated lipolysis (17). The up-regulation of *CD36* in our study is likely driven by *PPAR* activation. Indeed, *PPARγ* mRNA expression was elevated during the early VLCD phase, and this change correlated with the changes of mRNA expression of *CD36* and with changes of lipolytic genes (*HSL*, *ATGL*) in this study. Up-regulated *PPARγ* expression and activity might be associated by the enhanced availability of lipolysis-de-

rived fatty acids (PPARγ ligands), as suggested by Haemmerle et al (18, 19).

In contrast to the effect of 2 days of calorie restriction on lipolytic genes, the expression of lipogenic enzymes in SAAT was reduced. This reduction was likely linked to a down-regulation of *SREBP1c*, one of the lipogenic transcription factors, which is regulated by insulin (20). The improvement in IS during the early phase of the VLCD was not associated with the metabolic gene expression in SAAT, except a borderline correlation with several lipolytic genes (ie, *HSL*, *ATGL*). Thus, changes occurring in AT in response to short calorie restriction do not appear to play major role in the metabolic improvement induced by 2 days of a VLCD. Therefore, the potential role of other insulin -sensitive organs in the diet-induced metabolic changes must be taken into account. Lara-Castro et al (21) demonstrated that the decrease of the intramyocellular lipids after 6 days of a VLCD was closely related to insulin resistance. In contrast to that, Jazet et al (22) found no changes in the markers of insulin signaling and glucose transport in skeletal muscle after 2 days of a VLCD, but they found a diet-induced decrease of endogenous glucose production. The latter points to the role of the liver in the very short VLCD-induced metabolic changes.

Because no significant change in gene expression and secretion of proinflammatory (*IL-6*, *IL-8*, *TNFα*, *MCP-1*) and antiinflammatory (*IL-10*, *IL-1Ra*, *TGFβ*1) cytokines, and macrophage markers (*CD68*, *CD163*, *IRF5*, *MSR1*, *ACP5*, *CCR2*, *FCGBP*, *ITGAX*) in SAAT were observed after 2 days of the VLCD, it may be concluded that the early improvement of IS (metabolic changes) was not related to changes of immune status of adipose tissue. Indeed, several studies using opposite dietary intervention, ie, short overfeeding in healthy men, showed similar dichotomy between AT inflammation and IS under conditions of mild weight gain when IS was impaired despite no induction of inflammatory cytokines and macrophage activation in SAAT (23, 24).

In contrast to the changes induced by 2 days of a VLCD, after 28 days of severe calorie restriction mRNA expression of lipolytic genes (*ATGL*, *HSL*, *MGL*, *PLIN1*, *CGI58*) returned to the prediet levels. The no change in *ATGL* or *HSL* mRNA expression after 1 month of the VLCD, when compared with the prediet condition, is in agreement with some of the previous studies of our and other teams (1, 25). However, the decrease in expression of lipolytic genes on day 28 compared with day 2 does not essentially mean that lipolysis is attenuated. FFA levels in plasma were elevated at 28 day similarly to day 2, indicating maintenance of higher lipolytic rate. The decrease to baseline in expression of lipases at the late phase of the VLCD was accompanied by a decrease of *G0S2*, which has been shown as a dominant inhibitor of ATGL in adipocytes (26), and by a reduction in *DGAT1*, which is responsible for FFA reesterification. Reduced inhibition of *ATGL* together with reduced reesterification can thus still ensure the FFA release needful to cover the energy demand of the organism. Thus, our data support the hypothesis that G0S2 acts predominantly as a long-term regulator of ATGL, whereas CGI58 is more important for the regulation of acute lipolytic response (13). Furthermore, at day 28 a marked decrease of lipogenic genes, genes of β-oxidation, and insulin-stimulated glucose transport (*GLUT4*) in SAAT was observed. These processes probably prevent excessive breakdown as well as storage of FFAs in adipocytes during longer-term shortage and secure its adequate release from AT to serve as substrates for other organs.

The increase in a SAAT inflammatory state after 28 days of the VLCD is in agreement with published results of our and other groups (3, 11, 27, 28). Previously we have shown that 28 days of a VLCD was not associated with an increase in macrophage content in SAAT (29). Thus, it might be assumed that the observed increase of macrophage marker expression in response to strong calorie restriction reflects stimulation of macrophage activation rather than their accumulation. The increase of proinflammatory (*IL-6*, *IL-8*, *MCP-1*, *TNF*) but also antiinflammatory (*IL-10*, *IL-1Ra*) cytokines together with M1 (*IGTAX*, *CCR*) and M2 (*CD163*) macrophage markers is in accordance with the published findings that macrophages in SAAT are of a mixed phenotype (30). The role of the increase in the inflammatory state in SAAT after 28 days of severe calorie restriction is still unclear.

Interestingly, the only gene identified to be significantly down-regulated in both phases of the VLCD was *LOX*, one of the genes involved in fibrosis (and extracellular matrix remodeling. LOX catalyzes the cross-linking of collagens in AT and, thus, it is one of a key factors contributing to the fibrosis of AT observed in obesity (31). Inhibition of LOX also resulted in the improvement of several metabolic parameters, ameliorated glucose and insulin levels, decreased HOMA index, and reduced plasma triglyceride level in obese rats (31). Indeed, the decrease of *LOX* expression found in our study was associated with changes in SAAT metabolism genes (Supplemental Table 2). Importantly, the reduction of lipogenesis and fibrosis observed during weight loss in our study represent the opposite processes to those found in the overfeeding studies (23, 24).

Leptin appeared as the only adipokine for which a quantitative relationship with the diet-induced changes of HOMA-IR was found. In addition, leptin changes were correlated also with a number of metabolic-related genes

in AT and with plasma FGF21 levels. Leptin was shown to act on peripheral tissues by the regulation of fatty acid oxidation and energy expenditure through activation of AMP-activated protein kinase, induction of FFA oxidation genes, and increased transport of FFAs to mitochondria (32). It might be suggested that this adipokine is a sensor of metabolic changes in SAAT and the signal that mediates the metabolic interplay with other organs during calorie restriction. Further studies in this issue in obese humans should be warranted.

After 28 days of the VLCD, the diet-induced improvement of metabolic indices (TG, insulin, cholesterol) was correlated with changes in lipogenic genes (eg, *SCD1*, *FASN*, *DGAT2*). It was shown in mice that a decrease of *SCD1* expression (in liver or systemic level) was correlated with an improvement of the metabolic profile and insulin sensitivity (33, 34). Thus, we may hypothesize that the decrease of lipogenic genes in AT is paralleled with the decrease of lipogenesis in liver, which is probably one of the important contributors to the improvement of metabolic profile and IS during the VLCD.

FGF21 was shown to act as a metabolic regulator during fasting through stimulating ketogenesis and fatty acid oxidation in liver in mice (8). In addition, in mice and 3T3-L1 adipocytes, the role of FGF21 in the down-regulation of lipogenesis and lipolysis in SAAT was shown (8–10). In line with this, we observed negative correlation between the increase of plasma FGF21 during the 28 days of the VLCD and the changes in mRNA expression of genes involved in SAAT lipogenesis (*FASN*, *DGAT2*) and glucose uptake (*GLUT4*). Because FGF21 plasma levels are increased only in later phase of the diet (35), it might be suggested that FGF21 plays a role in the regulation of a switch from a short-term to a longer-term calorie restriction. The down-regulation of lipogenesis in association with insulin-stimulated glucose transport into AT supports the role of FGF21 in the saving of substrates for other organs during famine.

In conclusion, our findings show that the early (2 d) and later (28 d) phases of the VLCD differ with respect to metabolic and inflammatory response in SAAT. Although in both phases the effects of severe calorie restriction represent the reaction to shortage of calories/nutrients (ie, induced lipolysis, reduced storage of lipids), the expression of regulatory cofactors involved in these processes is different in the early and later phase of the VLCD. The diet-induced modifications in metabolic and inflammation-related functions of AT did not appear to play a pivotal role in the improvement of IS at the early phase of the VLCD. The processes observed after 28 days of the VLCD probably contribute to adaptation of SAAT to prolonged calorie restriction through the saving of substrates. Moreover, the correlation of the changes in metabolic genes in SAAT with metabolic indices and FGF21 suggest the possible cross talk of SAAT with liver function during the VLCD.

Acknowledgments

Address all correspondence and requests for reprints to: Michaela Šiklová, PhD, Department of Sports Medicine, Third Faculty of Medicine, Charles University in Prague, Ruská 87, 100 00 Prague 10, Czech Republic. E-mail: michaela.siklova@lf3.cuni.cz.

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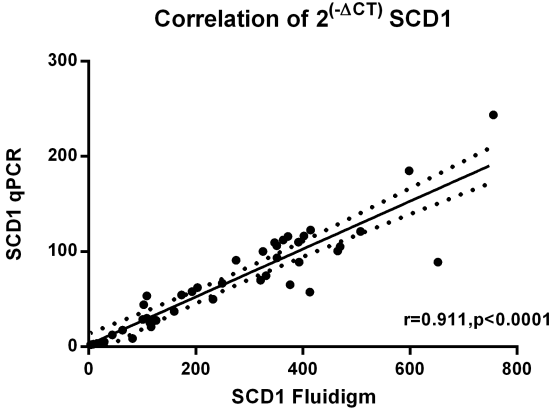
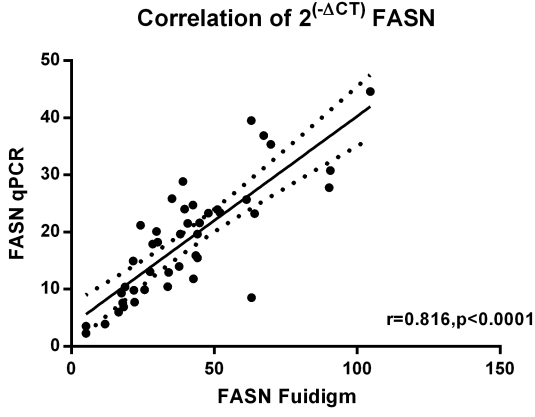
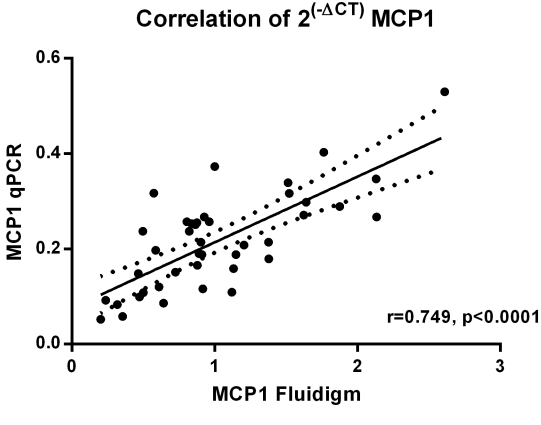
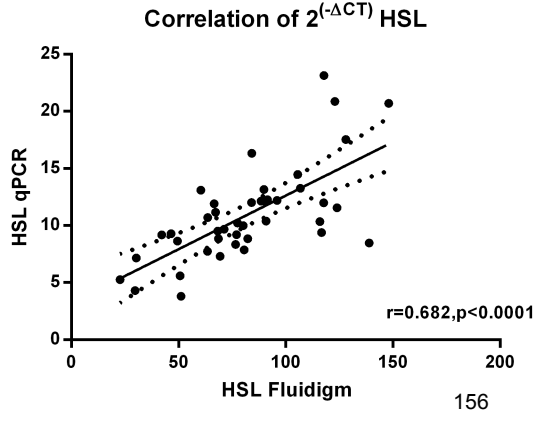
Supplemetal Figure for reviewers: Correlation between gene expression data measured by normal qPCR and by Fludigm/Biomark system



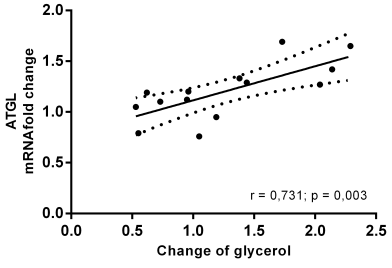


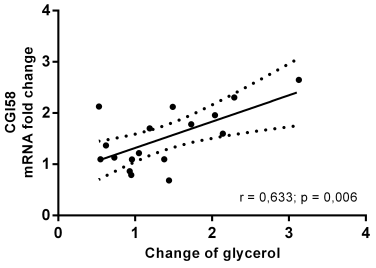


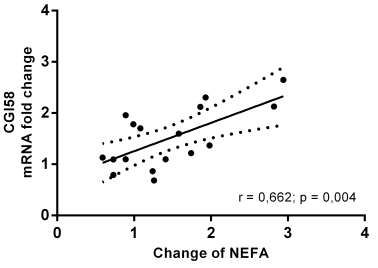


A

2 days changes

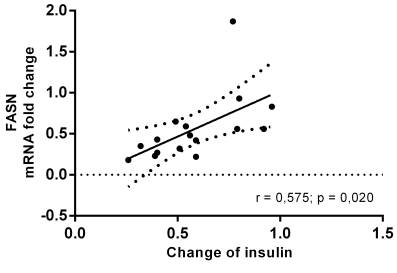


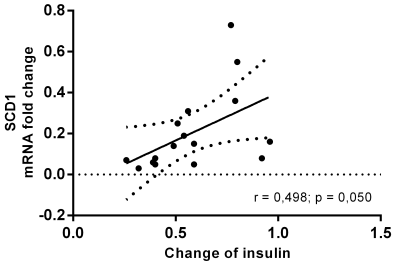


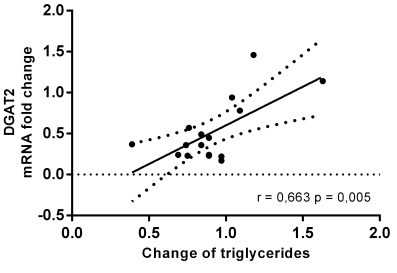


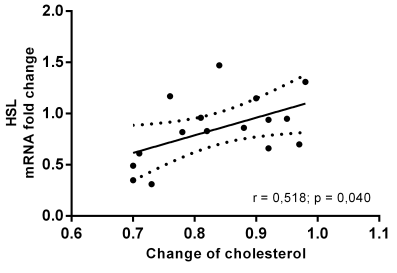
B

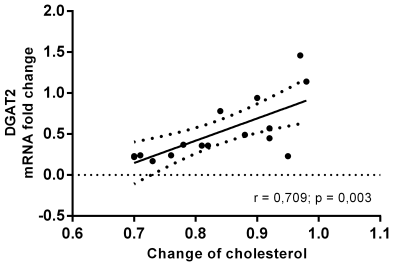
28 days changes

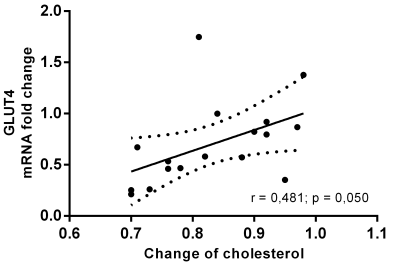


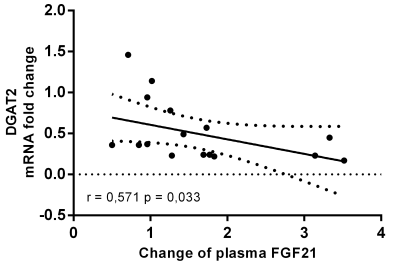


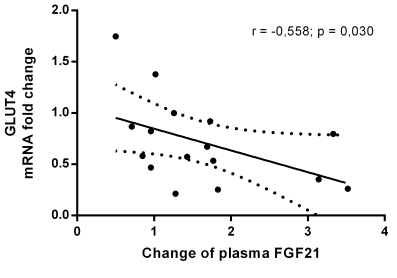


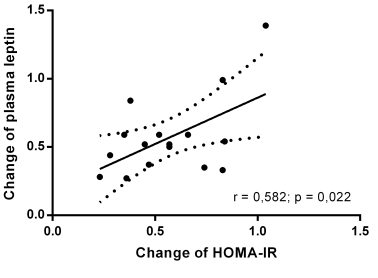












Supplementary figure 1: Correlations of selected mRNA gene expression in AT and fold changes of selected metabolic factors after A) 2 days and B) 28 days of VLCD. Analyzed by linear regression, Pearson correlation coefficient is presented (n=16).

Supplemental Table 1:List of analyzed genes

Gene symbol	Assay ID	Protein	Function
De novo lipogenesis			
ACLY	Hs00982738_m1	ATP citrate lyase	Synthesis of acetyl-CoA
ACACA	Hs01046047_m1	Acetyl-CoA carboxylase alpha	Synthesis of malonyl-CoA
FAS	Hs01005622_m1	Fatty acid synthase	Synthesis of palmitate
SCD1	Hs01682761_m1	Stearoyl-CoA desaturase 1	Insertion of a double bond into fatty acyl-CoA substrates
ELOVL6	Hs00225412_m1	ELOVL Fatty Acid Elongase 6	Elongation of 2 carbons to the chain of long- and very long-chain fatty acids
DGAT1	Hs01017541_m1	Diacylglycerol O-Acyltransferase 1	Re-esterification of fatty acids, triglyceride synthesis
DGAT2	Hs01045913_m1	Diacylglycerol O-Acyltransferase 2	Triglyceride synthesis
CHREBP	Hs00263027_m1	Carbohydrate-responsive element-binding protein	Transcription factor regulating de novo lipogenesis and glucose metabolism
SREBP1c	Hs01088691_m1	Sterol regulatory element binding protein 1c	Transcription factor regulating de novo lipogenesis and glucose metabolism
Lipolysis			
PNPLA2=ATGL	Hs00982040_g1	Patatin-like phospholipase domain containing 2 (=adipose triglyceride lipase)	Triglyceride hydrolysis
LIPE=HSL	Hs00943404_m1	Lipase E, Hormone Sensitive Type (=hormon sensitive lipase)	Diglyceride hydrolysis
MGL	Hs00200752_m1	Monoglyceride lipase	Monoglyceride hydrolysis
ABHD5=CGI58	s01104373_m1	Abhydrolase Domain Containing 5 (=lipid Droplet-Binding Protein CGI-58)	Cofactor of ATGL
PLIN1	Hs00160173_m1	Perilipin 1	Coating of lipid droplets, inhibitor of lipolysis
G0S2	Hs00274783_s1	G0/G1 Switch 2 (cofactor ATGL)	ATGL inhibitor (lipolysis inhibitor)
PPARγ	Hs01115513_m1	Peroxisome proliferator-activated receptor gamma	Master regulator of adipogenesis and glucose and lipid metabolism of adipocytes
FAT/CD36	Hs00169627_m1	Fatty acid translocase	Transport and regulation of fatty acid transport across the cell membranes
β-oxidation			
PPARα	Hs00947539_m1	Peroxisome proliferator-activated receptor alpha	Transcription factor regulating glucose and lipid metabolism
PPARGC1A=PGC1	Hs01016719_m1	Peroxisome proliferator-activated receptor gamma coactivator 1 alpha	Cofactor of PPARγ, involved in thermogenesis and energy metabolism
CPT1B	Hs03046298_s1	CarnitinePalmitoyltransferase 1B	Conversion of the long-chain acyl-CoA to long-chain acylcarnitine
ACOX1	Hs01074241_m1	Acyl-CoA Oxidase 1, Palmitoyl	First step of β-oxidation pathway of very long chain-fatty acids
ACADM	Hs00936580_m1	Acyl-CoA Dehydrogenase	First step of β-oxidation pathway of medium long chain-fatty acids
Insulin/Glucose receptors			
GLUT1	Hs00892681_m1	Glucose transporter 1	Basal glucose uptake
GLUT4	Hs00168966_m1	Glucose transporter 4	Stimulated glucose uptake
IRS1	Hs00178563_m1	Insulin receptor substrate 1	Transmits signal from insulin and IGF-1 receptor downstream
Fibrosis			
TGFb1	Hs00998133_m1	Transforming growth factor beta 1	Pro-fibrotic factor
TLR4	Hs01060206_m1	Toll-like receptor 4	Endotoxin receptor mediating the development of AT fibrosis
COL1A1	Hs00164004_m1	Collagen type I alpha 1	Major component of type I collagen

COL6A3	Hs00365098_m1	Collagen, type VI, alpha 3	Alpha chain of type VI collagen
LOX	Hs00942480_m1	Lysyl oxidase	Cross-links collagens and elastins
MMP9	Hs00234579_m1	Matrix metallopeptidase 9	Catalysis of ECM breakdown
Markers of monocyte/ macrophage			
CD68	Hs00154355_m1	Cluster of differentiation 68	Scavenger receptor
CD163	Hs00174705_m1	Cluster of differentiation 163	Scavenger receptor
MSR1	Hs00234007_m1	Macrophage scavenger receptor 1	Class A macrophage scavenger receptor
ACP5	Hs00356261_m1	Acid phosphatase 5, tartrate resistant	Belongs to the metallophosphoesterase superfamily
IRF5	Hs00158114_m1	Interferon regulatory factor 5	Transcription factor regulating induction of inflammatory cytokines
FCGBP	Hs00175398_m1	IgGFc-binding protein	Function unknown
ITGAX	Hs00174217_m1	Integrin, alpha X (complement component 3 receptor 4 subunit) (CD11C)	Mediates cell-cell interaction during inflammation
CCR2	Hs00356601_m1	MCP1 receptor	Mediates chemotactic perception for monocytes and basophils
Cytokines/adipokines			
Leptin	Hs00174877_m1	Leptin	Adipokine reflecting adipose tissue mass and regulating energy balance
IL6	Hs00985639_m1	Interleukin 6 (interferon, beta 2)	Cytokine stimulating immunologic response and regulating energy metabolism
IL1RN	Hs00893626_m1	Interleukin 1 receptor antagonist	Natural inhibitor of the pro-inflammatory IL1 cytokine
IL8	Hs00174103_m1	Interleukin 8	Causes chemotaxis of immune cells and induces phagocytosis
IL10	Hs00961622_m1	Interleukin 10	Regulation of inflammatory response
MCP1	Hs00234140_m1	Monocyte chemoattractant protein 1	Attracts inflammatory cells to the inflammatory site
TNFα	Hs00174128_m1	Tumor necrosis factor alpha	Proinflammatory cytokine regulating also lipid and carbohydrate metabolism
Reference genes			
PPIA	Hs04194521_s1	Peptidylprolylisomerase A (cyclophilin A)	Cis-trans isomerization of prolineimidic peptide bonds
GUSB	Hs00939627_m1	Glucuronidase Beta	Hydrolysis of β-D-glucuronic acid
PUM1	Hs00472881_m1	Pumilio homolog 1	Translational regulator

Table 2: Correlations between diet-induced changes of selected metabolic genes / indices and leptin, LOX, and CD36 during 2 days and 28 days of VLCD

Changes after 2 days of VLCD

mRNA (except of HOMA-IR)	Leptin (mRNA)	Leptin (secretion)	LOX (mRNA)	CD36 (mRNA)
ATGL	**0.662**[b]	0.276	0.284	**0.746**[c]
HSL	**0.647**[b]	0.135	0.469	**0.776**[c]
MGL	**0.636**[a]	-0.092	0.176	0.121
SCD1	**0.645**[c]	0.297	0.189	0.430
FASN	**0.668**[c]	0.250	0.513	**0.522**[a]
ACOX1	0.363	-0.194	0.207	0.051
ACADM	**0.551**[a]	0.335	0.254	0.256
PPARγ	**0.607**[b]	-0.104	**0.534**[a]	**0.841**[c]
PLIN	0.384	0.229	**0.593**[a]	0.239
GLUT4	0.386	0.408	**0.608**[a]	0.173
IRS1	0.300	0.300	**0.664**[b]	-0.248
SREBP	0.322	0.465	0.551	-0.047
ChREBP	**0.550**[a]	0.191	0.501	0.035
HOMA-IR	0.273	0.134	-0.064	0.182

Changes after 28 days of VLCD

mRNA (except of HOMA-IR)	Leptin (mRNA)	Leptin (secretion)	LOX (mRNA)	CD36 (mRNA)
ATGL	**0.706**[a]	**0.593**[a]	**0.658**[b]	**0.645**[b]
HSL	**0.712**[c]	**0.607**[a]	**0.641**[b]	**0.636**[b]
MGL	**0.709**[a]	**0.583**[a]	**0.756**[c]	0.248
SCD1	**0.702**[b]	**0.596**[a]	0.431	**0.507**[a]
FASN	**0.512**[a]	0.497	**0.589**[a]	0.488
ACOX1	0.414	0.332	**0.604**[a]	0.108
ACADM	0.372	0.347	**0.817**[c]	0.279
PPARγ	**0.641**[b]	**0.757**[a]	0.077	**0.819**[c]
PLIN	**0.594**[a]	0.423	**0.846**[c]	0.346
GLUT4	**0.593**[a]	**0.591**[a]	0.493	0.165
IRS1	**0.554**[a]	0.388	**0.668**[b]	0.280
SREBP	0.507	0.503	0.470	0.249
ChREBP	0.475	0.374	**0.689**[c]	0.242
HOMA-IR	0.445	**0.550**[a]	-0.426	0.439

Correlations were calculated as foldchange: (value at day 2/ baseline) or (value at day 28/ baseline).Pearson's correlation coefficient is presented (n=16). [a]$p<0.05$; [b]$p\leq0.01$; [c]$p\leq0.001$.