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Summary of the Ph.D. thesis

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

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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.

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 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.

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.

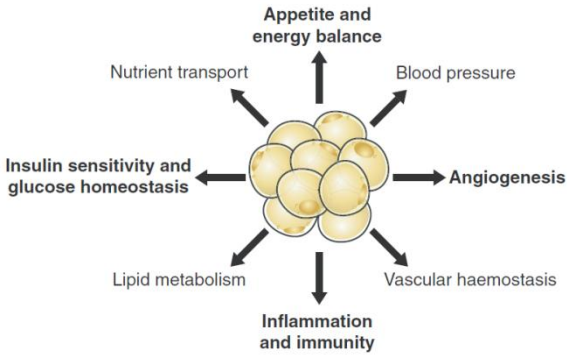

Figure 1: Adipose tissue is an organ with a plethora of functions. Adapted from (1).

In the introductory section of this thesis, we will first describe two elementary types of AT and its cellular composition. Next, the most important physiological functions of AT and adipocytes will be explained. 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.

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) (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 (4).

Fat distribution is markedly altered by many factors, such as sex, hormonal status, disease state and age (5). F.i., 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) (6). 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). Because of this, the proportion of body mass that is within fat depots remains constant. 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 (7). This is possible by the presence of uncoupling protein 1 (UCP1) that uncouples electron transport from ATP production, leading to the generation of heat (8). Because of the high mitochondria content and dense vascularisation, BAT appears *brown* compared to WAT. Recently, it was suggested that brown fat cells might be interspersed within the WAT (brown in white, i.e. *brite*, or *beige* AT). 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 (9).

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. 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 (10)**.**

1.1.3.2 Stromal vascular fraction

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 (11), 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) (12-14).

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*.

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 (15). However, peroxisome proliferator-activating receptor γ (PPARγ) was shown to be the both necessary and sufficient for adipogenesis.

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 (16; 17).

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 TAG 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 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) (18). 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.

1.2.1.2 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 (19; 20). The contribution of AT to DNL was considered for a long time negligible, but newer methods shown that DNL contributes approximately 20% of new TAG.

Carbohydrate metabolism, especially that of glucose, is the most commonly involved in the supplementation of carbon units for DNL. Therefore, glucose will be used as an example of a primary source to describe DNL pathway depicted on **Figure 2**. **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.**

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). Interestingly, although both of these DNL transcriptional factors regulate expression of key lipogenic genes such as *fasn* or *acc* (21), they are activated by different mechanisms. Experiments showed that SREBP1c gene expression is strongly stimulated by insulin (22-24), whereas ChREBP activity is regulated by glucose and other carbohydrates (25; 26). Nevertheless, the distinct regulation and functional roles of SREBP1c and ChREBP in AT are still being worked out.

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 (27). Recently, a novel class of AT-derived lipids was described: fatty acid-hydroxy-fatty acids (FAHFAs). These

lipids possess potent metabolic effects under the conditions of increased DNL in AT (28). 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 and exerted anti-inflammatory effects on AT-resident immune cells.

In addition to DNL products, many other lipid species as sphingolipids (29), cardiolipins (30) and prostaglandins (31; 32) also act as mediators of metabolism with both beneficial and deleterious effects and undoubtedly many more wait to be discovered.

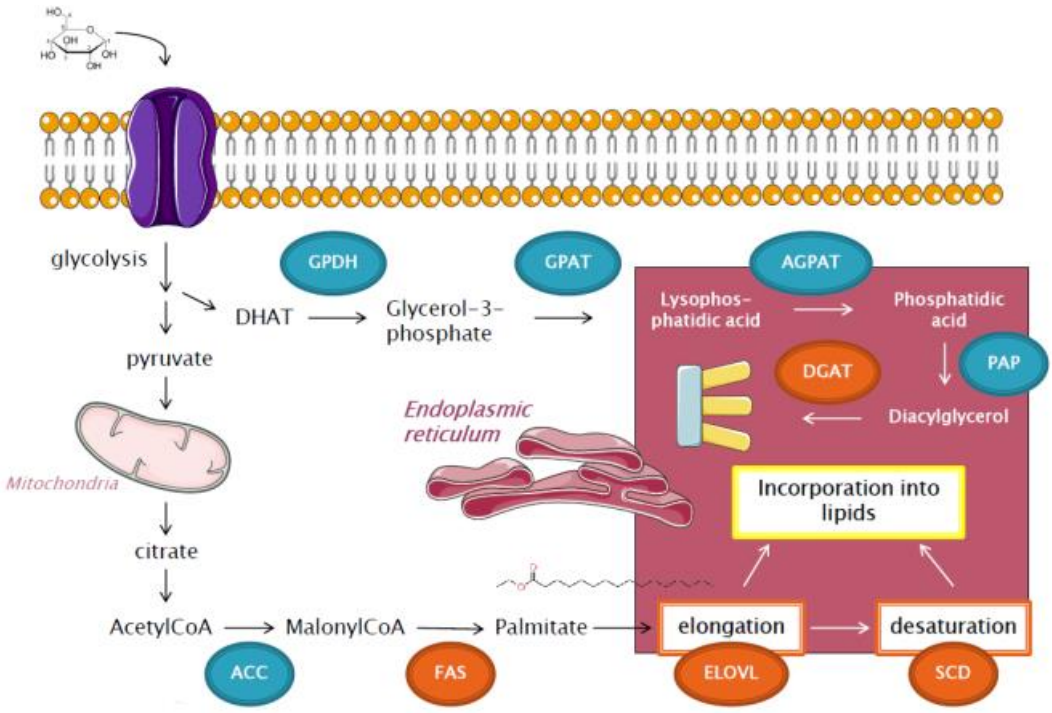

Figure 2: Simplified pathway of *de novo* lipogenesis. Glucose enteres inside the cell and undergoes glycolysis. Pyruvate enters mitochondria, where it is metabolised to citrate. In cytoplasm, citrate is converted to acetyl-CoA, metabolised by acetyl-CoA-carboxylase (ACC) into malonylCoA. MalonylCoA 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).

1.2.2 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. 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.

1.2.3 Secretory function of adipose tissue

AT functions as an active endocrine organ and releases multiple bioactive molecules known as lipokines (33) and adipokines (34-36). 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 (1.2.1.2), 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 and pro-inflammatory mediators. 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 (37). Leptin is the product of the obese gene (*ob*; also known as *lep*) (38). The major function of leptin is to regulate 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. In addition, leptin has multiple roles in the immune system (39). IL6 belongs to a group of pro-inflammatory cytokines. It is estimated that AT produces approximately one-third of total circulating IL6 (40). Plasma levels of IL6 are also increased in type 2 diabetic patients and elevated IL6 plasma concentration predicts the development of type 2 diabetes (41). If the expression of anti-inflammatory and pro-inflammatory adipokines is not balanced, the function of AT may be deteriorated. 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.

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* over 30 kg/m^2 (42; 43). World Health Organisation (WHO) reported that since 1980 obesity has doubled and now kills more people than undernourishment. The alarming point is that obesity spreads rapidly not only among adults, but also in children and elderly. This poses a major public health issue, since obesity is associated with an increased risk of developing insulin resistance (44) 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) (45; 46), atherosclerosis, hypertension, stroke, depression, infertility, obstructive sleep apnoea and several types of cancer (47; 48).

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 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 (49-51) and is an independent predictor for development of type 2 diabetes (52; 53).

1.3.2.2 Endoplasmic reticulum stress

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 in 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 (54-56).

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 (57). Importantly, high fat diet was also shown to cause ER stress.

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 UPR is to restore the normal functions of the ER and therefore the functions of the cell (58). 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). The overall process of UPR activation and its consequences on cell function is depicted in **Figure 3**. **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.**

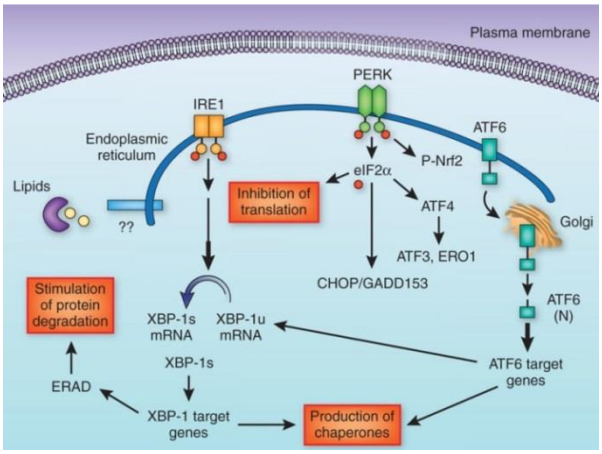

Figure 3: Activation of unfolded protein response. UPR is initiated through three paralelle signaling arms: IRE-1, PERK and ATF6. UPR activation stimulates protein degradation, selectively attenuates protein synthesis and increases the production of chaperons. When the ER stress is not resolved, the cell can undergo apoptosis. Adapted from (2).

1.3.2.3 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, calor, dolor and tumor. On the other hand, the pro-inflammatory mediators and signalling pathways are the same for both types of inflammation (59).

In obesity, stressed or damaged cells release damage-associated molecular patterns which are sensed by pattern-recognition receptors, thereby inducing inflammation (60). 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 induce

preadipocytes and endothelial cells to secrete monocyte chemoattractant protein 1 (MCP1) (61). MCP1 belongs to one of the critical factors attracting macrophages to adipocytes (62). Thus, as obesity develops, AT became progressively infiltrated by macrophages (61; 63). Local proliferation of macrophages also contributes to obesity-associated AT inflammation (64). Once pro-inflammatory macrophages are present in AT 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 (65). Furthermore, pro-inflammatory cytokines (such as IL6 and TNFα) and saturated free fatty acids belong to the major contributors to the establishment of insulin resistance (66; 67). These factors activate various ser/thr kinases such as nuclear factor-κB (NF-κB) signalling pathway, JNK or PKC (67; 68). Notably, proinflammatory cytokines not only interfere with insulin signalling, but also induce cellular senescence (69) which is considered as a hallmark of aging (70; 71).

1.3.2.4 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 (72). Thus, depending on the type of stressor, several "subtypes" of cellular senescence have been identified (71). 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, 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 reactive oxygen species (ROS) and the shift of pH optimum for lysosomal senescent associated β-galactosidase (SAβgal) activity (73; 74). 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) (75). In fact, histochemical detection of β-galactosidase activity at pH 6.0 is probably the most widely used assay for senescence detection (76). Nevertheless, to define senescence both in culture cells and in tissues, a collection of markers in combination should be used (77).

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) (78-81) The SASP is involved in the secretion of pro-inflammatory cytokines (71). 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 (82; 83).

Interestingly, even a low absolute number of senescent cells in a tissue may be able to exert systemic effects through the SASP (72). 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 (84-86). Thus, the accumulation of senescent cells within obese AT could represent another important step in development and progression of type 2 diabetes (87; 88).

Preadipocytes belong to the cells known to be susceptible to the development of cellular senescence (89; 90). 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

(89). Moreover, AT SA-βgal activity and p53 increase with BMI (89). 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 (91). 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 (92).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 (89). 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.**

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. SASP itself is believed to attract immune cells which carry out the clearance of senescent cells (82; 93). However, the functions of immune system decline with aging, which lead to progressive accumulation of senescent cells (94-96). 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 (89; 91; 97). 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, (98). Thus, strategies targeting senescent cells could improve metabolic function of obese AT, as well as aged AT.

1.3.2.5 Aging

Similarly as obesity, chronological aging is associated with ectopic fat accumulation causing lipotoxicity (99), chronic-low grade inflammation (100) and indeed, with the accumulation of senescent cells.

With aging, extensive changes in preadipocyte function occur (89). 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 (101) and show pro-inflammatory phenotype (89).

Preadipocytes from old individuals have lower expression of C/EBPα, PPARγ and their target genes than in preadipocytes from young individuals (102-104). Expression of these adipogenic transcription factors is also lower in serially passaged human preadipocytes when the cells are exposed to differentiation-inducing medium (105). Interestingly already six population doublings is sufficient to detectably impair adipogenesis (106; 107).

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 (108). 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 (109). 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.

1.4 Obesity management

Typically, strategies for obesity treatment can be non-pharmacological (diet and lifestyle intervention), pharmacological (anti-obesity drugs such as orlistat) and surgical (bariatric surgeries). Pharmacological treatment is recommended just for short-term use, (110) and patients undergoing any kind of bariatric surgery need lifelong medical monitoring and nutritional supplements (111; 112). 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 (113-116). The most striking decrease of weight is during the first week (2-8 kg), the later weight loss is moderate (maximally 2 kg/week) (117). There is a number of commercially available VLCDs, frequently in the form of liquid diet.

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 (118). This adaptation may be reached and evaluated in several months after the end of calorie restriction phase of diet.

These diets 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 (119-121). 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. 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 (122).

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 (111). Therefore, diets with 1200-1500 kcal/day and 1500-1800 kcal/day are often prescribed for women and men, respectively.

Lifestyle interventions typically prescribe 150-180 min per week of aerobic activity, such as brisk walking, or other types of moderate-intensity aerobic exercise (111). Regular

aerobic activity is associated with many health benefits such as improvements in lipid levels, decrease of blood pressure and visceral fat (123) and improved fitness (124).

Behaviour therapy provides a set of strategies and techniques to modify diet and patters of physical activity (111). The cornerstone of behaviour change is self-monitoring of food and calorie intake, along with recording physical activity and weight (122).

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 (125). 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 (126). Sarcopenia results in frailty, loss of independence, physical disability and increased mortality in older adults (127; 128). The combination of sarcopenia and obesity led to a term of *sarcopenic obesity* which is associated with more physical functional decline than simple obesity (129). 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. The weight loss could aggravate further sarcopenia and frailty. **Therefore, a clinical study described in Project D was designed with the aim to clarify the effects of weight loss on adipocytes in the elderly.**

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

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

3 Material and methods

Cohorts

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. AT and cells were derived from obese individuals that were recruited at the Third Faculty of Medicine of Charles University, University Hospital Kralovske Vinohrady, Czech Republic and Toulouse University Hospitals and Centre d'Investigation Clinique Inserm – Hôpitaux de Toulouse (CIC), France.

Project A: Cells were derived from obese women.

Project B: Two groups of women (aged 36.6±1.8 and 72.1±1.32, n=15 in each group) were recruited.

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.

Project D: Twenty two men (n=11 aged 35.8±0.7 and n=12 aged 64.5±1.1) were recruited.

Methods

Analysis of metabolites and cytokines in plasma
ELISA

Cell culture
Isolation and culture of stromal-vascular cells (**Figure 4**)
Wst-1 assay
Analysis of senescence on flow cytometer
Oil-Red-Oil staining

Gene expression
RNA isolation
Gene expression analysis [classical qPCR and 96.96 array (Fluidigm)]
Western blot

Analysis of metabolites
De novo lipogenesis
Separation of lipid species by thin layer chromatography
Glucose transport

Statistical analysis
Data were analysed using GraphPad Prism 6.0 software with appropriate statistical tests. The level of significance was set at ***p < 0.001, **p < 0.01, *p < 0.05.

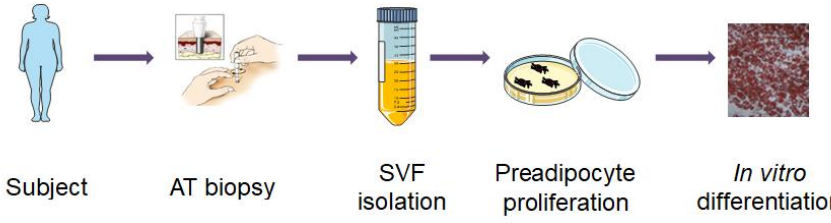

Figure 4: Isolation of stromal-vascular cells and culture. AT obtained by needle biopsy technique was digested with collagenase, enabeling dissociation of adipocytes and SVF. Culture media allowed the proliferation of preadipocytes and specific cocktail of factors their differentiation into adipocytes.

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 enzymatic cascade enabling fatty acid incorporation into TAG. Moreover, adipocytes are able to synthetize lipids *de novo*, from glucose (20). 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 (130; 131). Paradoxically, obesity impairs capacity of adipocytes to synthetize and store lipids (132; 133), which further contributes to high plasma free fatty acids levels, a putative cause of obesity-related hepatic and muscle insulin resistance (134). 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 (135; 136). The situation when the folding and other metabolic capacities of ER are overwhelmed is referred to as ERS. ERS activates a defense mechanism called UPR in order to enhance ER capacity and restore ER homeostasis (137). The signs of chronic ERS have recently been found in obese and insulin resistant subjects (138; 139). 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 (140). Importantly, ERS and consequently UPR were found to be important regulators of lipogenesis in liver (141). 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.

4.1.2 Results: experimental part A

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 hours. Both, 100 nM TG and 1 μg/ml TM (142), 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) (**Figure 5A**). The suppressive effect of ERS on lipogenesis was confirmed by a decreased capacity of adipocytes treated with TG to incorporate glucose carbon into lipids (**Figure 5B, 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 ERS 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 ERS inducer. This treatment resulted in approximately 60% reduction of neutral lipid content compared to control conditions 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.

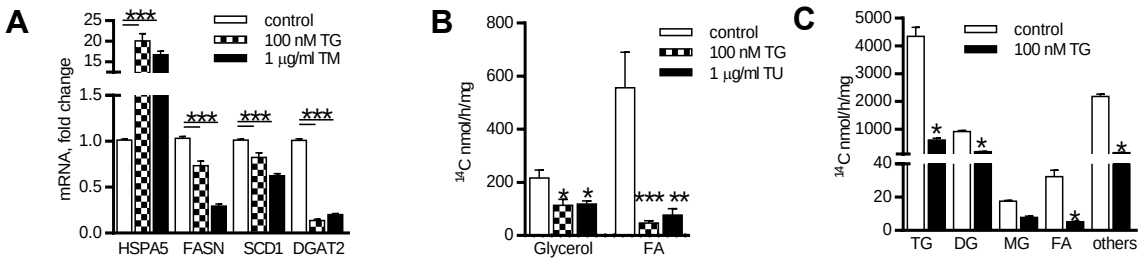

Figure 5: Acute ERS lowers lipogenesis in human adipocytes. Preadipocytes were differentiated for 12 days and then incubated for 24 hours with DMSO or 100 nM TG or 1μg/ml TM. **A**. mRNA expression of HSPA5, FASN, SCD1 and DGAT2 was measured by qRT-PCR and normalized to GUSB expression (n=15). **B**. 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). Data are means ±SEM.

Chronic low ERS impairs adipogenesis and associated lipogenesis

Obesity leads to chronic low intensity rather than acute high intensity ERS (138; 143). 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 (144). 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 unfolded protein response (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 UPR), was induced already by 2.5 nM TG within 1 hour, while an induction of expression of downstream ERS effectors (ATF6 arm- HSPA5(145), PERK arm- ATF4 (146), IRE1 arm- EDEM1, XBP1 splicing (147)) within 4 and 24 hours required higher TG concentrations (5 or 100 nM TG). 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. Capacity to accumulate neutral lipids was lowered by more than 50% in TG-treated adipocytes as detected by ORO staining. 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). mRNA expression of the lipogenic genes SCD1, DGAT2 and FASN was also lowered.

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. 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.

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, 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.

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 (148). 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 (28; 133; 149-151).

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 (89). 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 (73). 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 (72).

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 (152). 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 (153). Indeed, the experiments on rodents established ER stress as a trigger of insulin resistance and other metabolic disturbances caused by obesity (154). In line with this, we showed recently that ER stress impairs DNL in adipocytes and differentiation of preadipocytes (155). In addition, ER stress appears to be higher in subcutaneous AT from aged mice (156). 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. 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. Subcutaneous AT mRNA transcripts of *FASN*, a rate limiting enzyme in DNL, and *DGAT2*, an enzyme catalyzing final step of the TAG synthesis,

were decreased significantly in the elderly group ($p<0,05$). Levels of mRNA for these two genes were strongly correlated. 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 ($p<0,05$) and these two transcripts positively correlated. 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.

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. Expression of *HSPA5* and other chaperones correlated well with that of *FASN* and *DGAT2*. 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 and the expression of all lipogenic markers was strongly correlated with the expression of GDF15. 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.
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. 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.

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 (118). 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 (157). 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 (158; 159). 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 (160). 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 (118; 161)), the effects of a very short calorie restriction was studied rarely (158). Similarly, it was shown that the expression of metabolism-related genes in AT was reduced after 1 month of VLCD (162), 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 6**.

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 (163). Recently, it was shown, in mice and in cell cultures that FGF21 may affect adipose tissue metabolic pathways (lipogenesis, lipolysis) (164; 165). Thus, FGF21 levels and their association with changes in SAAT were also investigated.

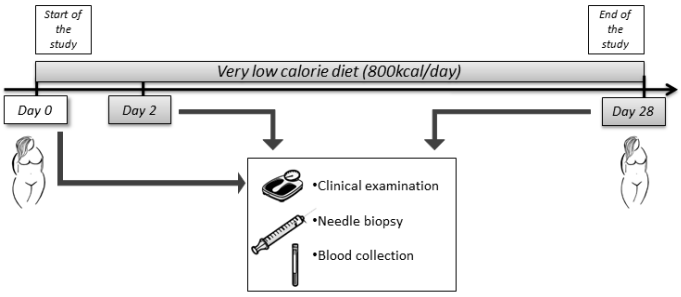

Figure 6: The design of the study. 17 obese pre-menopausal women were included in the study. Clinical examination, needle biopsy of SAAT and blood collection were performed at indicated days (day 0 - before the start of VLCD, day 2 – after 2days of VLCD, day 28 – at the end of one-month VLCD). Samples 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.

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), the genes involved in fibrosis (TLR4, collagens, TGFβ1, MMP9), and in inflammation (macrophage markers and cytokines), 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); 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); genes associated with β-oxidation of fatty acids – CPT1, ACOX1 and ACAD; insulin stimulated glucose transporter (GLUT4); leptin, and fibrotic enzyme - lysyl oxidase.

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.

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, transcription factor PPARα, PPARγ and PPARγ coactivator PGC1α, IRS1, and genes involved in fibrosis.

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. The changes of glycerol after 2 days of VLCD correlated with expression changes of HSL and ATGL. The changes of HOMA-IR after 2 days of VLCD tended to correlate with changes of HSL and ATGL expression.

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. The changes of HOMA-IR correlated with changes of plasma levels and secretion of leptin. Changes of cholesterol, insulin and TAG correlated with the

changes of expression of several lipolytic and lipogenic genes (i.e. HSL, SCD1, FASN, DGAT2). 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.

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 7**). Secretion of leptin was not changed after 2 days of VLCD but decreased significantly after 28 days of VLCD (**Figure 7**).

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. 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.

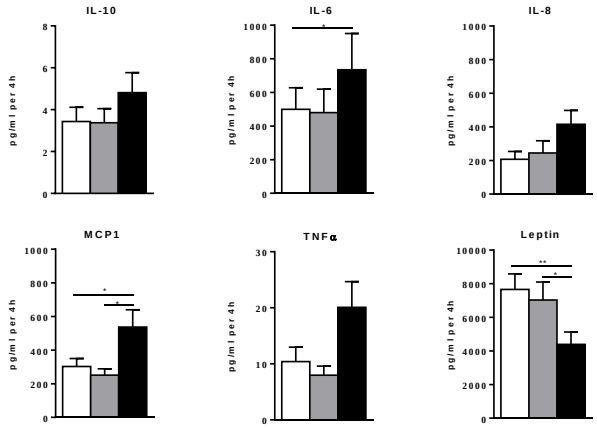

Figure 7: Secretion of cytokines / adipokines from subcutaneous adipose tissue of obese women before the diet (white), 2 days after VLCD (grey) and 28 days after VLCD (black). Data are presented as concentration of secreted protein (pg/ml per 4h) ± SEM (n=16); * p <0.05; ** p< 0.01; ***p< 0.001 compared 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).

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 (166; 167). In fact, the median age of the population is increasing almost as dramatically as obesity. In 2015, the worldwide population of 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 (168).

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 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 8**).

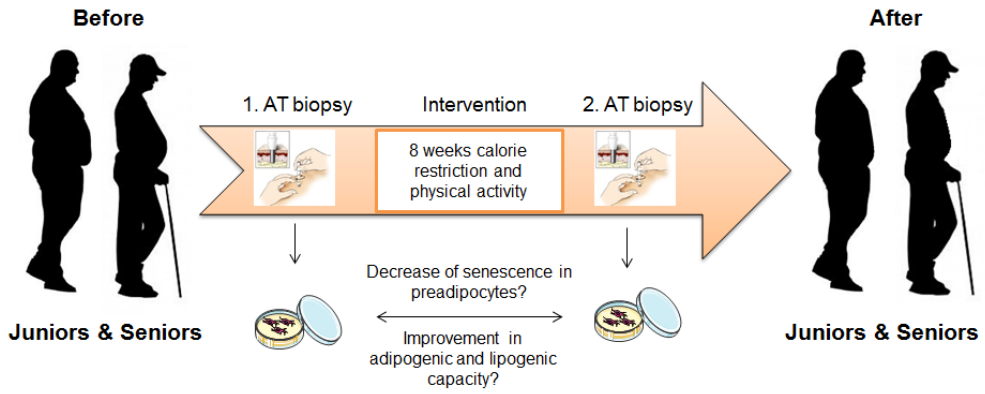

Figure 8: Design of the study. Obese juniors (age 30-40) and seniors (age 60-70) underwent eight weeks of calorie restriction (at least 20 % reduction of energetic need per day) in order to reach moderate weight loss. To prevent sarcopenia, subjects performed physical activity with professional sport 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

Clinical and laboratory characteristics of subjects

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 a 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 and was not affected by the intervention in either group. Also, levels of another marker of proliferative status of preadipocytes, i.e. Akt phosphorylation at S473 in response to growth hormone insulin, serum and other growth factors, were not different between the groups, either before and after the intervention.

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. No difference in the percentage of βgal positive and large cells was between juniors and seniors, and before/after the intervention. Similarly, no difference in mRNA expression of p16, a prototypical marker of senescence, was found between the groups and in response to the intervention.

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 a group of seniors (**Figure 9A,B**). 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 10**). However, the intervention improved the capacity of glucose uptake exclusively in a 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.

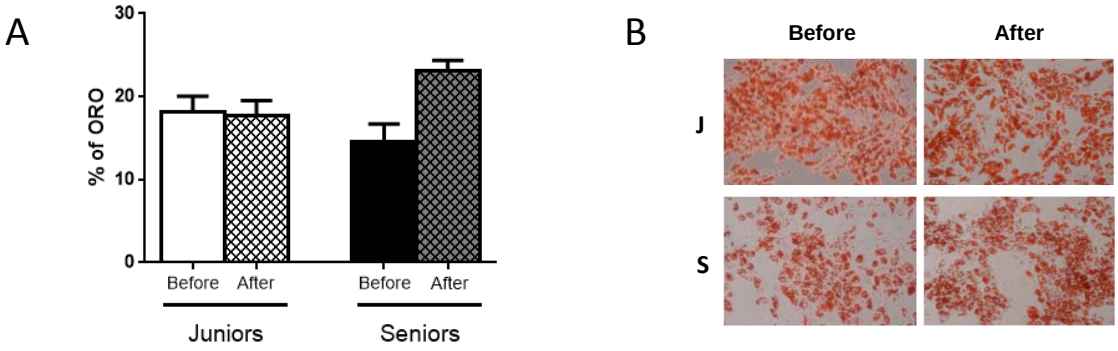

Figure 9: *In vitro* differentiation of preadipocytes. Accumulation of lipids was analysed using Oil-Red O staining. (A) Comparison of level of differentiation between juniors and seniors (n=7 in each group) and the impact of the intervention. (B) Representative photos (J=juniors, S=seniors). Analysed by Mann-two-way ANOVA, ±SEM.

Gene expression of adipocytes differentiated *in vitro*

In total, mRNA gene expression of 91 genes involved in adipogenesis and lipogenesis, fatty acid oxidation and lipolysis, senescence and inflammation, ER stress, anti-oxidant reaction and insulin sensitivity, browning/whitening, fibrosis and other genes 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 in a 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 a group of seniors. mRNA expression of the other genes stayed unchanged and expression of DLK1 and MMP9 was not detected.

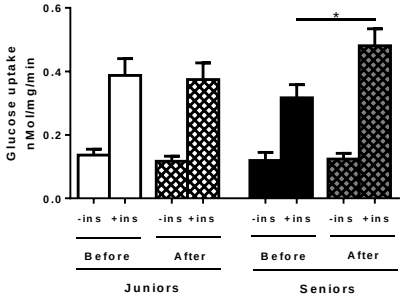

Figure 10: Glucose transport in adipocytes differentiated *in vitro*. Cells were differentiated for 12 days and deprived from insulin overnight. The next day, glucose transport in response to 100nM insulin was determined using radiolabelled 2-deoxyglucose. Comparison between juniors and seniors, before and after the intervention (n=7 in each group). Analysed by two-way ANOVA, ±SEM.

5 General discussion and future perspectives

Firstly, we showed that acute ER stress weakened lipogenic capacity of adipocytes and that chronic exposure of preadipocytes to ER stress impaired both, lipogenesis and adipogenesis. Indeed, lipogenesis is repressed in obese AT [290], whose demands for the synthetic and secretory activity are enhanced and result in persistent deficiency of ER capacity. ER stress could therefore contribute to the AT dysfunction often seen in obesity. However, in aging, ER stress in adipocytes is probably not the cause of observed reduction of lipogenic enzyme expression in subcutaneous AT. Nor the accumulation of senescent cells in subcutaneous AT from the elderly appeared to be linked to diminished expression of lipogenic enzymes. However, a reduction of senescent cell numbers by lifestyle modification could still provide beneficial effects, through the reduction of inflammatory cytokine production (169). On the other hand, our data have suggested that lipogenesis in the elderly might be reduced as a consequence of mitochondrial dysfunction. Indeed a down regulation of mitochondrial enzymes in adipose tissue with aging was shown in mice [33].

Next, we showed that already 2 days of VLCD decreased mRNA levels of lipogenic genes in obese individuals. This result complements and refines previous observations that activity of lipogenic enzymes (particularly those involved in DNL) is very sensitive to the nutritional status of the body (170). The impact of VLCD on AT was further intensified in later phase of the diet. Changes in adipocyte metabolism were induced also by moderate calorie restriction in seniors suggesting that such lifestyle intervention could bring metabolic benefits for obese seniors.

6 Conclusions

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.

7 List of publications

- ***which are part of the thesis:***

Šrámková V, Rossmeislová L. Krauzová E, Kračmerová J, Koc M, Langin D, Stich V, Siklova M. Comparison of early (2 days) and later (28 days) response of adipose tissue to very-low calorie diet in obese women. The Journal of Clinical Endocrinology & Metabolism. 2016; 101(12):5021-5029. ***IF: 5.531***

Koc M, **Mayerová V**, Kračmerová J, Mairal A, Mališová L, Štich V, Langin D, Rossmeis lová L.Stress of endoplasmic reticulum modulates differentiation and lipogenesis of huma n adipocytes. Biochem Biophys Res Commun. 2015; 460(3):684-90. ***IF: 2.371***

- ***which are not part of the thesis:***

Vlková V, Štěpánek I, Hrušková V, Šenigl F, **Mayerová V**, Šrámek M, Šímová J, Bieblov á J, Indrová M, Hejhal T, Dérian N, Klatzmann D, Six A, Reiniš M. Epigenetic regulations in the IFNγ signalling pathway: IFNγ-mediated MHC class I upregulation on tumour cells is associated with DNA demethylation of antigen-presenting machinery genes. Oncotarget. 2014; 5(16):6923-35. ***IF: 6.359***

Mráz M, Doležalová D, Plevová K, Staňo Kozubík K, **Mayerová V**, Černá K, Musilová K, Tichý B, Pavlová S, Borský M, Verner J, Doubek M, Brychtová Y,Trbušek M, Hampl A, Mayer J, Pospíšilová Š. MicroRNA-650 expression is influenced by immunoglobulin gene rearrangement and affects the biology of chronic lymphocytic leukemia.
Blood. 2012;119(9):2110-3. ***IF: 9.060***

SLABÝ, Ondřej, Marek SVOBODA, Andrej BEŠŠE et al. MikroRNA v onkologii. MikroRNA v onkologii. 1.vyd. Praha: Galén, 2012. 324 s. ISBN 978-80-7262-587-1. (*chapter book*)

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