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FACULTEIT GENEESKUNDE EN LEVENSWETENSCHAPPEN

master in de biomedische wetenschappen

Masterproef

Understanding cancer cachexia: adipose tissue under the loupe

Promotor :
Dr. SANDER RENSEN
Prof.dr. STEVEN OLDE DAMINK

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Scriptie ingediend tot het behalen van de graad van master in de biomedische wetenschappen

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List of abbreviations

APR	Acute-phase response
ASC	Adipose-derived stromal/stem cells
ATGL	Adipose triglyceride lipase
ATP	Adenosine triphosphate
BAT	Brown adipose tissue
BM	Basal growth medium
BMI	Body mass index
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CM	Conditioned medium
COX-2	Cyclooxygenase-2
Cq	Quantitation cycle
CRP	C-reactive protein
DAB	3,3'-Diaminobenzidine
DAG	Diacylglycerol
ddH_2O	Double-distilled water
DEXA	Dual energy X-ray absorptiometry
DMEM	Dulbecco's modified Eagle's medium
DMI	Differentiation medium I
DMII	Differentiation medium II
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-related kinase
FA	Fatty acid
FABPs	Fatty acid binding proteins
FBS	Fetal bovine serum
FFA	Free fatty acid
GEMMs	Genetically engineered mouse models
Gs proteins	Stimulatory guanine nucleotide-binding proteins
HRP	Horseradish peroxidase
HSL	Hormone sensitive lipase
IBMX	3-isobutyl-1-methylxanthine
IL-6	Interleukin-6
MAG	Monoacylglycerol
MGL	Monoglyceride lipase
MPTC	Maastricht Pathology Tissue Collection
ORO	Oil red o
P/S	Penicillin/streptomycin
P2RX5	Purinergic receptor P2X, ligand-gated ion channel 5
PAT2	Proton assistant amino acid transporter-2
PBS	Phosphate buffered saline
PI3-K	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
qRT-PCR	Quantitative real-time polymerase chain reaction
REE	Resting energy expenditure
TAG	Triacylglycerol
TBS	Tris buffered saline
TNF-α	Tumor necrosis factor-α
UCP1	Uncoupling protein 1
WAT	White adipose tissue
ZAG	Zinc-α2-glycoprotein

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Summary

Pancreatic cancer patients often suffer from cachexia, a syndrome characterized by involuntary weight loss, attributable to a reduction in skeletal muscle and adipose tissue mass. While the skeletal muscle aspect of this condition is extensively investigated, much less is known about the role of adipose tissue biology in the development of cancer cachexia. Recent studies show that cachectic mice display so-called 'browning' of white adipose tissue (WAT), causing heat production and slimming. Activation of adipose tissue lipolysis has also been suggested to be one of the principal factors causing adipose tissue loss in cancer patients. Therefore, we hypothesized that WAT browning and increased lipolysis occur in cachectic pancreatic cancer patients.

Peritumoral WAT samples obtained from resected tumor specimens of pancreatic cancer patients were stained for Uncoupling protein 1 (UCP1), a brown adipose tissue marker. WAT samples derived from non-cachectic patients who underwent surgery because of obesity, gallbladder malfunctioning, or an increased risk for developing breast cancer (BRCA gene mutation), were included for comparison. Differentiated murine 3T3-L1 adipocytes were incubated for 6 and 24 hours with conditioned medium (CM) derived from human pancreatic cancer cell lines (PK-1, KLM-1, PANC-1, and PK-45H). CM of two human breast cancer cell lines, MCF-7 and T47D, was included as non-cachectic cancer control. UCP1 expression and the degree of lipolysis were determined by quantitative RT-PCR and analysis of glycerol concentration in the cell culture supernatant, respectively.

Immunohistochemistry showed positive UCP1 staining in all adipose tissue sections. Staining was variable between patients, and heterogeneously distributed within WAT. More specifically, areas with stronger and less stained adipocytes were observed. In line with the universal UCP1 expression in WAT of pancreatic cancer patients, gene expression analysis revealed that pancreatic CM significantly increased UCP1 expression in murine 3T3-L1 adipocytes with a high passage number after 24h ($P=0.03$), whereas breast CM did not ($P=0.11$). A similar trend could be observed in adipocytes with a lower passage number. However, incubation with both pancreatic and breast CM for 6h had no effect on the release of glycerol (resp. $P=0.39$ and $P=0.31$).

In conclusion, our data support the occurrence of WAT browning in pancreatic cancer patients. However, staining was not limited to this subpopulation, since WAT derived from other types of patients also displayed positive staining. Extensive correlation analyses of these data to available clinical patient data will give us more insight into the significance of WAT browning in cancer cachexia. Furthermore, this study revealed that pancreatic tumor cells are able to modulate UCP1 expression at the mRNA level in 3T3-L1 adipocytes, while the levels of lipolysis remained unaffected.

In general, repetition of the experiments and follow-up research is required to gain a better understanding of the significance of alterations in adipose tissue biology in the development of cancer cachexia.

Samenvatting

Patiënten met pancreaskanker lijden vaak aan cachexie, een syndroom dat gekarakteriseerd wordt door onvrijwillig gewichtsverlies, toe te rekenen aan een reductie in skeletspier- en vetweefselmassa. Terwijl het aspect van de skeletspier in deze conditie uitgebreid onderzocht wordt, is er veel minder bekend over de rol van vetweefsel in de ontwikkeling van kanker cachexie. Recente studies tonen aan dat cachectische muizen zogenoemde 'verbruining' van wit vetweefsel (WAT) vertonen, met productie van warmte en vermagering tot gevolg. Activatie van lipolyse in het vetweefsel wordt ook beschouwd als één van de voornaamste factoren in het verlies van vetweefsel in kankerpatiënten. Daarom hypothetiseerden we dat verbruining van WAT en verhoogde lipolyse optreden in cachectische patiënten met pancreaskanker.

Om deze hypothese te onderzoeken, werden peritumorale WAT stalen, verkregen van gereseceerde tumor specimens afkomstig van patiënten met pancreaskanker, gekleurd voor Uncoupling protein 1 (UCP1), een marker voor bruin vetweefsel. WAT stalen afkomstig van niet-cachectische patiënten geopereerd omwille van obesitas, slecht functioneren van de galblaas of een verhoogd risico op het ontwikkelen van borstkanker (BRCA gen mutatie), werden geïncludeerd ter vergelijking. Gedifferentieerde muis 3T3-L1 adipocyten werden gedurende 6 en 24 uur blootgesteld aan geconditioneerd medium (CM) afkomstig van humane pancreaskanker cellijnen (PK-1, KLM-1, PANC-1 en PK-45H). CM van twee humane borstkanker cellijnen, MCF-7 en T47D, werd geïncludeerd als niet-cachectische kanker controle. UCP1 expressie en de mate van lipolyse werden bepaald door middel van respectievelijk kwantitatieve RT-PCR en analyse van de glycerol concentratie in celkweek supernatant.

Immunohistochemie toonde een positieve UCP1 kleuring in alle vetweefselcoupes aan. De kleuring varieerde tussen de patiënten en was heterogeen verdeeld in het WAT. Meer bepaald, gebieden met sterker en minder gekleurde adipocyten werden waargenomen. In overeenstemming met de universele UCP1 expressie in WAT van patiënten met pancreaskanker, onthulde gen expressie analyse dat pancreas-CM de UCP1 expressie in muis 3T3-L1 adipocyten met een hoog passage nummer significant verhoogde na 24u ($P=0.03$), terwijl borst-CM geen effect veroorzaakte ($P=0.11$). Een gelijkaardige trend kon worden waargenomen in adipocyten met een lager passage nummer. Echter, incubatie met zowel pancreas- als borst-CM gedurende 6u had geen effect op de afgifte van glycerol (resp. $P=0.39$ en $P=0.31$).

Concluderend ondersteunen deze data het idee dat WAT verbruining in patiënten met pancreaskanker optreedt. WAT verbruining was echter niet beperkt tot deze subpopulatie, aangezien WAT afkomstig van andere soorten niet-cachectische patiënten ook een positieve kleuring vertoonde. Uitgebreide correlatie analyses van deze data met beschikbare klinische patiëntgegevens zal ons meer inzicht verschaffen over het belang van WAT verbruining in kanker cachexie. Bovendien onthulde deze studie dat pancreas tumorcellen in staat zijn om UCP1 mRNA expressie in 3T3-L1 adipocyten te verhogen, terwijl de lipolyse levels onveranderd bleven.

In het algemeen is de herhaling van experimenten en navolgend onderzoek vereist om een beter inzicht te krijgen in het belang van veranderingen in vetweefsel biologie in de ontwikkeling van kanker cachexie.

1 Introduction

1.1 Pancreas and its disorders

The pancreas is a large gland located in the upper abdomen. It is composed of both an exocrine and endocrine part. The exocrine pancreas covers the largest tissue volume of the organ, accounting for 84%, and is responsible for the production of enzymatic proteins, necessary for proper digestion of nutrients. The endocrine pancreas is composed of clusters of cells, the islets of Langerhans, which produce the hormones glucagon and insulin, key players in the regulation of blood glucose levels [1]. Disorders of the pancreas are associated with significant morbidity and mortality, and include diabetes mellitus, pancreatitis (acute or chronic), and pancreatic cancer. Worldwide, pancreatic cancer is the twelfth most common type of cancer, with 338.000 newly diagnosed cases in 2012 [2]. In the Dutch population, pancreatic cancer was the eighth most frequent type of cancer amongst females last year, while it was the tenth in the male population. Moreover, almost 2500 Dutch patients died from pancreatic cancer in 2013, while more than 2300 new cases were reported in that year, characterized by a general 1- and 5-year survival rate of resp. 19% and 5% [3]. Since there are usually no noticeable symptoms in the early phase of pancreatic cancer development, this disease is in many cases already advanced at diagnosis and is frequently fatal [4]. One of the main reasons is that pancreatic cancer patients often suffer from cachexia, a syndrome of severe muscle wasting and weight loss. More specifically, up to 30% of all cancer-related mortality is due to cachexia [5].

1.2 Cancer cachexia

1.2.1 Definition and classification

According to the international consensus definition, cancer cachexia is defined as a multifactorial syndrome characterized by an ongoing loss of skeletal muscle mass, with or without loss of fat mass, that cannot be fully reversed by conventional nutritional support and leads to progressive functional impairment [6]. A negative energy and protein balance is the consequence of a variable interplay of decreased food consumption, aberrant metabolism, and inflammation. Development of cachexia is dependent on tumor type, but seems to be more common in cancers of the upper gastrointestinal tract (such as pancreatic cancer), lung, and colorectal cancer, while unusual in breast cancer [7].

The criteria for diagnosis of cancer cachexia are weight loss >5% over a period of 6 months, or weight loss of >2% in combination with a body mass index (BMI) <20 kg/m^2 or sarcopenia (i.e. loss of skeletal muscle mass). The latter is defined as appendicular skeletal muscle index <5.45 kg/m^2 in females and <7.26 kg/m^2 in males as determined by dual energy X-ray absorptiometry (DEXA). Cancer cachexia is characterized by progressive development through a spectrum, with 3 clinically relevant stages, i.e. precachexia, cachexia, and refractory cachexia. Precachectic patients show early metabolic and clinical signs, including impaired glucose tolerance and anorexia, which precede substantial involuntary weight loss (i.e. ≤5%).

The risk of progression to the cachectic stage depends on several factors, including low food intake, type of cancer and stage, unresponsiveness to anti-cancer therapy in some cases, and systemic inflammation. Cachectic persons are assigned to the refractory stage when they have a highly advanced cancer (pre-terminal) or when their progressive cancer is unresponsive to therapy. Active catabolism is present in this phase and patients show impaired physical performance with an expected survival of <3 months [6,8].

1.2.2 Impact of cancer cachexia

Almost 50% of all cancer patients will develop cachexia and this percentage even rises to 86% in the last 2 weeks of the patient's life. Furthermore, 45% of cancer patients will lose >10% of their initial body weight throughout progression of the disease [9]. This has severe implications with regard to prognosis; a loss of weight reaching 30% of the former stable body weight is usually associated with death [9,10]. While involuntary weight loss is the main characteristic of cancer cachexia, there are also other symptoms that can arise in (a part of) cachectic individuals. These include loss of appetite (anorexia), early satiety, tiredness, depression, pain, and loss of normal functioning, which greatly reduce quality of life. Other complications are reduced responsiveness to chemotherapy and radiotherapy, and substantial loss of muscle-dependent functioning, including cardiovascular and respiratory functionality, which have a significant impact on the patient's outcome and well being as well [9,11].
Loss of mobility and independence creates not only distress for the patient, but also a possible burden on family members. In addition, cancer cachexia is accompanied by a high economic burden, since cachectic persons require regular and long hospital admissions for cachexia-associated complications, and high therapy-associated costs [9,12].

1.2.3 Understanding cancer cachexia

In order to be able to find effective treatment options for the fight against cancer cachexia, the mechanisms underlying this syndrome have to be clarified. In this section, several important processes previously proposed to be associated with the pathophysiology of cancer cachexia will be addressed. In particular, the disturbance in energy balance, inflammatory and tumor-secreted factors, and finally the management of cancer cachexia will be discussed.

A. Disturbed energy balance

An imbalance between energy intake/expenditure and protein synthesis/breakdown is the main characteristic of the pathophysiology of cancer cachexia. More specifically, the drivers for this imbalance are a reduction in food intake and increased metabolism, favoring energy expenditure and protein breakdown. A complex interplay between tumor and host is thought to be involved in this process.

Cancer patients often suffer from anorexia. A decrease in nutrient intake in these patients can lead to a sustained loss in their body weight. Loss of appetite can be temporary, being the result of therapeutic factors and can be reversed when the therapy is finished.

However, a study of Tranmer *et al.* demonstrated that anorexia can also develop by the action of the tumor, independently of the treatment [13]. Even though anorexia often goes along with cachexia, it is not the causative factor for the development of cachexia. Whereas most of the weight loss in anorectic patients can be attributed to loss of adipose tissue and only little loss of muscle due to decreased energy intake, there is a significant loss of both muscle and adipose tissue in cachectic cancer patients, which cannot be restored by increasing caloric consumption [9,14].

Loss of body mass can also be the result of increased energy expenditure, which is a direct consequence of the disturbed metabolism seen in cachectic cancer patients. The resting energy expenditure (REE) in sedentary living people comprises up to 70% of the total energy expenditure. In cancer patients, the type of tumor strongly determines their REE. While the REE in people with pancreatic and lung cancer is elevated, colorectal and gastric cancer patients show no increase [9,14]. A proposed mechanism for this elevated REE is an increased thermogenesis in skeletal muscle and adipose tissue, and futile metabolic cycles, which require high amounts of energy [9].

B. Inflammatory factors

Inflammation is another process that appears to play a role in the pathophysiology of cancer cachexia. Increased levels of acute-phase response (APR) proteins, such as C-reactive protein (CRP>10 mg/l), indicate the presence of systemic inflammation and are associated with increased weight loss and a poor outcome in cancer patients [9,15]. Cytokines derived from the cancer cells or secreted by the host in reaction to the tumor are suggested to affect certain pathways resulting in the development of anorexia and hypercatabolism. For instance, pro-inflammatory Interleukin-6 (IL-6) is considered to be an important cytokine involved in pancreatic cancer cachexia, because of its relation with weight loss, lipolysis, and a decreased survival rate amongst patients [8]. In addition, recent studies in cachectic mice revealed the possible contribution of IL-6 to activated thermogenesis and white adipose tissue browning [7]. Furthermore, Tumor Necrosis Factor-α (TNF-α) is another factor which has been shown to affect lipid metabolism. More specifically, TNF-α appears to have the capacity to inhibit lipogenesis, while promoting lipolysis and muscle degradation. However, its relevance in the development of cancer cachexia is still a subject of debate, because there are also human studies showing no correlation between levels of TNF-α, anorexia, and weight loss [8,9].

C. Tumor-released factors

Several tumor-released substances, including cytokines and catabolic agents, have been suggested to have detrimental effects on host tissue, giving rise to metabolic defects eventually leading to the development of cancer cachexia. One of the most well studied tumor-derived factors likely contributing to the development of pancreatic cancer cachexia is the lipid mobilizing factor Zinc-α2-glycoprotein (ZAG). While ZAG is normally expressed by healthy tissue, such as adipose tissue, it is overexpressed by certain types of cancer [16]. *In vivo* studies have demonstrated the relation between ZAG and weight loss.

When ZAG was administered to mice, body fat was rapidly reduced and levels of free fatty acids (FFAs) in the serum were increased, indicating ongoing lipolysis [17]. Besides its role in lipid mobilization, ZAG is also responsible for increased substrate utilization and activation of oxidative mechanisms in mitochondria in brown adipose tissue leading to increased energy consumption and hypercatabolism [8].

IL-6 is not only secreted during inflammation by immune cells, other tissues, including adipose tissue, and even cancer cells are able to release this substance. As described previously, a role for IL-6 in modulating lipid metabolism has been suggested. An *in vitro* study showed that IL-6 caused a significant reduction in the number of lipids within differentiated murine adipocytes and that it significantly increased the amount of glycerol released by these cells [18]. Furthermore, Van Hall *et al.* also identified IL-6 as a lipolytic factor, since IL-6 infusion in human subjects resulted in an increase in fatty acid (FA) concentration and whole body fat oxidation [19].

D. Management of cancer cachexia

To date, the search for a potential therapy for cachexia is a difficult challenge and an effective treatment is still lacking. The most efficient therapy would be the elimination of the cancer itself, but this is something that is not always easy to achieve, especially in advanced cancers. Nowadays, management of cancer cachexia largely focuses on nutritional support in order to counteract the substantial weight loss seen in these patients. However, since anorexia itself is not responsible for the development of cachexia, increasing nutritional intake is not sufficient to overcome the problem. Because of the multifactorial pathogenesis of cachexia, multi-target therapies are required, which address all the different aspects contributing to this syndrome, including inflammation, anorexia, and muscle atrophy [20]. Currently, various clinical trials are being conducted regarding cancer cachexia, which will hopefully result in finding an effective candidate treatment for this devastating syndrome in the near future.

1.3 Adipose tissue under the loupe

1.3.1 Adipose tissue: key regulator of body weight

Adipose tissue, also known as body fat, is a central player in body weight regulation and can be divided into two major types: white adipose tissue (WAT) and brown adipose tissue (BAT). WAT is composed of unilocular adipocytes, containing one central lipid droplet, and is distributed throughout nearly all body parts. The density and distribution of the tissue is dependent on sex and age [1]. The traditional function of WAT is to accumulate and store excess energy in the form of lipids, mainly triacylglycerols (TAGs or triglycerides), which can be utilized in times of food deprivation. In addition, WAT also functions as an endocrine and secretory organ by releasing adipokines (e.g. leptin), which are responsible for the communication between WAT and some metabolically important organs (e.g. liver), and may promote inflammation [21]. BAT on the other hand has long been thought to operate only in rodents and human newborns as cold adaptation machinery, but recent studies show its presence in the human adult body.

BAT is composed of multilocular or multiple small lipid droplets-containing brown adipocytes, which are capable of accumulating and storing lipids, and which contain numerous mitochondria providing its brown color [21]. The most important function of BAT is to keep the body temperature in a stable range while exposed to cold stress. It can do this by the generation of heat, through so-called thermogenesis. Thermogenesis is mediated by expression of mitochondrial uncoupling protein 1 (UCP1), or thermogenin, which is able to uncouple mitochondrial respiration in the direction of thermogenesis rather than adenosine triphosphate (ATP) synthesis [22,23]. This process consumes a great amount of energy, thereby burning a lot of nutrients, potentially contributing to syndromes of weight loss.

1.3.2 Alterations in adipose tissue biology during cancer cachexia

A. White adipose tissue browning

BAT is considered the principal organ of heat production in mammalian organisms and has awakened interest as therapeutic option in promoting weight loss for several decades. Recently, literature showed emergence of groups of UCP1-expressing, heat-producing adipocytes within WAT when stimulated by various factors [24]. These beige or brite ('brown in white') adipocytes show similar morphology as brown adipocytes in mice, namely the presence of intracellular multilocular lipid droplets, but they are thought to originate from a distinct precursor population. In addition, beige adipocytes are characterized by an abundance of mitochondria and express several BAT-specific genes, including UCP1 [24]. This so-called process of WAT browning, which is characterized by a phenotypic switch from WAT to BAT, has been considered biomedically interesting to counteract metabolic disorders, such as obesity, and is related to leanness in human individuals.

Interestingly, mouse models of cachexia have recently been demonstrated to show WAT browning [22]. There are some processes which are proposed to activate browning. These include adrenergic stimulation, extended cold exposure, the recently identified myokine Irisin, and the prostaglandin-synthesis-catalyzing enzyme cyclooxygenase-2 (COX-2). Interestingly, the β-adrenergic pathway seems to be operational during cachectic conditions and increased BAT thermogenic activity was demonstrated in cachectic mice. Furthermore, WAT browning is suggested to develop early, even prior to skeletal muscle atrophy, during the cachectic disease course. More specifically, this process could already be detected in precachectic mice and deteriorated as their condition progressed to cachexia [22].

B. Increased lipolysis

As mentioned previously, WAT is capable of storing TAGs, which represent the major reserve of energy in the human body. In times of fuel deprivation, this reserve can be consumed which results in the hydrolysis of TAGs into glycerol and FFAs. These substances are then released into the bloodstream and act as energy substrates for other organs [25]. Lipolysis is regulated by several enzymes which are active at fixed steps during the process (Figure 1). The first step of lipolysis, in which TAG is hydrolyzed to form diacylglycerol (DAG) and one FFA, is regulated by adipose triglyceride lipase (ATGL or desnutrin).

Hormone sensitive lipase (HSL) is responsible for the second step of lipolysis, namely hydrolysis of DAG to monoacylglycerol (MAG) and the additional release of one FFA. Finally, the enzyme which is responsible for the last step of lipolysis, namely hydrolysis of MAG to generate free glycerol and the last FFA, is monoglyceride lipase (MGL). Chaperone proteins, namely fatty acid binding proteins (FABPs), facilitate the transfer of these released FFAs outside the adipocyte. Another set of proteins which are associated with the surface of lipid droplets, called perilipins, are also thought to play a crucial role in the breakdown of lipids. In the inactive state, perilipins form a barrier between the intracellular lipids and HSL, but phosphorylation of these proteins results in movement of perilipins away from the lipid droplet and enables HSL to reach the intracellular triglycerides, thereby enhancing lipolytic breakdown [25].

Adipocyte lipolysis is subjected to hormonal and neural control. Sensory and sympathetic nerve fibers reach the adipose tissue and thereby can influence the lipolytic process. The catecholaminergic neurotransmitters epinephrine and norepinephrine, together with insulin, are the primary hormonal regulators of human lipolysis, though inflammatory factors such as IL-6 have also been shown to influence lipolysis. The lipolytic cascade gets activated when catecholamines are released from sympathetic nerve endings and subsequently bind to β-adrenergic receptors on the adipocyte plasma membrane. These receptors are linked to stimulatory guanine nucleotide-binding proteins (Gs proteins), which transmit a signal that triggers activation of the enzyme adenylyl cyclase. As a result, ATP is converted to cyclic adenosine monophosphate (cAMP), which binds to and thereby activates protein kinase A (PKA), which is responsible for the phosphorylation of lipolysis-associated enzymes, such as HSL and perilipins [26,27]. As mentioned earlier, insulin is also capable of influencing cellular lipolytic rates, but in contrast to catecholamines, insulin acts as an inhibitor. The antilipolytic action of insulin can be mainly attributed to the degradation of cAMP via activation of phosphatidylinositol 3-kinase (PI3-K), leading to reduced signaling towards HSL [27].

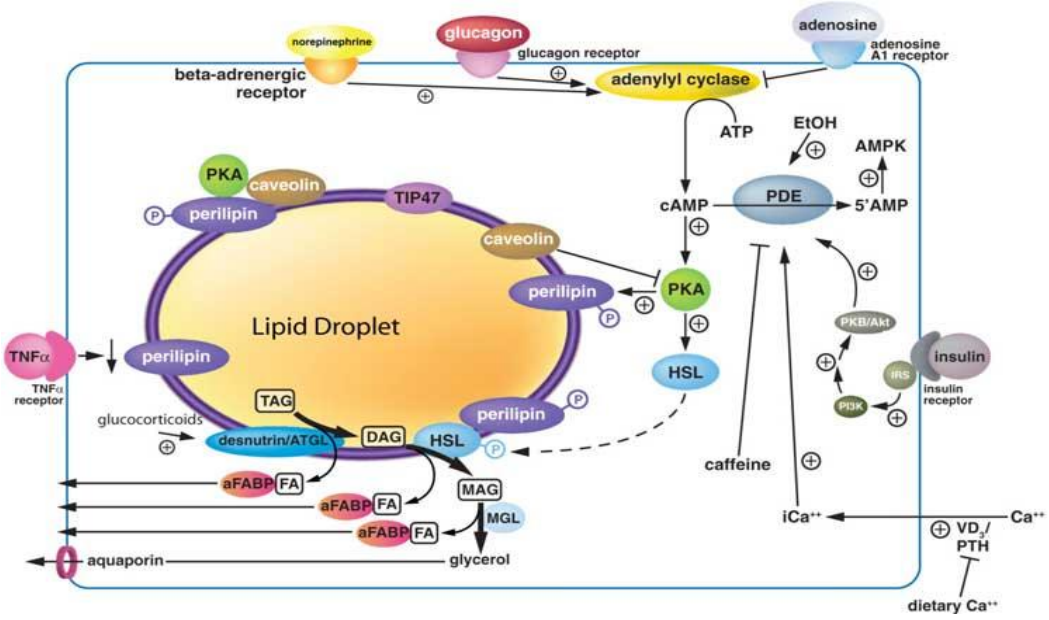


Figure 1. Regulation of lipolysis [25]

The lipolytic process comprises the breakdown of TAGs into glycerol and FFAs, which requires the action of several enzymes inside the adipocyte. The first step of lipolysis, in which TAG is hydrolyzed to form DAG and one FFA, is regulated by ATGL or desnutrin. Next, DAG is hydrolyzed to form MAG and the subsequent release of one FFA. This step is catalyzed by HSL. Finally, MGL is active at the last step in the process, namely the hydrolysis of MAG to generate free glycerol and the last FFA. Fatty acid binding proteins facilitate the transfer of these released FFAs outside the adipocyte. Perilipins are associated with the surface of lipid droplets and form a barrier between the intracellular lipids and HSL in the inactive state. Phosphorylation of perilipins results in movement of these proteins away from the lipid droplet and enables HSL to reach the intracellular triglycerides, thereby enhancing lipolytic breakdown. The catecholamines epinephrine and norepinephrine, together with insulin, are the primary hormonal regulators of human lipolysis. Whereas the former act as lipolytic agonists, the latter operates as inhibitor of the lipolytic process. TAG: triacylglycerol; FFA: free fatty acid; DAG: diacylglycerol; ATGL: adipose triglyceride lipase; MAG: monoacylglycerol; HSL: hormone sensitive lipase; MGL: monoglyceride lipase; aFAPB: adipose fatty acid binding protein; PKA: protein kinase A; cAMP: cyclic adenosine monophosphate; ATP: adenosine triphosphate; PI3-K: phosphatidylinositol 3-kinase.

Weight loss seen in cancer patients can be attributed to a reduction in skeletal muscle and adipose tissue mass. While the skeletal muscle aspect is extensively investigated, there is much less known about the loss of adipose tissue as contributor to the development of cancer cachexia. Increased lipolysis is one of the proposed mechanisms engaged in the reduction of adipose tissue. Although there are several studies demonstrating an increased rate of lipolysis in cancer patients, the drivers behind this increase still remain unclear. Suggested mechanisms are a compensatory response for the decrease in energy intake or the production of adipose tissue- (e.g. IL-6) or tumor-derived (e.g. ZAG) lipolytic substances [30,31]. These factors could explain the extreme leanness observed in cachectic cancer patients, but still need further research. Mechanisms involved in the modulation of the lipolytic process by IL-6 are still not completely understood. Literature described that IL-6-induced lipolysis probably not proceeds via the usual cAMP/PKA-pathway, but via activation of the extracellular signal-related kinase (ERK) pathway, presumably leading to increased mRNA levels of HSL, ATGL, and genes involved in FFA oxidation [28,29].

1.4 Research aims

Adipose tissue lipolysis and WAT browning appear to play important roles in cancer cachexia, a condition which is very prominent in pancreatic cancer patients. However, the direct impact of pancreatic tumor cells on adipocyte biology has not been studied so far.

We hypothesized that WAT browning and increased lipolysis occur in cachectic cancer patients and that these processes contribute to the weight loss observed in these individuals. We aimed to: (1) determine whether WAT browning occurs in pancreatic cancer patients by means of immunohistochemical staining of peritumoral adipose tissue for the brown fat marker UCP1; (2) explore whether WAT browning occurs in a murine adipocyte cell line exposed to conditioned medium of different human pancreatic cancer cell lines by analyzing UCP1 mRNA expression via quantitative real-time PCR; (3) investigate whether there is increased lipolysis after incubation of a murine adipocyte cell line with conditioned medium of different human pancreatic cancer cell lines by performing a lipolysis assay.

2 Materials and methods

2.1 Immunohistochemistry

After approval to gain access to the Maastricht Pathology Tissue Collection (MPTC, protocol number 2013-16), peritumoral WAT samples were obtained from tumor specimens of pancreatic cancer patients resected between 2008 and 2013 that were archived at the Department of Pathology (N=60; Table 1). WAT samples derived from patients who underwent surgery because of obesity, gallbladder abnormality, or an increased risk of developing breast cancer (BRCA gene mutation) were included for comparison (N=2). In these patients, WAT browning is not expected to occur, since they do not have cachexia. Human BAT was considered as positive control tissue.

The tissues were fixed with 10% formalin, embedded in paraffin, and sectioned at a thickness of 4-5 µM. For immunohistochemical analysis, sections were deparaffinized with xylene and rehydrated with a series of decreasing ethanol concentrations, respectively 100%, 96%, and 70% ethanol. Sections were exposed to 0.3% hydrogen peroxide in methanol for 30 minutes at room temperature in order to block endogenous peroxidase activity. Sections were rinsed with 1x Tris buffered saline (TBS) supplemented with 0.1% TWEEN® 20 (Sigma-Aldrich, St. Louis, Missouri) during intermediate washing steps. Heat induced antigen retrieval was performed by means of 10% citrate buffer (Dako, Glostrup, Denmark) above 85°C for 10 minutes. Tissue sections were first blocked with 10% normal swine serum (Centrale Proefdier Voorzieningen/Maastricht University, Maastricht, The Netherlands) for 30 minutes at room temperature. Next, samples were incubated overnight with rabbit polyclonal anti-UCP1 antibody (1:250; ab10983, Abcam, Cambridge, United Kingdom) or dilution buffer, composed of 1x TBS, 0.1% TWEEN® 20 and 0.1% Bovine Serum Albumin (BSA; PAA Laboratories GmbH, Pasching, Austria), for blank control sections. The next day, sections were incubated with biotinylated secondary swine-anti-rabbit antibody (1:250; Dako) for 1 hour at room temperature. Antibody dilutions were made in dilution buffer. Next, amplification of target antigen signal was performed by using Streptavidin-Avidin Biotin/Horseradish Peroxidase (HRP) complex (1:50; Vector Laboratories, Burlingame, California) for 1 hour at room temperature. In the next step, the DAB (3,3'-Diaminobenzidine) substrate chromogen system (Dako North America Inc., Carpinteria, California) was used for visualization of bound antibody. Sections were washed with distilled water and counterstaining was performed by means of hematoxylin. Thereafter, tissue samples were washed with tap water and dehydrated again with a series of increasing ethanol concentrations, respectively 70%, 96%, 100% ethanol, and xylene. Representative pictures of selected patients were made at 200x and 400x magnifications with a Nikon Eclipse E800 microscope (Nikon, Tokyo, Japan). Two other primary UCP1 antibodies were tested to see if the same results were obtained; one was a gift from the Department of Human Biology (1:250; B. Cannon) and the other was purchased from Abcam (ab155117). The latter did not work in our positive control tissue and the former showed similar results as our ab10983 antibody. Therefore, experiments were continued with the ab10983 antibody.

Table 1. Patient characteristics

	Group (N=60)
Sex	
Male	28 (46.7%)
Female	32 (53.3%)
Age (years)	66.4±10
BMI	24.7±3.7
CRP (mg/l)	16.8±36.6
Weight loss	
0-2%	11 (18.3%)
2-5%	2 (3.3%)
≥5%	28 (46.7%)
n.r.	19 (31.7%)

Weight loss (%) was registered over a period of 6 months. Age, BMI, and CRP are represented as mean±SD. n.r: not registered; BMI: body mass index; CRP: c-reactive protein.

2.2 Cell culture

Murine 3T3-L1 embryonic fibroblasts/pre-adipocytes were kindly provided by Dr. Petra Niessen from the Department of Internal Medicine (Maastricht). The human pancreatic cancer cell lines PK-1, KLM-1, PANC-1, and PK-45H were kindly provided by Dr. Dipok Kumar Dhar from University College London. The two human breast cancer cell lines, MCF-7 and T47D, were obtained from MAASTRO clinic (Maastricht). All the cells were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Bleiswijk, The Netherlands) supplemented with Fetal Bovine Serum (FBS; PAA Laboratories GmbH), antibiotics Penicillin/Streptomycin (P/S; Life Technologies), and Sodium Pyruvate (Life Technologies) (= Basal growth medium, BM, Table 2). Cells were split at 80% confluence with 0.25%/0.3% Trypsin/EDTA (Life Technologies). Conditioned medium (CM) of the cancer cell lines was prepared by incubating the cells with BM for 48 hours, after which the medium was collected and stored at -20°C.

2.3 Differentiation of 3T3-L1 fibroblasts into adipocytes

When cultured 3T3-L1 pre-adipocytes reached 100% confluence, they were incubated an additional 48h with BM before differentiation was initiated (Figure 2A). This time is needed for the cells to stop growing. Next, adipocyte differentiation was stimulated by incubating the cells with insulin, dexamethasone and 3-isobutyl-1-methylxanthine (IBMX; all purchased from Sigma-Aldrich) (= Differentiation medium I, DMI, Table 2). After 72h, the medium was changed and cells were incubated with insulin only (= Differentiation medium II, DMII, Table 2). This medium was refreshed every other day until further experiments. Intracellular lipid droplets were visible around day 7 and became larger and more abundant over the following days. Cells were differentiated until day 12 when used in subsequent experiments (Figure 2B and C).

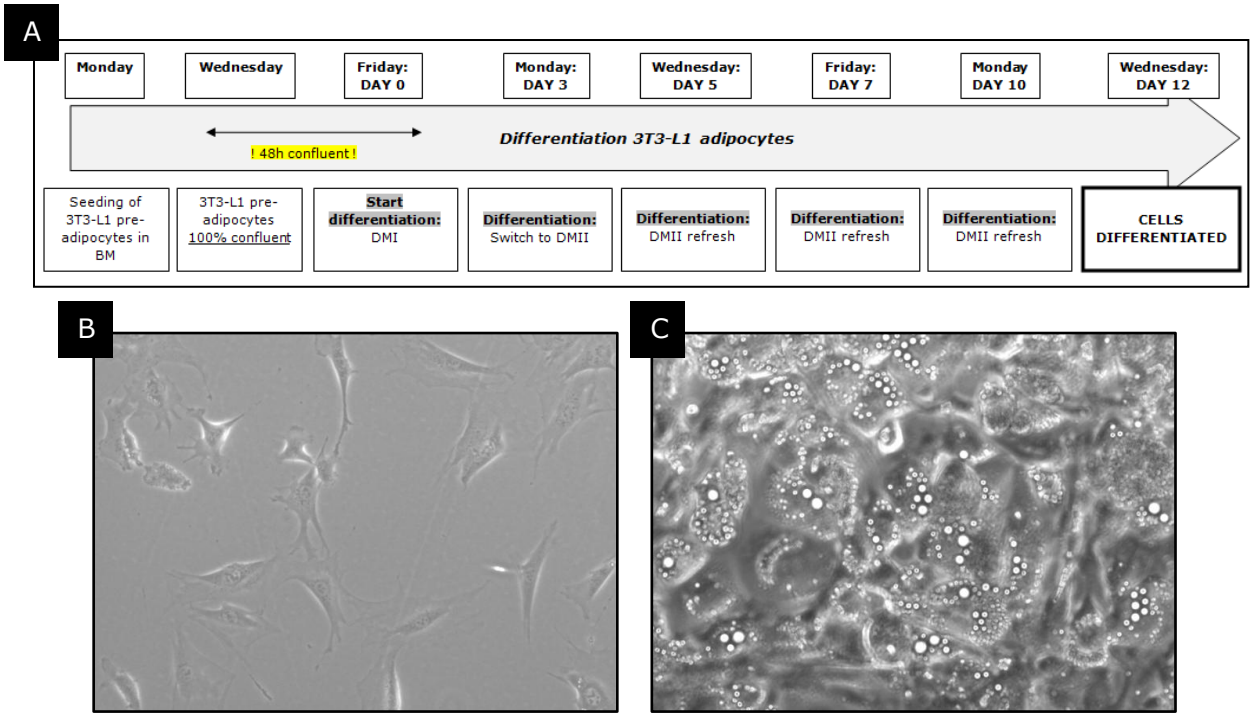


Figure 2. Murine 3T3-L1 adipocyte differentiation

(A) Time schedule of 3T3-L1 adipocyte differentiation from 3T3-L1 pre-adipocytes (B) to mature adipocytes (C). Pictures were made at 100x magnification with an Axio Observer.A1 inverted light microscope (Zeiss, Oberkochen, Germany). BM: basal growth medium; DMI: differentiation medium I; DMII: differentiation medium II (see Table 2).

2.3.1 Oil Red O staining

An Oil Red O (ORO) staining was performed to show the accumulation of red lipid droplets inside the adipocytes after differentiation of pre-adipocytes. In brief, a 0.5% Oil Red O stock solution (Sigma-Aldrich) was prepared in isopropanol and stirred overnight for complete dissolution, at least one day before start of the experiment. On the day of the experiment, a fresh Oil Red O working solution was made by mixing 60% Oil Red O stock solution with 40% double-distilled water (ddH_2O). This working solution was filtered before use. Cells were washed with phosphate buffered saline (PBS) and fixed with 4% formalin at room temperature for 30-60 minutes. Next, cells were washed with ddH_2O and incubated with 60% isopropanol for 2-5 minutes. Hereafter, cells were incubated with filtered Oil Red O working solution for 5 minutes. Subsequently, cells were washed with tap water and counterstained by means of hematoxylin for 1 minute. Finally, cells were washed once again with tap water, dried, mounted with Dako Aqueous Mounting medium, and photographed with a Nikon Eclipse E800 microscope (Figure 3).

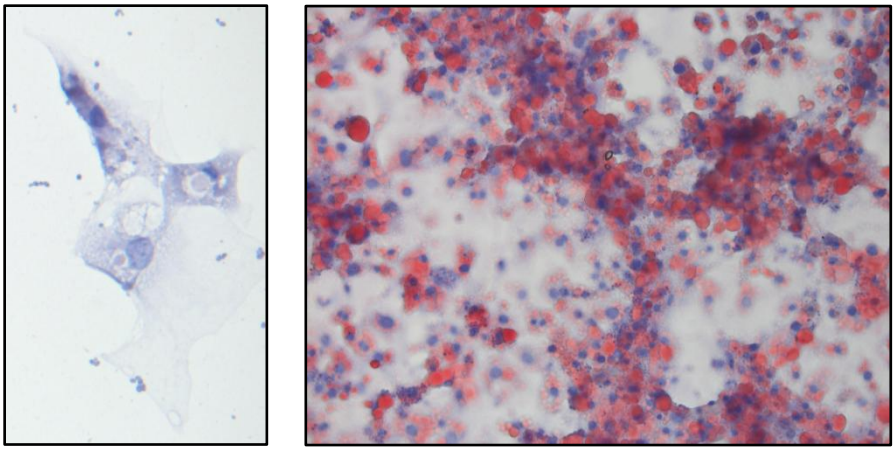


Figure 3. Oil Red O staining of undifferentiated 3T3-L1 murine pre-adipocytes (left) and differentiated adipocytes (right)

Pictures were made at 200x magnification with an Eclipse E800 microscope (Nikon).

Table 2. Differentiation media and compounds

Medium type	Compounds
Basal growth medium (BM)	DMEM, high glucose (no. 42430-025) Supplemented with: ▪ 10% FBS ▪ 1% P/S ▪ 1% Sodium pyruvate
Differentiation medium I (DMI)	DMEM, high glucose (no. 42430-025) Supplemented with: ▪ 0.5 mM IBMX ▪ 10 µg/ml Insulin ▪ 0.444 µg/ml Dexamethasone
Differentiation medium II (DMII)	DMEM, high glucose (no. 42430-025) Supplemented with: ▪ 2.5 µg/ml Insulin

DMEM-high glucose medium, P/S, and Sodium pyruvate were all purchased from Life Technologies (Bleiswijk, The Netherlands). Insulin, dexamethasone and IBMX were purchased from Sigma-Aldrich (St. Louis, Missouri). FBS was purchased from PAA Laboratories GmbH (Pasching, Austria). FBS: fetal bovine serum; P/S: penicillin/streptomycin; IBMX: 3-isobutyl-1-methylxanthine.

2.4 Gene expression analysis

2.4.1 Experimental setup

3T3-L1 pre-adipocytes were cultured and differentiated in 12-well cell culture plates (Eppendorf, Hamburg, Germany). Adipocytes with a relatively high and low passage number were used in order to investigate whether the same results could be achieved in the two separate experiments, independently from time and batch of cells. After 12 days of differentiation, cells were exposed for 24 hours to 50% CM of different human pancreatic cancer cell lines, namely PK-1, KLM-1, PANC-1, and PK-45H.

In addition, CM of two human breast cancer cell lines, MCF-7 and T47D, was included as non-cachectic cancer control. Cells exposed to regular BM instead of CM were considered as reference. CM and BM were both mixed with 50% DMII (Table 2).

2.4.2 RNA isolation

Differentiated 3T3-L1 adipocytes were lysed in the cell culture plate by using TRI Reagent® Solution (Sigma-Aldrich). Next, chloroform was added to the samples, mixed, and incubated at room temperature for 5-15 minutes. Samples were centrifuged at 13.000 rpm for 15 minutes at 4°C by which 3 phases were generated. The top layer or the aqueous phase, which contained the RNA, was transferred to a new tube. Isopropanol was added and solutions were mixed and incubated at room temperature for 5-15 minutes, which caused the RNA to precipitate. Samples were centrifuged again at 13.000 rpm for 15 minutes at 4°C, after which the supernatant was discarded. RNA was washed twice with 75% ethanol and centrifuged at 13.000 rpm for 5 minutes. Finally, the ethanol was removed and the RNA pellet was air dried and dissolved in nuclease free water. Subsequently, the RNA concentration of the samples was determined by means of the NanoDrop 1000A spectrophotometer (Thermo Scientific, Waltham, Massachusetts) at an absorbance of 260 nm.

2.4.3 cDNA synthesis

750 ng RNA was converted to cDNA by means of the SensiFAST™ cDNA Synthesis Kit (Bioline, Luckenwalde, Germany) according to the manufacturer's instructions. The set program of the thermal cycler MJ Research PTC-200 DNA Engine (BIOzym Group, Landgraaf, The Netherlands) was as follows: 5 minutes at 25°C, 30 minutes at 42°C, and 5 minutes at 85°C.

2.4.4 Quantitative real-time PCR

5 ng of the produced cDNA was used as a template strand in the quantitative real-time polymerase chain reaction (qRT-PCR). In addition, SensiMix™ SYBR & Fluorescein MasterMix (Bioline) and 150 nM of reverse and forward primers, synthesized by Sigma-Aldrich, were included in the reaction mixture (Table 3). Expression of the UCP1 gene was investigated by means of the MyiQ system (Bio-Rad, Hercules, California). cDNA samples underwent the following PCR conditions: 40 cycles at 95°C for 1 minute (denaturation), 60°C for 1 minute (annealing), and 72°C for 10 seconds (elongation). Amplification was followed by melt curve analysis by using the iQ5 software (Bio-Rad). Relative gene expression could be calculated by means of LinRegPCR software (version 2013.0; Amsterdam, The Netherlands). Normalization of results was performed by using β-actin as reference gene (Table 3). Data are represented as fold expression in relation to the mean expression in the negative control group.

Table 3. Primers used for quantitative RT-PCR

Gene	Forward primer	Reverse primer
UCP1	5′ – CACGGGGACCTACAATGCTT – 3′	5′ – TAGGGGTCGTCCCTTTCCAA – 3′
β-actin	5′ – GACAGGATGCAGAAGGAGATTACTG – 3′	5′ – CCACCGATCCACACAGAGTACTT – 3′

UCP1: uncoupling protein 1

2.5 Lipolysis assay

2.5.1 Experimental setup

3T3-L1 adipocytes were cultured and differentiated in 12-well cell culture plates (Eppendorf). After 12 days of differentiation, cells were exposed for 6 hours to 50% CM of the same human pancreatic and breast tumor cell lines used for the qRT-PCR experiment. CM of each cell line was mixed with 50% BM, containing 0.6% FA free BSA (Sigma), resulting in a BSA end concentration of 0.3%. The reason for the use of BM instead of DMII in this experiment is because DMII contains insulin, which is a negative regulator of lipolysis. BSA is responsible for the conjugation of FAs during the whole experiment, which prevents the resynthesis of triglycerides from free glycerol and FAs. Adipocytes incubated with 0.3% BSA-BM, and not with CM, were regarded as negative control.

Literature already indicated that Isoprenaline at a concentration of 10 μM is able to induce lipolysis in 3T3-L1 adipoctyes at its maximum level [32]. In order to find the most optimal Isoprenaline concentration as positive control for this experiment, a dose-response curve was constructed, including 0, 1, 5, 10, 25, and 50 μM (N=2). The remainders of CM/BSA-BM media which were not exposed to the cells were included in the assay in order to investigate the initial glycerol concentration in CM of the used tumor cells.

2.5.2 The assay

The purpose of the lipolysis assay is to measure the accumulation of free glycerol (μM) in the cell culture supernatant, which reflects the degree of ongoing lipolysis in the adipocytes. The lipolysis assay was performed by using the EnzyChrom™ Adipolysis Assay Kit (BioAssay Systems, Hayward, California) according to the manufacturer's protocol. Optical density of the samples was read at 570 nm by means of a Spectramax M2 microplate reader (Molecular Devices, Sunnyvale, California) and data were analyzed with the Soft Max Pro 6.2.1 software (Molecular Devices).

2.6 Statistical analysis

Statistics were performed using GraphPad Prism 6.0 software (Graphpad, San Diego, California). Non-parametric Kruskal-Wallis and Mann-Whitney tests were conducted to analyze possible differences between groups. Statistical significance among the values was considered at a p-value<0.05. Results are shown as mean±SEM.

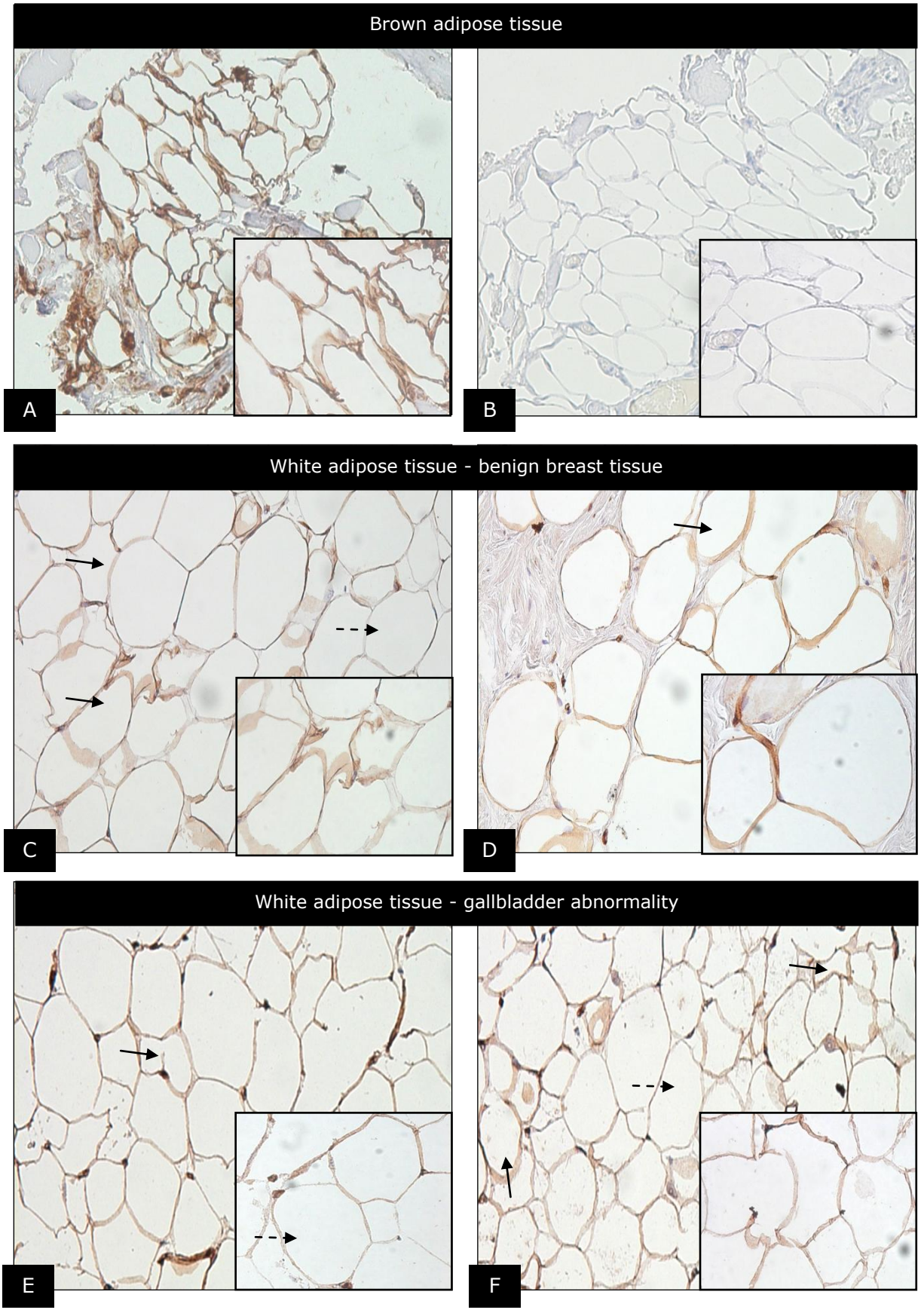
3 Results

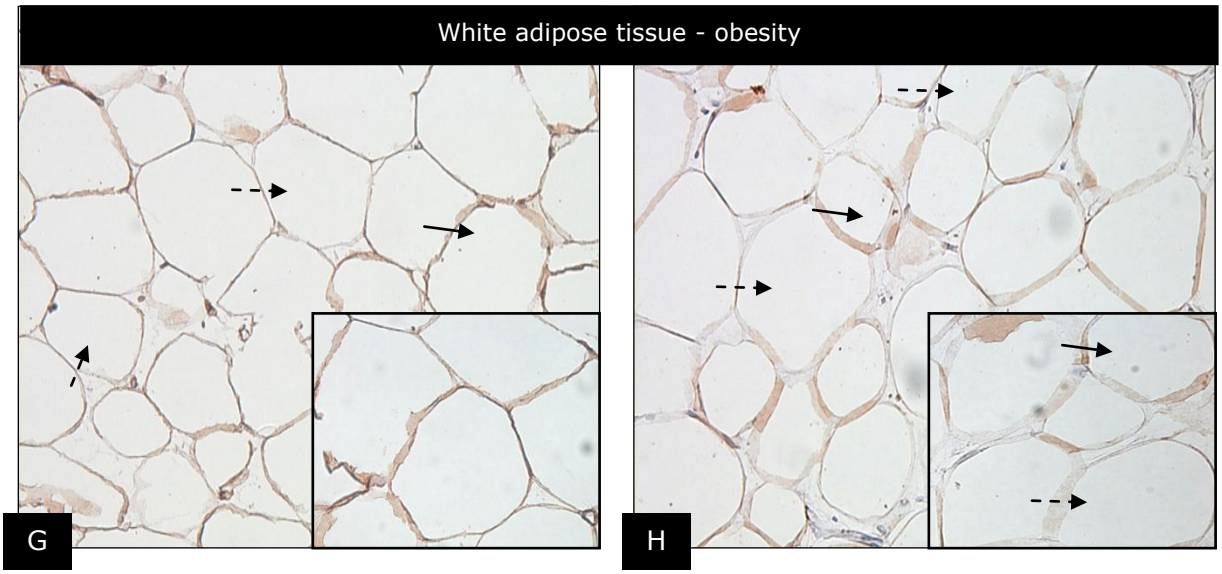
This study aimed at exploring whether changes in adipose tissue or adipocyte biology occur resp. in pancreatic cancer patients or by the impact of pancreatic tumor cells. To this end, WAT browning and activation of lipolysis were studied using several model systems in the context of pancreatic cancer. Peritumoral WAT of pancreatic cancer patients was stained for the BAT marker UCP1, investigating the occurrence of browning of WAT at the protein level. In addition, both UCP1 gene expression and the extent of lipolysis were studied in differentiated murine 3T3-L1 adipocytes after exposure to CM of the human pancreatic cancer cell lines PK-1, PK-45H, PANC-1, and KLM-1.

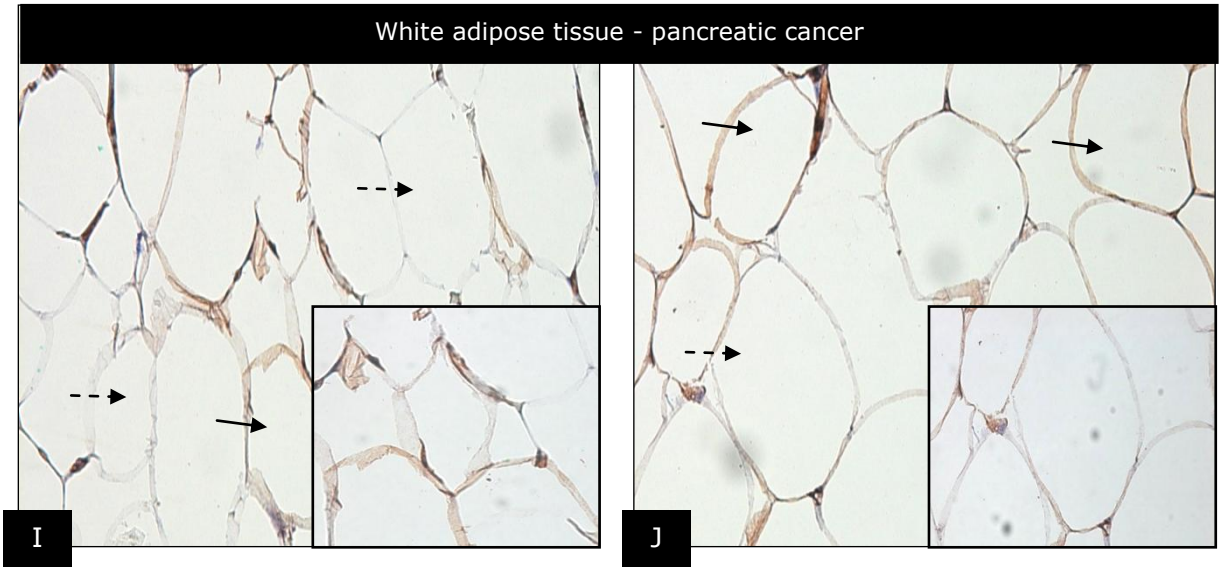
3.1 Peritumoral WAT of pancreatic cancer patients stains positive for UCP1

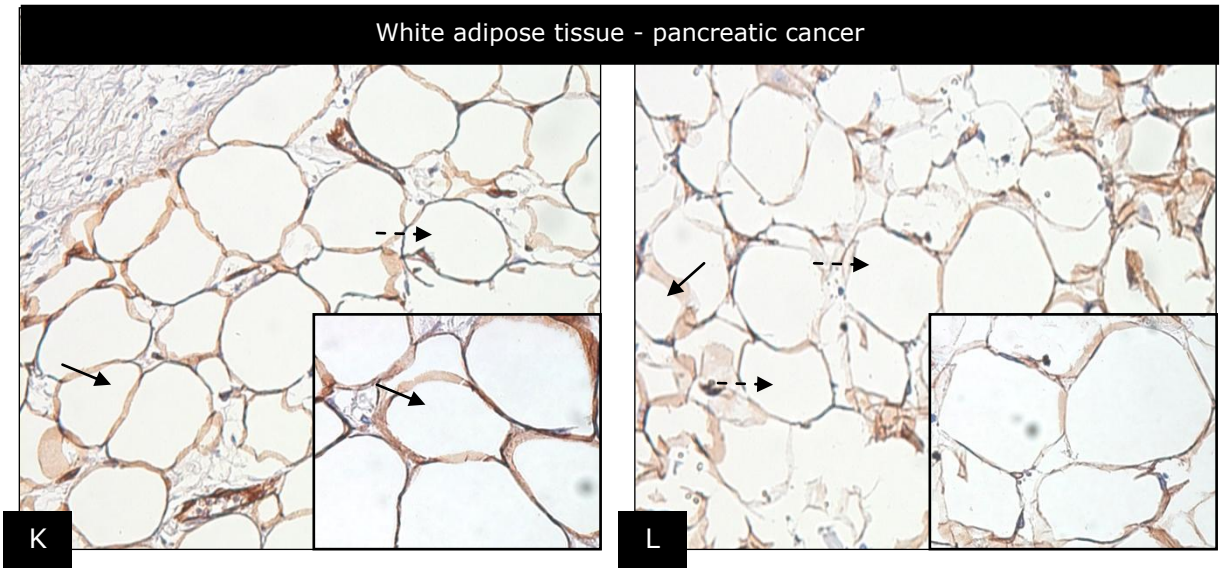
To determine whether browning of WAT occurs in patients suffering from pancreatic cancer compared to some other medical conditions that are not associated with cachexia, immunohistochemical stainings for UCP1 were performed on archived WAT samples derived from these different patients. As expected, a clear positive staining signal was observed in human BAT (positive control; Figure 4A), whereas a blank control section of BAT, which was not incubated with primary UCP1 antibody, demonstrated no staining (Figure 4B). WAT sections from individuals with distinct types of medical conditions without cachexia, such as an increased risk of developing breast cancer due to a BRCA gene mutation (Figure 4C and D), gallbladder abnormality (Figure 4E and F), and obesity (Figure 4G and H), were included for comparison and were expected to produce a negative result. Representative stainings of six different pancreatic cancer patients were selected and are represented here (Figure 4I-N). A positive staining could be observed in adipose tissue samples of all different patients, although there was no clear pattern. Staining was variable between patients, and heterogeneously distributed within WAT. More specifically, tissue areas with stronger (indicated by full arrows) and less stained (indicated by dashed arrows) adipocytes were observed (Figure 4C-N). Blank control WAT sections consistently showed a negative staining (data not shown). When comparing the immunohistochemical results with some clinical parameters of the 6 selected pancreatic cancer patients, no marked difference in staining results were observed between patients with increased weight loss (I, J, and K) and no weight loss (M and N) assessed over a period of six months (Table 4).

Together, these findings indicate that peritumoral WAT of pancreatic cancer patients stains positive for UCP1, but that there seems to be no difference with WAT of other subpopulations, since they also display a positive staining.


Brown adipose tissue
A
B
White adipose tissue - benign breast tissue
C
D
White adipose tissue - gallbladder abnormality
E
F


White adipose tissue - obesity
G
H


White adipose tissue - pancreatic cancer
I
J


White adipose tissue - pancreatic cancer
K
L

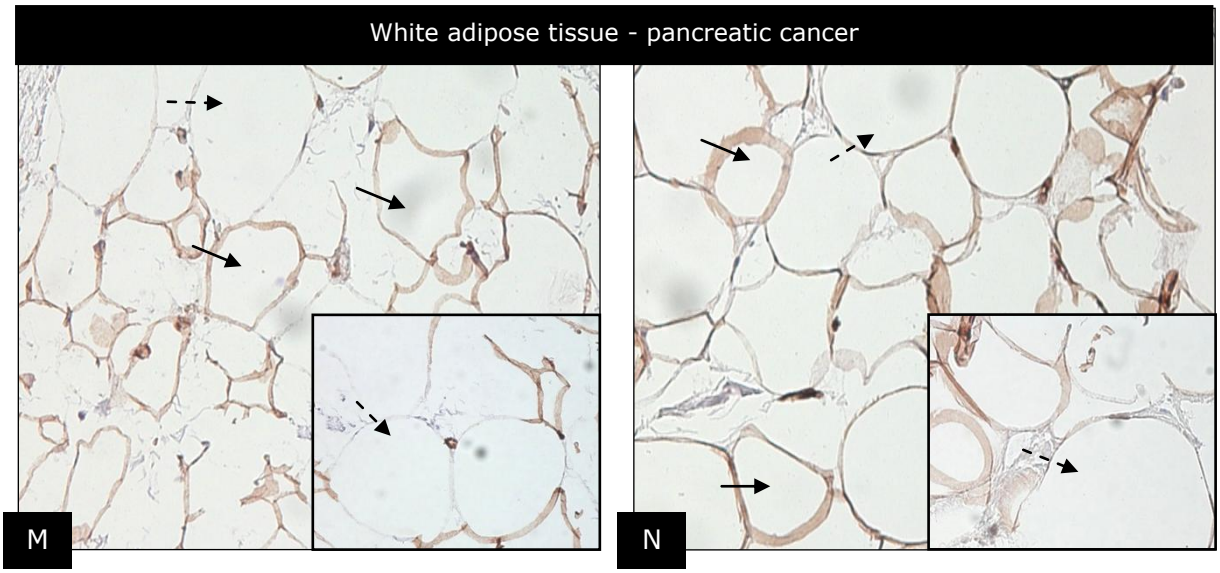


Figure 4. Immunohistochemical staining UCP1 on adipose tissue samples from different subpopulations

In order to investigate whether browning of WAT is associated with pancreatic cancer, archived WAT samples derived from pancreatic cancer patients and patients with several other medical conditions without cachexia were stained for UCP1 for comparison. (A) Human BAT as positive control; (B) Human BAT as negative blank control (no incubation with primary antibody); (C,D) WAT of two healthy patients who have an increased risk of developing breast cancer, since they possess a BRCA gene mutation; (E,F) WAT of two patients who underwent surgery because of gallbladder malfunctioning; (G,H) WAT of two obese patients; (I-N) Peritumoral WAT of six selected pancreatic cancer patients. In BAT, a consistent and clear immunohistochemical staining of UCP1 was demonstrated (brown color). In the blank control BAT section, no staining could be observed. WAT of all patients displayed a positive staining, although there was no clear pattern; the degree of staining varied between patients, and staining was heterogeneously distributed within WAT. More specifically, tissue areas with stronger (full arrows) and less stained (dashed arrows) adipocytes were detected. The blank control WAT sections consistently showed a negative staining (data not shown). Representative pictures of selected patients were made at 200x and 400x (inset) magnifications with a Nikon Eclipse E800 microscope. WAT: white adipose tissue; BAT: brown adipose tissue; UCP1: uncoupling protein 1.

Table 4. Patient characteristics of 6 selected pancreatic cancer patients

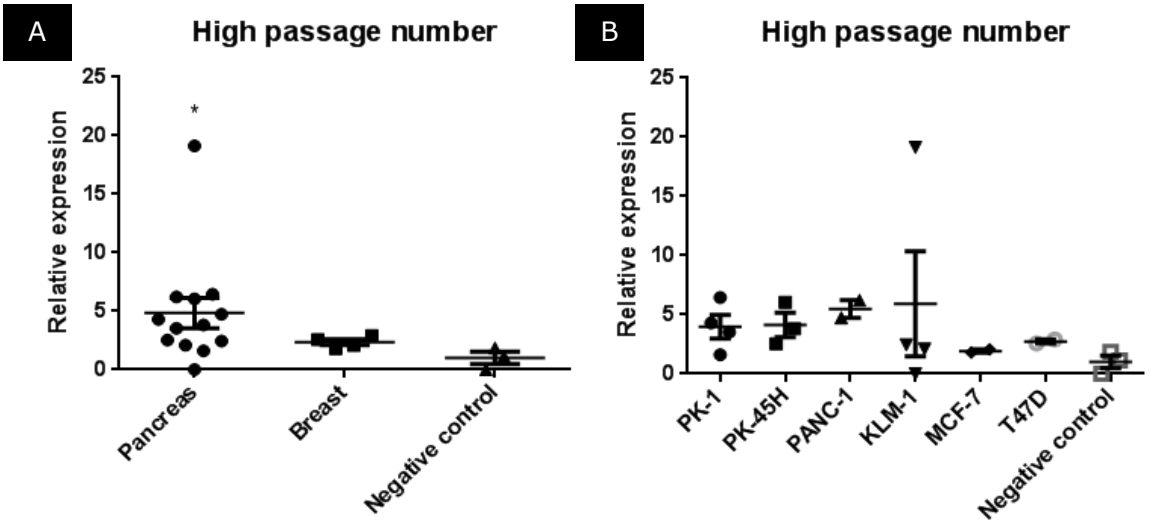
Picture	Age	Sex	BMI	CRP (mg/l)	% Weight loss
I	66	F	21.3	2	10.77
J	69	M	24.5	n.r.	11.63
K	78	F	23.4	n.r.	16.67
L	65	M	22	1	n.r.
M	67	M	27.1	2	0
N	60	F	23.1	5	0

CRP levels>10 mg/l indicate systemic inflammation. Weight loss (%) was assessed over a period of 6 months. F: female; M: male; n.r.: not registered; BMI: body mass index; CRP: c-reactive protein.

3.2 Conditioned medium of human pancreatic cancer cell lines affects UCP1 expression in murine 3T3-L1 adipocytes

To investigate whether UCP1 expression in murine 3T3-L1 adipocytes is affected by CM derived from human pancreatic or breast cancer cell lines, gene expression analysis of UCP1 was performed by qRT-PCR. The cancer cell lines were pooled in order to obtain enough data points for statistical analysis. CM of pancreatic cancer cell lines significantly upregulated UCP1 in differentiated adipocytes with a relatively high passage number (P=0.03; Figure 5A). CM of breast cancer cell lines had no effect on UCP1 expression compared to the negative control (=cells incubated with BM instead of CM; P=0.11). Although there were insufficient observations for each cancer cell line separately to perform proper statistics, CM of PK-1, PK-45H, and PANC-1 seemed to be more potent to upregulate UCP1 mRNA expression compared to CM of KLM-1, MCF-7, and T47D (Figure 5B). A trend towards increased UCP1 expression could be observed in differentiated adipocytes with a lower passage number after incubation with CM of pancreatic cancer cell lines as well (Figure 5C). However, the induction was much less pronounced compared to the experiment performed with more frequently passaged cells. CM of breast cancer cell lines was not included in this experiment, since there was no sufficient amount of cells for inclusion of all conditions. When looking at the individual cell lines, UCP1 expression seemed to be increased after incubation with CM of PK-1, PK-45H, and PANC-1 compared to the control condition (Figure 5D). However, expression levels of UCP1 were still relatively low in both experiments as demonstrated by the relatively high quantitation cycle (Cq) values during the analysis.

Taken together, these findings demonstrate that CM derived from human pancreatic cancer cell lines upregulates UCP1 expression in differentiated murine 3T3-L1 adipocytes, although this expression level is relatively low given the high Cq values. CM of breast cancer cell lines seems not to be able to induce this effect.



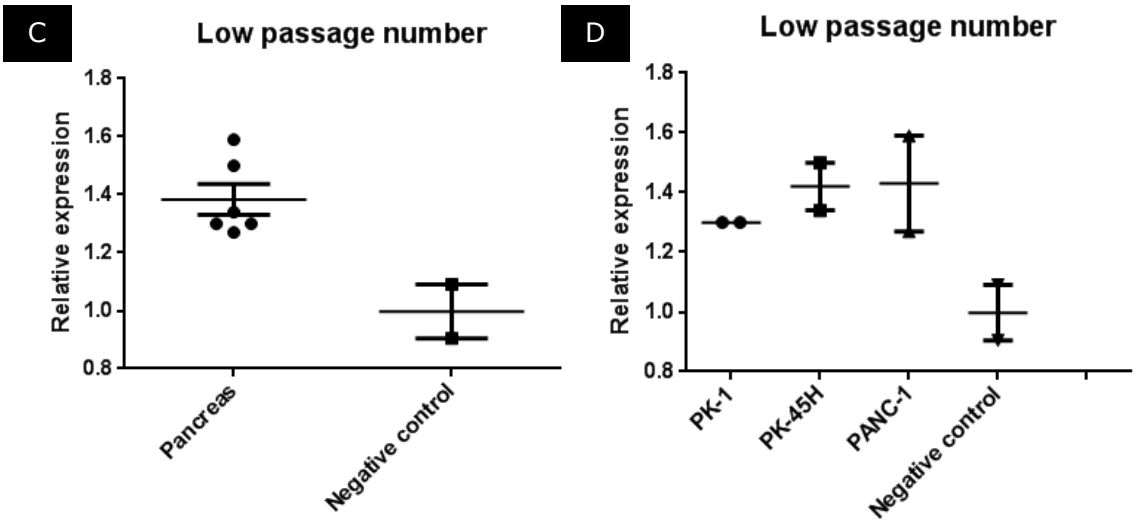


Figure 5. UCP1 expression in murine 3T3-L1 adipocytes after exposure to CM derived from human cancer cell lines

UCP1 gene expression in differentiated 3T3-L1 adipocytes was investigated after an incubation of 24 hours with CM of human pancreatic and breast cancer cell lines. Experiments with (A) frequently and (B) less passaged adipocytes were conducted in order to investigate whether the same results could be achieved in the two separate experiments, independently from time and batch of cells. (A) Cancer cell lines were pooled in order to obtain enough data points for statistical analysis. The results demonstrate that CM of pancreatic cancer cell lines (N=13) significantly increased UCP1 expression in adipocytes with a high passage number (P=0.03). CM of breast cancer cell lines (N=4) was not able to induce this effect when compared to the negative control (= cells exposed to BM instead of CM; N=3; P=0.11). (B) Although there were not enough observations in each cancer cell line group separately to perform proper statistics, PK-1, PK-45H, and PANC-1 seemed to be more potent to increase UCP1 mRNA expression compared to KLM-1, MCF-7, and T47D. (C) In less passaged cells, a trend towards increased UCP1 expression could be observed after exposure to CM of pancreatic cancer cell lines (N=6) compared to the negative control (N=2). However, these values were in a lower range compared to the experiment with more frequently passaged cells. CM of breast cancer cell lines was not included in this experiment, since there were not enough cells for inclusion of all conditions. (D) When looking at the individual cell lines, UCP1 mRNA expression seemed to be increased after incubation with CM from PK-1, PK-45H, and PANC-1 compared to the control condition. Significance was tested by means of a Kruskal-Wallis test, followed by a Mann-Whitney U test to compare possible differences between two groups. *P-value<0.05 compared to the negative control. Data are represented as mean±SEM. UCP1: uncoupling protein 1; BM: basal growth medium; CM: conditioned medium.

3.3 Conditioned medium of human pancreatic cancer cell lines does not affect lipolysis in murine 3T3-L1 adipocytes

As already demonstrated by the previous experiment, UCP1 mRNA expression was upregulated by CM of pancreatic cancer cell lines. Since the association of UCP1 with lipolysis has already been described, the impact of pancreatic tumor cells on adipocyte lipolysis was questioned [33]. To explore whether CM derived from human pancreatic or breast cancer cell lines induces activation of lipolysis in differentiated murine 3T3-L1 adipocytes, a lipolysis assay was performed which measured the concentration of glycerol released in the culture medium as a consequence of triglyceride lipolysis.

Firstly, the optimal dose of Isoprenaline as lipolytic control was determined by means of a dose-response curve experiment. Although the number of observations in each group was too small to perform any statistical analysis, an approximate 3-fold increase of [glycerol] in the cell culture supernatant could be observed following incubation with different concentrations of Isoprenaline, compared to the baseline (= 0 µM; Figure 6). Consequently, a concentration of 1 µM Isoprenaline was used as positive control in subsequent experiments. The results demonstrate that the initial glycerol concentration was already elevated in CM of both pancreatic and breast tumor cells. Particularly PANC-1 and MCF-7 showed higher glycerol concentrations in their CM (Figure 7). For the next experiment, cancer cell lines were pooled in order to obtain enough data points for statistical analysis. Incubation with CM derived from human pancreatic and breast cancer cell lines had no significant effect on the concentration of glycerol in the culture medium of differentiated 3T3-L1 adipocytes after 6 hours compared to the negative control (= cells exposed to BM instead of CM; resp. P=0.39 and P=0.31; Figure 8A). Furthermore, there was no significant difference between CM of human pancreatic and breast cancer cell lines regarding their effect on glycerol concentration in the culture medium (P=0.37). Isoprenaline at a concentration of 1 µM was able to increase [glycerol] by approximately 3-fold compared to the baseline (negative control; P<0.001). Individual pancreatic and breast cancer cell lines showed no difference in lipolysis compared to the negative control condition (P=0.40; Figure 8B).

Together, these data indicate that CM of human pancreatic cancer cell lines is not able to induce lipolysis in murine 3T3-L1 adipocytes, and that there is no significant difference compared to the non-cachectic cancer control cell lines MCF-7 and T47D.

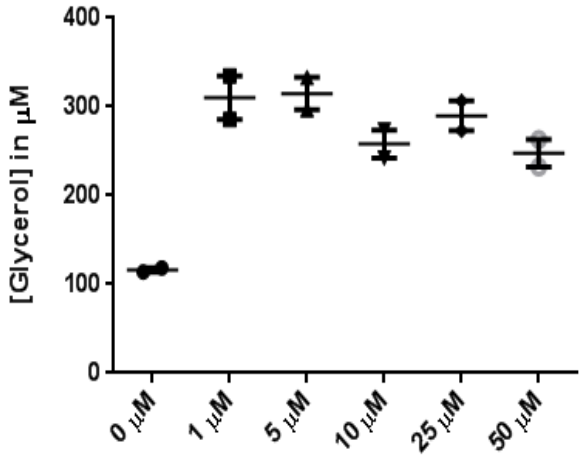


Figure 6. Dose-response curve for Isoprenaline

The optimal concentration of Isoprenaline as lipolytic control was determined by means of a dose-response curve, including 0, 1, 5, 10, 25, and 50 µM (N=2). Although no statistical analysis could be performed due to the small number of observations in each group, the results indicate that Isoprenaline increased [glycerol] about three times compared to the baseline (negative control, 0 µM). Consequently, a concentration of 1 µM Isoprenaline was used as positive control in subsequent experiments. Data are represented as mean±SEM.

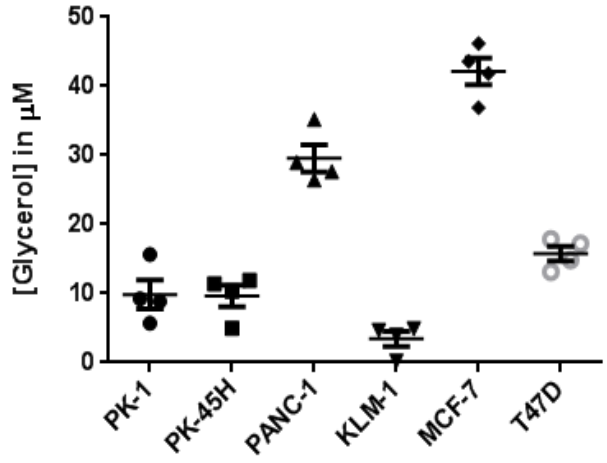


Figure 7. Glycerol concentration in CM of the tumor cells

The concentration of glycerol (µM) in CM which was derived from tumor cells and which did not have contact with the 3T3-L1 adipocytes was measured. Both pancreatic and breast cancer cell lines (N=4 per cell line) showed initially elevated levels of glycerol in their CM. Especially CM derived from PANC-1 and MCF-7 contained higher concentrations of glycerol. CM: conditioned medium. Data are represented as mean±SEM.

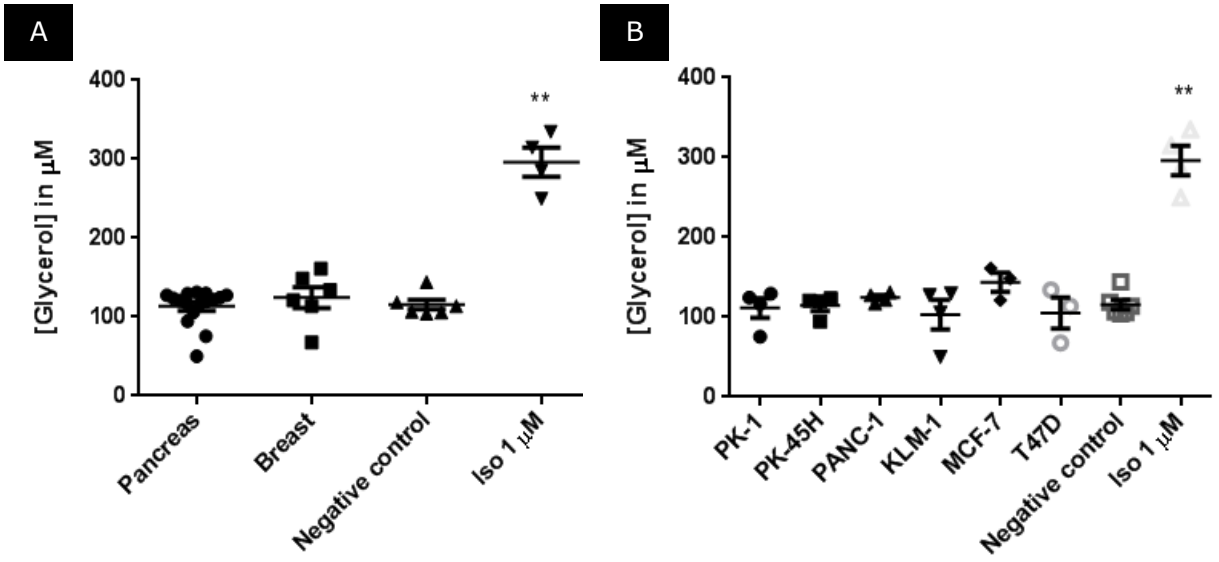


Figure 8. The extent of lipolysis in murine 3T3-L1 adipocytes after exposure to CM derived from human cancer cell lines

The concentration of released glycerol (µM) in the culture medium of differentiated 3T3-L1 adipocytes was measured as indication of the extent of lipolysis after 6 hours exposure to CM of human pancreatic (N=16) and breast (N=6) cancer cell lines. (A) Cancer cell lines were pooled in order to obtain enough data points for statistical analysis. The results show that incubation with CM of pancreatic and breast cancer cell lines had no significant effect on the degree of lipolysis compared to the negative control (= cells exposed to BM instead of CM; resp. P=0.39 and P=0.31). Furthermore, there was no significant difference between the two types of cancer cell lines regarding their effect on lipolysis (P=0.37). Isoprenaline (1 µM) as positive control (N=4) was able to increase [glycerol] about three times compared to the baseline (negative control; N=6; P<0.01). (B) There was no difference regarding stimulation of lipolysis between individual pancreatic and breast cancer cell lines and the negative control (P=0.40). Significance was tested by means of a Kruskal-Wallis test, followed by a Mann-Whitney U test to compare possible differences between two groups. **P-value<0.01 compared to the negative control. Data are represented as mean±SEM. BM: basal growth medium; CM: conditioned medium.

4 Discussion and outlook

Cachexia is a syndrome of involuntary loss of skeletal muscle and adipose tissue mass, which is very common in pancreatic cancer patients, and most unusual in individuals with breast cancer. In the present study, we focused on possible alterations in adipose tissue characteristics which can develop as a result of the existence of cancer, more specifically pancreatic cancer, and which may contribute to the onset/progression of cachexia. Two proposed mechanisms involved in the development of pancreatic cancer-related cachexia, due to their engagement in energy regulation, were explored in this project, namely WAT browning and stimulation of lipolysis. In WAT browning, WAT adopts characteristics of BAT, thereby gaining the thermogenic capacity of BAT, resulting in heat production, energy expenditure, and may induce weight loss. Lipolysis is marked by the breakdown of triglycerides through which excess energy is expended. Increased activation of lipolysis leads to a shift in the energy balance towards increased energy consumption, possibly resulting in unfavorable conditions of weight loss. Our data support the occurrence of WAT browning in pancreatic cancer patients. Immunohistochemistry revealed that UCP1 is expressed in peritumoral WAT of pancreatic cancer patients. However, the results also showed that this positive staining was not limited to WAT of pancreatic cancer patients, but that also WAT derived from obese individuals or patients operated for malfunctioning of the gallbladder or benignity of breast tissue stained positive for UCP1. The staining varied between patients, and was not distributed in a homogeneous way within WAT. More particularly, areas with stronger and less stained adipocytes were observed throughout the tissue. In line with the universal UCP1 expression in WAT of pancreatic cancer patients, pancreatic CM induced a significant upregulation of UCP1 after 24 hours of incubation in differentiated murine 3T3-L1 adipocytes with a high passage number. In contrast, CM of breast cancer cell lines was not able to stimulate UCP1 expression in these cells. In adipocytes with a lower passage number, the same trend could be observed after incubation with pancreatic CM, although the values were in a lower range. Activation of lipolysis was also investigated in differentiated murine 3T3-L1 adipocytes after 6 hours of incubation with CM derived from the same human pancreatic and breast cancer cell lines as used in the previous experiment. These data indicate that the level of lipolysis in the adipocytes was neither affected by CM of pancreatic cancer cell lines nor by CM of breast cancer cell lines.

The immunohistochemical analysis demonstrated that UCP1 is expressed at the protein level within peritumoral WAT derived from pancreatic cancer patients, but that this observation is not restricted to this subpopulation, since WAT of patients with other medical conditions not assumed to be associated with WAT browning also showed a positive staining. This is not what we expected in the first place, because obesity represents the opposite condition of cachexia regarding body weight and energy expenditure. In addition, WAT located in benign breast tissue was also assumed to stain negative, since this tissue was derived from a healthy donor, who did not have any type of cancer and no cachexia. Furthermore, the gallbladder patients were also expected to produce a negative result, because they did not have any background of cachexia, but rather had a generally high body weight. Since we therefore doubted the specificity of the primary anti-UCP1 antibody, we tested two other anti-UCP1 antibodies. One did not work and the other yielded similar staining patterns.

A possible explanation for the positive staining in all patients could be the cooling of the body during surgery. As described previously, BAT activation and UCP1 expression are triggered by cold exposure [22,34]. Since all these patients were exposed to a colder environment for a certain period during surgery, it could be that ectopic UCP1 expression in WAT was already induced to some degree. In addition, the obese donor patients included in this experiment were already subjected to a slimming protocol before surgery in order to lose weight. Weight loss has already been demonstrated to be associated with activation of BAT, and therefore may also induce browning of WAT [35]. In addition, literature previously described a role for bile acids in regulating BAT energy expenditure via TGR5-mediated signaling [36]. Furthermore, animal studies have shown that elevated plasma levels of bile acids induced UCP1 expression in WAT [37]. In relation to our data, gallbladder malfunctioning could lead to altered levels/functioning of bile acids, which may influence the expression levels of UCP1 in WAT of our analyzed patients. A study of Petruzzelli *et al.* revealed that mouse models of cancer cachexia showed consistent browning of subcutaneous WAT, as confirmed by immunohistochemical analysis [22]. More specifically, subcutaneous WAT of genetically engineered mouse models (GEMMs) of pancreatic and lung cancer, which displayed >15% total loss of body weight after 6-8 months, showed depots of small multilocular adipocytes and positive UCP1 staining, which was not observed in littermate controls. Furthermore, Petruzzelli and colleagues also investigated UCP1 expression within adipose tissue in a human setting. WAT samples from cachectic cancer patients were taken from distinct sites within the body. In accordance with our data, almost 88% of the patients displayed positive UCP1 staining (N=8), including intestinal and peripancreatic WAT derived from patients with pancreatic and lung cancer, respectively. Intestinal WAT from colon cancer patients without cachexia showed no expression of UCP1. Another recent study of Wang *et al.* demonstrated increased UCP1 expression in WAT surrounding breast tumors compared to WAT neighboring benign breast lesions as analyzed by means of immunohistochemistry [38].

These data indicate that UCP1 may not be exclusively expressed in adipose tissue from pancreatic cancer patients, suggesting that expression of this protein, considered to be an excellent marker of BAT, is more widespread than previously thought. However, a method for quantification of the immunohistochemical results is required, which allows making a link between the extent of UCP1 expression and clinical parameters of the patients. Although UCP1 is the most established marker of BAT, other proposed surface markers of beige and brown adipocytes, e.g. purinergic receptor P2X, ligand-gated ion channel 5 (P2RX5) and proton assistant amino acid transporter-2 (PAT2), could also be included in future immunohistochemical analyses [39]. Another option for the future is to perform Western blot analysis for UCP1 by using tissue material of newly recruited patients. In this manner, more quantitative data about UCP1 protein expression will be acquired, and at the same time, the correct operation of the used antibody can be demonstrated.

Gene expression analysis revealed that there was a significant upregulation of UCP1 in differentiated murine 3T3-L1 adipocytes with a high passage number following exposure to CM of human pancreatic cancer cell lines. CM of human breast cancer cell lines did not affect UCP1 expression compared to the negative control. In differentiated murine 3T3-L1 adipocytes with a low passage number, a similar trend was observed regarding UCP1 expression after exposure to CM of human pancreatic cancer cell lines, although the values were lower. The latter may be explained by the unfavorable culture condition of the cells with a lower passage number. After 12 days, the degree of differentiation was very low and many dead cells were present. The adipocytes with a higher passage number showed numerous differentiated cells, as demonstrated by the large number of lipid droplets inside these cells. In accordance with our findings, Petruzzelli *et al*. demonstrated an increased level of UCP1 protein in axillary WAT of their pancreatic cancer mouse model compared to control mice [22]. In addition, that study indicated an upregulation of UCP1 mRNA in axillary and inguinal WAT derived from cachectic mice with skin cancer compared to littermate controls. These findings confirm that UCP1 expression is increased at both the mRNA and protein level in murine cancer-related cachexia. In addition, immunohistochemical analysis of UCP1 in WAT samples derived from several cachectic cancer patients also demonstrated the presence of this marker at the protein level in the same study. Unfortunately, the degree of cachexia of the donor patients from whom the pancreatic and breast cancer cell lines used in this project were generated, is unknown. In line with the previous data and assuming that 80% of pancreatic cancer patients develop cachexia, and breast cancer patients usually not, the increased versus unaffected UCP1 mRNA expression in adipocytes after incubation with pancreatic respectively breast CM corresponded with our expectations. It should be noted that 3T3-L1 adipocytes are derived from mice, whereas the pancreatic and breast cancer cell lines we used originate from human subjects. This species difference may influence the final outcome. This issue is already addressed by ongoing experiments with human adipose-derived stromal/stem cells (ASC), but will not be finished in time for inclusion in this thesis.
In conclusion, our data indicate that UCP1 expression can be modulated by human pancreatic tumor cells, suggesting that pancreatic tumor cell-derived factors induce browning of these white adipocytes. However, it is important to repeat these experiments and to include a positive control condition, in which UCP1 expression is definitely upregulated.

Lipolysis was not affected by incubation with CM derived from either human pancreatic or breast cancer cell lines. Furthermore, the degree of lipolysis in the cells did not differ significantly between pancreatic and breast CM. Lipolysis has been shown to be the main causative factor for the reduction of adipose tissue mass observed in cancer patients [40]. Analogous to our experiment, Chung and colleagues subjected mature 3T3-L1 adipocytes to CM of murine colon-26 adenocarcinoma cells, which resulted in a decrease in lipid staining and elevated FFA levels in the culture medium [41]. In contrast, these researchers incubated the adipocytes for a longer time period with CM, namely 2 days, which could explain the difference between their and our results. Agustsson *et al.* demonstrated an increased lipolytic activity in adipose tissue from cachectic gastrointestinal cancer patients, as indicated by increased mRNA and protein levels of HSL, a key regulator of lipolysis [30]. However, investigation of enzyme activity has to be considered, which will eventually determine the real impact of the protein.

Another study by Zuijdgeest-van Leeuwen *et al.* also confirmed an increased whole-body lipolysis in cancer patients who were losing weight compared to healthy controls [31]. In line with these data, we expected that incubation with pancreatic CM resulted in elevated lipolysis levels in the adipocytes. Although the high passage number of the 3T3-L1 adipocytes could be associated with low lipolytic potential, the positive control showed that the cells were still lipolytically active, excluding the possibility that this negative outcome was attributable to the passage number. What should be noted is that CM in this experimental setup was diluted by half, resulting in decreased concentrations of potential lipolytic factors, and therefore possibly leading to a decreased effectiveness. Again, the species difference may here be a reason for the lipolytic inactivity observed in the adipocytes, which will be addressed by performing a similar experiment with human ASC. The results also showed initially increased levels of glycerol in CM derived from the different tumor cells, particularly PANC-1 and MCF-7. These data suggest that tumor cells are able to release glycerol in the medium. Literature already revealed that cancer cells can adapt their lipid metabolism in favor of survival and progression. More specifically, increased lipogenesis within the cancer cells contributes to substantial levels of FAs, facilitating tumor growth [42]. More recently, the ability of cancer cells to stimulate their lipolytic machinery in order to gain additional levels of FAs has also been suggested [43]. In conformity with our data, enhanced lipolysis in the tumor cells can lead to the release of both glycerol and FAs in the culture medium [43].

In summary, this study revealed that peritumoral WAT derived from pancreatic cancer patients showed evidence of browning, as demonstrated by positive UCP1 immunohistochemical staining. However, staining was not limited to this patient population, since WAT derived from other types of patients also displayed positive staining. Extensive correlation analyses of these data to available clinical patient data will give us more insight into the significance of WAT browning in cancer cachexia. For future experiments, it is important to further optimize the staining protocol and that WAT samples from distinct patient groups will be analyzed in order to find real negative control tissue to compare with. Another possible future option is to include other markers of brown/beige adipocytes in the immunohistochemical analysis, in order to gain a broader view of WAT and BAT characteristics. Next, CM of human pancreatic cancer cell lines showed the ability to modulate UCP1 expression, but not the levels of lipolysis in murine 3T3-L1 adipocytes. The incubation times between these two experiments differed (24h vs. 6h) and may indicate that a longer time is required for the lipolytic substances within the culture medium to affect the lipolytic cascade. As already indicated, the species difference between human- and mouse-derived cells could be influencing the outcome. Therefore, ongoing experiments with human ASC will counteract this problem. Because of the small number of observations in the different groups at this point, repetition of experiments is necessary in order to draw proper conclusions. Follow-up research, such as investigating the expression of UCP1 and enzymes engaged in lipolysis (e.g. HSL and ATGL) at the protein level by means of Western blot analysis, should be considered. Furthermore, literature already described substances secreted by the tumor as lipolytic factors. Therefore, enzyme-linked immunosorbent assay (ELISA) should be considered to assess the concentration of proposed lipolytic factors within the conditioned medium of the cancer cell lines, such as ZAG and IL-6.

In general, further research regarding the alterations affecting adipose tissue biology during cancer cachexia is required to gain more insight into their contribution to the cachectic process. Understanding the detrimental drivers behind cancer cachexia will be of significant importance in order to address the extreme weight loss observed in cancer patients, potentially resulting in lower mortality rates, better prognosis, more effective treatment options, and improved quality of life.

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