KATRIN RADLOFF

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A thesis submitted in fulfilment of the requirements for the degree of Doctor of Science (Doctor rerum naturalium) in the graduate program Cell and Tissue Biology at the Institute of Biomedical Sciences of the University of São Paulo.

São Paulo/Potsdam
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Supervisors:

Prof. Dr. Marília Cerqueira Leite Seelaender (São Paulo)

Prof. Dr. Gerhard Paul Püschel (Potsdam)

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A caquexia associada ao câncer é caracterizada por perda progressiva de massa muscular acompanhada ou não de perda de tecido adiposo. Clinicamente, a caquexia está associada com aumento de morbidade e mortalidade. Embora seja acompanhada por inflamação sistêmica, atualmente acredita-se que, a caquexia seja parcialmente resultado dessa inflamação. O aumento de citocinas pró-inflamatórias, como TNF- ¿, IL-6, IL-1ß e IFN-¿, são marcadores da inflamação sistêmica, e estão envolvidos em diferentes alterações metabólicas associadas à caquexia, incluindo prejuízo na ingestão alimentar e perda tecidual. Especificamente no tecido adiposo, a caquexia funciona como estímulo catabólico aumentando significativamente a lipólise, resultando na liberação de diferentes ácidos graxos (AG), que funcionam como precursores para a produção de compostos bioativos, como prostaglandinas, resolvinas e lipoxinas. Em algumas condições clínicas, como peritonite, resolvinas e lipoxinas estão associadas com a resolução da inflamação. O tecido adiposo é importante contribuidor para inflamação sistêmica e caquexia, por meio do aumento da infiltração de macrófagos e liberação de fatores inflamatórios. Nossa hipótese considerou que o processo inflamatório no tecido adiposo falha em resolver a inflamação sistêmica. Tal fato, pode estar associado com aumento da inflamação no próprio tecido, com reflexo sistêmico e nos sintomas da caquexia. Assim, o objetivo do presente estudo é avaliar

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Assinatura

PATROCINADOR PRINCIPAL

Não se aplica.

Para as Pan(a)kets

Obrigada por tudo

“An expert is a person who has made all the mistakes that can be made in a very narrow field.”

— Niels Bohr

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SUMMARY

RADLOFF, K. **The role of the fatty acid profile and its modulation by cytokines in the systemic inflammation in cancer cachexia.** 2018. 159 f. Thesis (Ph.D. in Cell and Tissue Biology) – Institute of Biomedical Sciences, University of São Paulo, São Paulo, 2018 and University of Potsdam, Potsdam, 2018.

Systemic inflammation is a hallmark of cancer cachexia. Among tumor-host interactions, the white adipose tissue (WAT) is an important contributor to inflammation as it suffers morphological reorganization and lipolysis, releasing free fatty acids (FA), bioactive lipid mediators (LM) and pro-inflammatory cytokines, which accentuate the activation of pro-inflammatory signaling pathways and the recruitment of immune cells to the tissue. This project aimed to investigate which inflammatory factors are involved in the local adipose tissue inflammation and what is the influence of such factors upon enzymes involved in FA or LM metabolism in healthy individuals (Control), weight stable gastro-intestinal cancer patients (WSC) and cachectic cancer patients (CC). The results demonstrated that the inflammatory signature of systemic inflammation is different from local adipose tissue inflammation. The systemic inflammation of the cachectic cancer patients was characterized by higher levels of circulating saturated fatty acids (SFA), tumor-necrosis-factor-α (TNF-α), interleukins IL-6, IL-8 and CRP while levels of polyunsaturated fatty acids (PUFAs), especially n3-PUFAs, were lower in CC than in the other groups. In vitro and in adipose tissue explants, pro-inflammatory cytokines and SFAs were shown to increase the chemokines IL-8 and CXCL10 that were found to be augmented in adipose tissue inflammation in CC which was more profound in the visceral adipose tissue (VAT) than in subcutaneous adipose tissue (SAT). Systemic inflammation was negatively associated with the expression of PUFA synthesizing enzymes, though gene and protein expression did hardly differ between groups. The effects of inflammatory factors on enzymes in the whole tissue could have been masked by differentiated modulation of the diverse cell types in the same tissue. In vitro experiments showed that the expression of FA-modifying enzymes such as desaturases and elongases in adipocytes and macrophages was regulated into opposing directions by TNF-α, IL-6, LPS or palmitate. The higher plasma concentration of the pro-resolving LM resolvin D1 in CC cannot compensate the overall inflammatory status and the results indicate that inflammatory cytokines interfere with synthesis pathways of pro-resolving LM. In summary, the data revealed a complex inter-tissue and inter-cellular crosstalk mediated by pro-inflammatory cytokines and lipid compounds enhancing inflammation in cancer cachexia by feed-forward mechanisms.

Key words: cancer cachexia, inflammation, adipose tissue, cytokines, chemokines, SFA, PUFA

RESUMO

RADLOFF, K. **O papel e a modulação do perfil de ácidos graxos por citocinas na inflamação da caquexia associada ao cancer.** 2018. 159 f. Tese (Doutorado em Biologia Celular e Tecidual) - Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo e Universidade de Potsdam, Potsdam, 2018.

Titulo da Dupla-Titulação: Efeito dos ácidos graxos sobre a atividade inflamatória de macrófagos na cachexia associada ao cancer: consequências sobre as funções celulares.

A inflamação sistêmica é uma das características que marcam o diagnóstico da caquexia associada ao câncer. Entre as interações tumor-hospedeiro, o tecido adiposo branco contribui à inflamação, uma vez que ele sofre uma reorganização morfológica e lipólise, liberando ácidos graxos livres (AGLs), mediadores lipídicos (LMs) e citocinas pró-inflamatórias, que acentuam a ativação de vias de sinalização pró-inflamatória e o recrutamento de células do sistema imunológico para o tecido. O objetivo deste projeto foi investigar quais fatores inflamatórios sistêmicos estão envolvidos na inflamação do tecido adiposo e qual é a influência desses fatores sobre as enzimas envolvidas no metabolismo dos AGs ou LMs em indivíduos saudáveis (Controle), pacientes com câncer gastrointestinal com peso estável (WSC) e pacientes com câncer e caquexia (CC). Os resultados demonstraram que a resposta inflamatória sistêmica é diferente da resposta encontrada no tecido adiposo. A inflamação sistêmica dos pacientes com câncer e caquexia (CC) foi caracterizada por níveis circulantes mais elevados de ácidos graxos saturados (SFAs), tumor-necrosis-factor-α (TNF-α), Interleukin IL-6, IL-8 e proteina C-reativa (PCR), enquanto os níveis de ácidos graxos poliinsaturados (PUFAs), especialmente n3-PUFAs, foram menores em CC que nos demais grupos. *In vitro* e em explantes de tecido adiposo, citocinas pró-inflamatórias e SFAs aumentaram a expressão das quimiocinas IL-8 e CXCL10. E também observamos um aumento na expressão destas quimiocinas na inflamação do tecido adiposo no CC, que era mais profundo no tecido adiposo visceral (VAT) quando comparado ao tecido adiposo subcutâneo (SAT). A inflamação sistêmica foi negativamente associada com a expressão de enzimas sintetizadoras dos PUFAs, embora a expressão gênica e protéica mostraram somente pequenas diferencias entre os grupos. Os efeitos dos fatores inflamatórios sobre as enzimas no tecido adiposo podem ter sido mascarados pela modulação diferenciada dos diversos tipos celulares constituintes desse tecido. Experimentos *in vitro* mostraram que a expressão de enzimas que modificam os AGs, tais como as dessaturases e elongases em adipócitos e macrófagos, foram reguladas em direções opostas por TNF-α, IL-6, LPS e palmitato. Mesmo os pacientes CC demonstrando uma maior concentração plasmática da Resolvina D1, que é um mediador lipídico de resolução da inflamação, ainda assim, a inflamação sistêmica é maior nesses pacientes, e os resultados indicam que as citoquinas inflamatórias interferem com as vias de síntese das LMs da resolução. Concluímos que, os dados revelaram um *crosstalk* inter-tecidual e intercelular complexo mediado por citocinas pró-inflamatórias e compostos lipídicos

que aumentam a inflamação na caquexia associada ao câncer por mecanismos autoregulação.

Palavras chaves: caquexia associada ao câncer, inflamação sistêmica, tecido adiposo, citocinas, quimiocinas, ácidos graxos saturados, ácidos graxos poliinsaturados.

ZUSAMMENFASSUNG

RADLOFF, K. **Die Rolle des Fettsäure-Profils und dessen entzündungsbedingten Veränderungen in der Tumorkachexie.** 2018. 159 f. Doktorarbeit (Doctor rerum naturalium, Ph.D.-Programm *Cell and Tissue Biology*) – Institut für Biomedizin, Universität São Paulo, São Paulo und Unversität Potsdam, Potsdam, 2018.

Systemische Entzündung ist ein grundlegendes Merkmal der Tumorkachexie. Bei den entzündungstreibenden Wechselwirkungen zwischen Tumor und Wirt spielt das weiße Fettgewebe eine besondere Rolle, da es, bedingt durch morphologische Veränderungen und Lipolyse, freie Fettsäuren, bioaktive Lipidmediatoren (LM) und pro-inflammatorische Cytokine freisetzt. Diese verschiedenen Substanzen verstärken die Aktivierung entzündungsfördernder Signalwege und eine Rekrutierung von Immunzellen in das Gewebe. Das Ziel dieser Arbeit war es daher zu untersuchen, welche Faktoren an der Entwicklung der lokalen Fettgewebsentzündung beteiligt sind und wie diese Faktoren Syntheseenzyme von Fettsäuren und Lipidmediatoren beeinflussen könnten. Dazu wurden Plasma und Fettgewebeproben von gesunden Kontrollpersonen (Control) und normalgewichtigen (WSC) sowie kachektischen Magen-Darm-Krebs-Patienten (CC) untersucht. Die Ergebnisse zeigten, dass sich die inflammatorischen Charakteristiken der systemischen Entzündung von denen der lokalen Fettgewebsentzündung unterscheiden. Die systemische Entzündung war gekennzeichnet durch höhere Spiegel gesättigter Fettsäuren (SFA), Tumor-necrosis-factor alpha (TNF-α), Interleukin IL-6, IL-8 und C-reactive protein (CRP) während die Konzentrationen von mehrfachungesättigten Fettsäuren (PUFA) –besonders n3-Fettsäuren- geringer in CC waren als in den anderen Gruppen. *In vitro* und in *ex vivo* kultivierten Fettgewebssegmenten konnte gezeigt werden, dass die Inkubation mit pro-inflammatorischen Cytokinen und gesättigten Fettsäuren zu einem Anstieg der Chemokine IL-8 sowie CXCL10 führte. Erhöhte Spiegel dieser Moleküle wurden auch in der Fettgewebsentzündung bei kachektischen Patienten beobachtet, welche im viszeralen Fettgewebe ausgeprägter war als im subkutanen. Systemische Entzündungsmarker waren negativ mit der Expression PUFA-synthetisierender Enzyme assoziiert, obwohl sich Gesamt-mRNA-sowie Proteingehalt kaum zwischen den Studiengruppen unterschieden. Die Effekte von Entzündungsfaktoren auf diese Enzyme im Gesamtgewebe könnten durch eine differenzielle Modulierung in diversen Zelltypen des Gewebes maskiert sein. Denn in *in vitro*-Experimenten zeigte die Inkubation mit TNF-α, IL-6, LPS oder Palmitat, dass die GeneExpression von Fettsäure-modifizierenden Enzymen wie Desaturasen oder Elongasen in Adipozyten und Makrophagen in entgegengesetzte Richtungen reguliert wird. Die höhere Plasmakonzentration des entzündungslösenden LM Resolvin D1 in CC konnte dem inflammatorischen Zustand nicht entgegenwirken und die Ergebnisse deuten darauf hin, dass inflammatorische Cytokine in die Synthesewege von entzündungslösenden LM eingreifen. Zusammenfassend demonstrieren die Daten das komplexe Zusammenspiel zwischen verschiedenen Geweben und Zelltypen, in dem Cytokine und Lipidverbindungen aus dem Blutkreislauf die Entzündung der Tumorkachexie durch selbst-verstärkende Mechanismen vorantreiben.

Schlüsselwörter: Tumorkachexie, Entzündung, Fettgewebe, Cytokine, Chemokine, gesättigte Fettsäuren, mehrfachungesättigte Fettsäuren

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ABBREVIATIONS

13-HODE	13-Hydroxyoctadecadienoic acid
12S-HETE	12-(S)-Hydroxyeicosatetraenoic acid
15S-HETE	15-(S)-Hydroxyeicosatetraenoic acid
17S-HDHA	17(S)-hydroxy Docosahexaenoic Acid
18S	18S ribosomal RNA
AIDS	Acquired immune deficiency syndrome
ATGL	Adipose triglyceride lipase
BCAA	Branched Chain Amino Acids
BMI	Body mass index
BRAF	Serine/threonine-protein kinase B-Raf
CCL2	Chemokine (C-C motif) ligand 2
CI	Confidence interval
CXCR1 and CXCR2	C-X-C motif chemokine receptor 1 and 2
DEXA	Dual-energy X-ray absorptiometry
EDTA	Ethylenediaminetetraacetic acid
EORTC	European Organisation for Research and Treatment of Cancer
GPR120	G-protein coupled receptor 120
HMB	beta-hydroxy-beta-methylbutyrate
HR	Hazard ratio
HSL	Hormone sensitive lipase
IFN-γ	Interferon gamma
IL-10	Interleukin 10
IL-13	Interleukin 13
IL-1Ra	Interleukin 1 receptor antagonist
IL-4	Interleukin 4
INCA	Instituto Nacional de Câncer
JAK	Janus kinase
KRAS	Kirsten RAt Sarcoma virus associated oncogene
LPS	Lipopolysaccharide

MAPK	Mitogen-activated protein kinase
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
PBS	Phosphate-buffered saline
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α)
PI3K	Phosphoinositide 3-kinase
PTHRP	Parathyroid hormone-related protein
PVDF	Polyvinylidene fluoride or polyvinylidene difluoride
QoLQ	Quality of Life Questionnaire
qPCR	Quantitative Polymerase chain reaction
RPL27	60S ribosomal protein L27
SDS	Sodium dodecyl sulfate
STAT3	Signal transducer and activator of transcription 3
TBS-T	Tris-buffered saline with Polysorbate 20 (Tween 20)
TNM	Classification of Malignant Tumors (tumor size and invasion, involvement of lymph nodes, metastasis)
TP53	Tumor protein p53
Tris base	Tris(hydroxymethyl)aminomethane
USA	United States of America
WHO	World health organization

CONTENT

1 INTRODUCTION

1.1 CANCER CACHEXIA - STATISTICS AND DIAGNOSIS

Cancer is according to the WHO the second leading cause of death. In 2015 8,8 Million people worldwide died from cancer (GLOBOCAN, 2018). In Europe, USA and Japan cancer affects about 0.5% of the population and among all types of cancer 30% of patients will manifest cachexia during the progression of the disease (von Haehling et al., 2016). Cancer patients with cachexia are particularly at risk of premature death as they present the highest mortality rate with up to 80% compared to non-cachexia cancer patients (von Haehling et al., 2016, Del Fabbro et al., 2012).

Cancer cachexia is defined as ‘a multifactorial syndrome characterized by ongoing loss of skeletal muscle mass and fat mass that cannot be fully reversed by conventional nutritional support and leads to progressive functional impairment’ (von Haehling and Anker, 2010, Evans et al., 2008, Fearon et al., 2011). This definition was established in December 2006 by leading scientists and clinicians of the area (Springer et al., 2006). The pathophysiology of cachexia is characterized by systemic inflammation indicated by CRP plasma concentrations above >5mg/l, anorexia, fatigue, weakness with low physical performance and metabolic imbalances (Evans et al., 2008, Fearon et al., 2011). In 2008 Evans et al. defined parameters for the diagnosis of cachexia. The group suggested that a patient is cachectic when unintentional weight loss is accompanied with 3 or more of 5 parameters, namely decreased muscle strength, fatigue, anorexia, low fat-free mass index and abnormal biochemistry (inflammation, anemia, low serum albumin). The benchmark of weight loss of a cachectic patient was established as at least a reduction of 5% bodyweight over the previous 12 months or BMI <20 kg/m^2 (figure 1) (Evans et al., 2008).

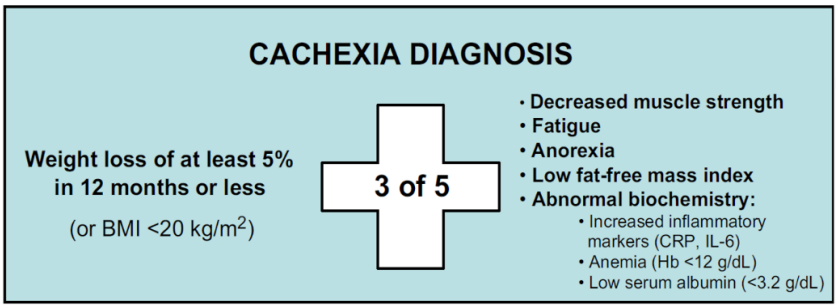


Figure 1: Diagnosis of cachexia by Evans 2008

For cancer cachexia, Fearon and Strasser et al. advanced the classification of cachexia by identifying three stages to determine the severity of the syndrome. The first stage is termed pre-cachexia typically characterized by factors observed in anorexia, a weight loss of ≤5% over the past 6 months with the additional presence of early cachexia-derived metabolic changes. The second stage is termed cachexia and presents a weight loss of more than 5% in 12 months or a BMI <20 or sarcopenia accompanied with weight loss and systemic inflammation, whereas the final stage, 'refractory cachexia' is characterized by irreversible catabolism leading to a low performance status and low expectation of long-term survival (Fearon et al., 2011), (Figure 2). In addition to these criteria, it was suggested to evaluate physical functioning and psychosocial effects, for example with the European Organization for Research and Treatment of Cancer [EORTC] Quality of Life Questionnaire [QoLQ]-C30 or patient-completed Eastern Cooperative Oncology Group questionnaire) (Fearon et al., 2011).

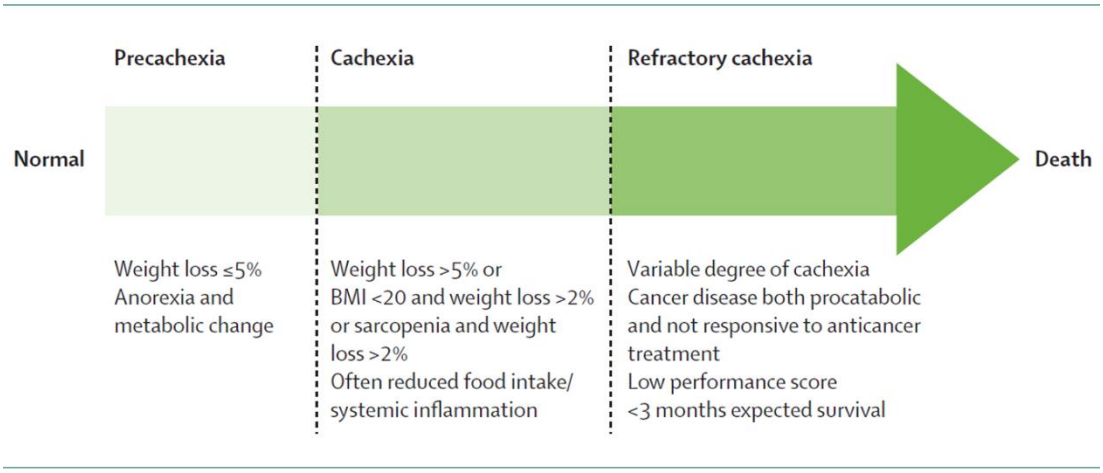


Figure 2: Stages of cancer cachexia by Fearon 2011

The stage approach based only on weight loss and BMI may not be sufficient to predict survival according to Blum et al. who recommend measuring of muscle loss with objective methods such as dual energy X-ray absorptiometry (DEXA), magnetic resonance imaging or computed tomography (Blum et al., 2014). However, in a Canadian and European sample of 8160 cancer patients, Baracos et al. (Martin et al., 2015) developed a BMI-adjusted weight loss (%) grading system instead of using only weight loss or BMI for staging. Five weight loss categories and 5 BMI categories are combined in a 5 x 5 matrix and with survival and hazard ratio (HR) information for each of the 25 possibilities, the matrix provides 5 scores (0-4) predicting survival (0=longer survival, 4=short survival) (figure 3) (Martin et al., 2015). Although grading systems taking into account more parameters might be more precise in predicting survival rates, the current study employed the guidelines of Fearon et. al. because it combines high accuracy with very good feasibility

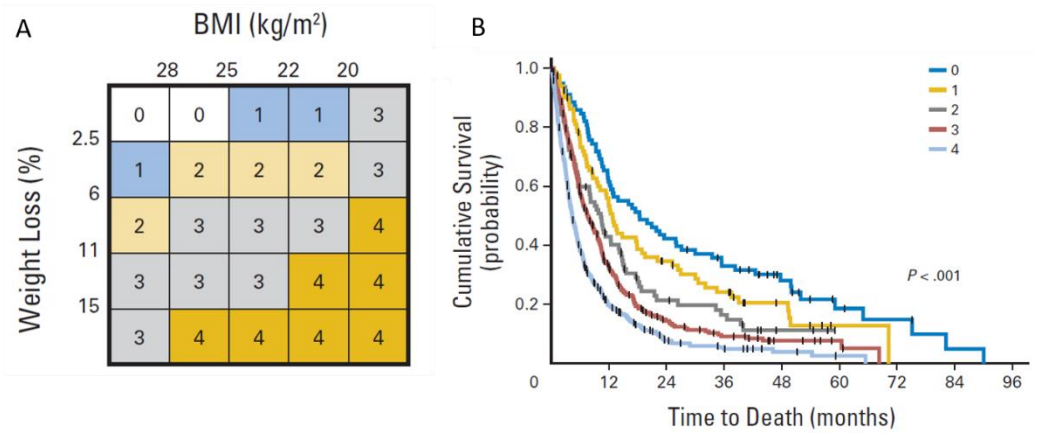


Figure 3: BMI-adjusted WL grading system (grades 0 to 4) applied on cumulative survival time. A: Median survival times by grade were as follows: grade 0, 20.9 months (95% CI, 17.9 to 23.9 months; unadjusted HR, 1.0); grade 1, 14.6 months (95% CI, 12.9 to 16.2 months; HR, 1.3); grade 2, 10.8 months; 95% CI, 9.7 to 11.9; HR, 1.5); grade 3, 7.6 months (95% CI, 7.0 to 8.2 months; HR, 2.0), and grade 4, 4.3 months; 95% CI, 4.1 to 4.6 months; HR, 3.1; P .001). B: Cumulative survival curves from the subgroup analysis of the training sample for (A) gastroesophageal cancer.

Although stages of severity indices may help to predict patient's survival, prevention and treatment of cachexia is still a challenge for the health care system. Cancer cachexia should be treated with a multimodal strategy (Aversa et al., 2017). Three strategies have been established with some evidence for efficacy: nutrition, physical exercise and pharmacological approaches. Nutritional monitoring and intervention should be started from the moment of cancer diagnosis (Muscaritoli et al., 2011).

The recommendations are an appropriate intake of a high quality protein source rich in essential amino acids. The ingestion of branched chain amino acids BCAA showed beneficial effects in cancer cachexia but more studies are needed to confirm these effects (reviewed in (Aversa et al., 2017). In this context the role of amino acid derivatives such as beta-hydroxy-beta-methylbutyrate (HMB) that inhibits protein degradation or L-carnitine is worth to be investigated more in attenuating muscle wasting (Molfino et al., 2013, Madeddu et al., 2012).

In addition to nutritional support, physical exercise has been shown to maintain muscle mass or reduce the typical pattern of muscle wasting by modulating inflammation, insulin sensitivity or cellular homeostasis and promoting myogenesis and enhancing the degradation of damaged mitochondria (Aversa et al., 2017). Endurance training stimulates oxidative metabolic adaptation while resistance training activates anabolic mechanisms in muscle (Camera et al., 2016). However, exercise capacity has been shown to vary significantly between patients due to differences in the severity of the syndrome and comorbidities such as chronic fatigue, anemia or cardiac dysfunction (Argilés et al., 2012).

Therefore, there are no universal recommendations for exercise modality and intensity (Argilés et al., 2012). Pharmacological treatments target appetite, inflammation and the balance of anabolic and catabolic pathways in muscle (Aversa et al., 2017). Some agents have already reached phase II and III in clinical trials and showed the potential to actually increase body lean mass (Kaasa et al., 2015, Cohen et al., 2015, Temel et al., 2016). Nevertheless, more studies are needed to better understand the therapeutic potential of each pharmaceutic to optimize its effectiveness for the individual patient.

1.2 GASTRO-INTESTINAL CANCER

The incidence of cancer cachexia varies between different tumor sites. In colorectal cancer (CRC) about 28% present cachexia and half of these patients show a weight loss of more than 10%. However, gastric-cancer cancer, the cachexia incidence is higher with up to 67% and among those, 30-39% present a severe weight loss of more than 10% (Tan and Fearon, 2008). With 1.4 million new cases in 2012, CRC is the third common cancer worldwide accounting for 700,000 deaths in the same year and the incidence may increase by 60% by 2030 (Ferlay et al., 2015). Gastric cancer is the fourth leading cause of cancer-related deaths after CRC worldwide and although its incidence has decreased, a 5-year survival rate of 30% still very low relative to other cancer sites (Bosetti et al., 2013, Park et al., 2014).

There are several risk factors involved in the etiology of both CRC and gastric cancer. The main risk factor of gastric cancer is H. pylori infection. This gram-negative bacterium is found in about 50% of the human population, it acts by epigenetic effects on gastric epithelial cells and it indirectly drives inflammatory responses (Cheng et al., 2016). In CRC, this microbiome has also been shown to play a role in a mice model where a combination of a high fat diet with a rise in firmicutes and ruminococcaceae was assumed to induce a gene expression profile similar to that observed in human CRC (Qin et al., 2018). In another study a pro-inflammatory diet (rich in red/processed meat and fast food) was associated with the presence of colon carcinomas containing Fusobacterium nucleatum (Liu et al., 2018). Genetic factors indicated in the progression of CRC are mutations in PIK3CA, BRAF, KRAS and TP53 genes whereas in gastric cancer genetic polymorphisms in IL1RN and IL10 are associated with tumor incidence (Yamauchi et al., 2012, Russo et al., 2005, Persson et al., 2011).

Many lifestyle factors have been linked to cancer prevalence though a few key types are related to CRC and gastric cancer. Obesity accounts for 15-30% of the cancer deaths in the US (Hursting and Dunlap, 2012) and a European study revealed that higher BMI, waist circumference and waist-to-hip-ratio were related to a greater risk of CRC (Murphy et al., 2018). The risk of gastric cancer also rises in obese individuals (Yang et al., 2009). Various obesity-related mechanisms include; increased cell survival and

proliferation due to chronic inflammation, insulin and leptin resistance, ER-stress and insulin-like growth factors (Yang et al., 2009, Tarasiuk et al., 2018). Heavy alcohol consumption and smoking are related to an increased risk for both CRC and gastric cancer while levels of physical activity are inversely related to gastrointestinal cancer incidence (Murphy et al., 2018, Cheng et al., 2016, Sjödahl et al., 2008).

1.3 SYSTEMIC INFLAMMATION IN DEVELOPMENT AND MANIFESTATION OF CANCER CACHEXIA – CROSSTALK BETWEEN TUMOR AND HOST

A striking feature of tumor induced cachexia is the presence of systemic inflammation that impacts the control of energy balance, modulation of food intake and metabolism of the muscle and adipose tissue (figure 4) (Tsoli and Robertson, 2013, Schcolnik-Cabrera et al., 2017). In the scenario of chronic inflammation, elevated levels of pro-inflammatory cytokines perpetuate and enhance the inflammatory response and favor the shift from a local to a systemic process (Seelaender et al., 2012). Cytokines mediate the immune responses by paracrine intercellular actions. Within the tumor microenvironment, cancer cells and interacting host immune cells, such as macrophages, T-lymphocytes or natural killer cells or non-immune cells e.g. adipocytes and hepatocytes, produce a battery of pro-inflammatory cytokines, such as tumor necrosis factor α (TNF-α), Interleukin 1 (IL-1), and Interleukin 6 (IL-6) leading to acute phase immune response (Schcolnik-Cabrera et al., 2017, Onesti and Guttridge, 2014).

Acute phase proteins synthesized in the liver, such as C-reactive protein (CRP), contribute to systemic inflammation and the loss of protein stores as their synthesis requires large amounts of essential amino acids (for 1g of fibrinogen 2.6g muscle protein must be catabolized) (Reeds et al., 1994). Consequently CRP concentration correlates positively with parameters of the clinical outcome of cachexia such as hypoalbuminemia, other inflammation related parameters (cytokine-, adipokine plasma levels), weight loss and metabolic changes (Reeds et al., 1994). Thus, an inflammation-based prognostic score for patients, the “Glasgow Prognostic Score”, was proposed (McMillan, 2008). TNF-α and IL-1 activate the transcription factor nuclear factor kappa beta (NF-κβ) leading to

muscle protein degradation due to a higher activity of ubiquitinin ligases (Argilés et al., 2011, Guttridge et al., 2000, Miyamoto et al., 2016). Furthermore, TNF-α as well as interferon-gamma (IFN-γ) repress the myosin heavy chain IIb mRNA in skeletal muscle (Acharyya et al., 2004). IL-6 contributes to muscle wasting by the disruption of mitochondrial biogenesis through the activation of the JAK/STAT3 pathway (Onesti and Guttridge, 2014). TNF-α and IL-6 promote anorexia by the induction of corticotrophin releasing factor, which decreases neuropeptide Y (NPY) resulting in early satiety and lower food intake (Bing and Trayhurn, 2008, Morley et al., 2006).

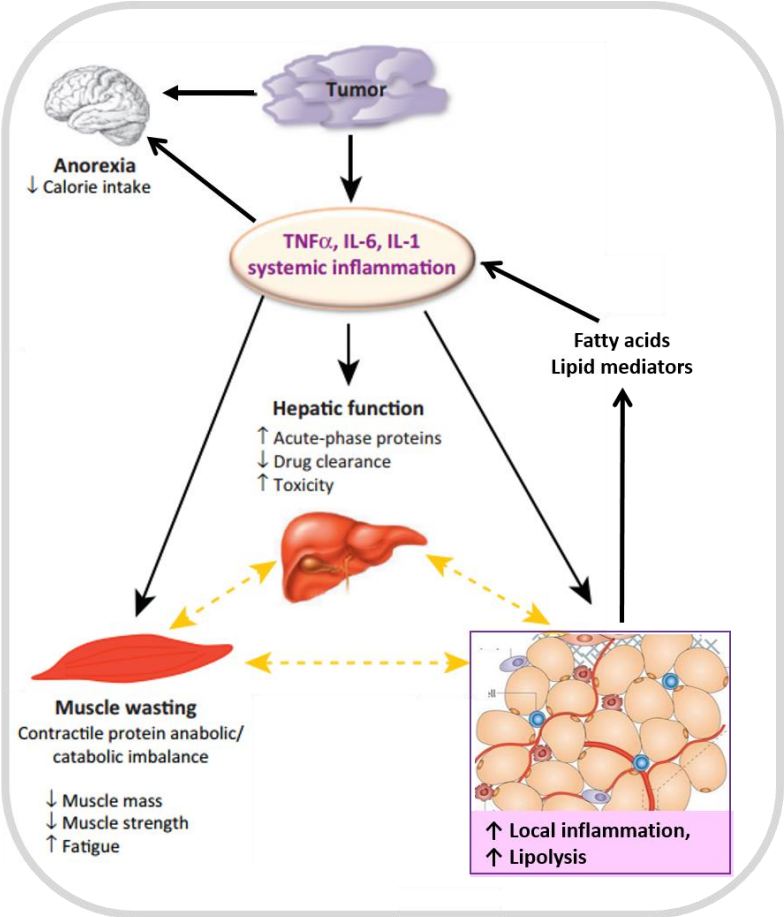


Figure 4: After Tsoli et al. 2013 Interplay between tumor, systemic inflammation and host organs.

In the adipose tissue cytokines lead to fat depletion by the inhibition of adipocyte differentiation, promoting of lipolysis and the induction of browning (figure 4) (Schcolnik-Cabrera et al., 2017). The protein degradation in skeletal muscle and triglyceride breakdown in the adipose tissue leads to the release of glutamine and alanine as well as glycerol and non-esterified fatty acids (NEFA). Alanin and glycerol are used in the liver for gluconeogenesis. The glucose is by the tumor for its energy homeostasis, in turn the tumor releases lactate deriving mostly from anaerobic glycolysis (Warburg effect), which is again used for gluconeogenesis in the liver (figure 4) (Argilés et al., 2014, Liberti and Locasale, 2016).

1.4 THE ROLE OF THE ADIPOSE TISSUE IN CANCER CACHEXIA

The white adipose tissue (WAT) is a highly metabolically active organ responsible for the storage and release of energy in the form of lipids. In a healthy adult these lipids consists to 90% of triacylglycerides that are stored in lipid droplets (Bing and Trayhurn, 2008, Sethi and Vidal-Puig, 2007). The balance of storing and dispersing of the fuel reserves is critical to maintain a healthy energy homeostasis (Rohm et al., 2013, Frayn et al., 2003) and the negative energy balance in cancer cachexia contributes to the loss of WAT (Tsoli and Robertson, 2013).WAT is also a major endocrine organ that secretes more than 100 adipokines and pro- and anti-inflammatory cytokines (Neves et al., 2016, Batista et al., 2013, Amor et al., 2016). That makes WAT not only a target organ, which is affected by the syndrome; it also contributes to the progression of cachexia.

1.4.1 ADIPOSE TISSUE INFLAMMATION

Adipose tissue inflammation plays a role in metabolic extremes. In obesity the development of a low grade inflammation leads to comorbidities such as insulin resistance and a higher tumor risk (Tarasiuk et al., 2018, Restifo et al., 2018). While during cachexia, the local adipose tissue inflammation contributes to the progression of the systemic inflammation and muscle wasting (Schcolnik-Cabrera et al., 2017, Ebadi and Mazurak, 2014). The local adipose tissue inflammation generates a positive feedback loop where activated resident immune cells and adipocytes secrete chemotactic cytokines that attract circulating immune cells to the adipose tissue, which become more activated and mediate further inflammatory responses (Frasca and Blomberg, 2017). In healthy adipose tissue several cell types maintain the physiological function, in this scenario resident macrophages act as immunologic surveillance (Garrow., 1989, Weisberg et al., 2003). The percentage of macrophages under physiological conditions in WAT ranges is about 10% while during metabolic extremes immune cell infiltration increases substantially (Weisberg et al., 2003).

There are two main types of adipose tissue macrophages (ATM) M1 and M2 macrophages. M1 are activated by IFN-γ and they secrete the pro-inflammatory cytokines TNF-α, IL-1, IL-6 and CCL2, whereas M2 are activated by T-cell derived IL-4 and IL-13 (Lumeng et al., 2008, Gordon, 2003). After activation, M2 act in tissue repair as they produce immune suppressive factors such as IL-10 or IL-1Ra (Gordon and Taylor, 2005). In adipose tissue of obese and cachectic patients a shift from M2 to M1 macrophages was described (Lumeng et al., 2007).

Other resident cell types in the adipose tissue are invariant natural killer T-cells or regulatory T-cells that play a role in preventing unfavorable events such as the infiltration of circulating T-effector cells (Lynch et al., 2012, Vignali et al., 2008). T-effector cells drive adipose tissue inflammation by releasing IFN-γ which activates M1 macrophages (Nishimura et al., 2009). In high fat diet-fed mice, it was found that B-cells are infiltrating the adipose tissue before the T effector cells (Duffaut et al., 2009). The B-cells, once back in the circulation present anti-genes to T-cells and therefore promote their infiltration to the adipose tissue (Frasca and Blomberg, 2017, Frasca et al., 2017).

The adipocyte itself is also capable to secrete pro-inflammatory and chemotactic stimuli such as TNF-α, IL-6, CCL2, Interleukin 8 (IL-8) and C-X-C motif chemokine 10 (CXCL10) that again increase immune cell infiltration (Sewter et al., 1999, Fain et al., 2005, Herder et al., 2007). For obesity the effects of immune cell infiltration and low grade inflammation is well studied (Cancello and Clément, 2006, Catalán et al., 2013, Cozzo et al., 2017). In cancer cachexia pro-inflammatory cytokines such as TNF-α, IL-6 and Interleukin 1 beta (IL-1β) play a crucial role in fat depletion and local adipose tissue inflammation (Onesti and Guttridge, 2014). Enhanced IL-6/STAT3 signaling was associated with adipose tissue wasting in cachectic mice bearing C26 colon tumor cells (Ebadi and Mazurak, 2014). In cachectic gastro intestinal cancer patients the gene expression of the NF-κB targets TNF-α, IL-1β, CCL2 was enhanced compared to non-cachectic patients, pointing to NF-κB as important regulator during the development of cachexia (Camargo et al., 2015). In cachectic Walker 256 tumor bearing rats, it was found that also the inflammasome pathway contributes to adipose tissue inflammation by an increase of IL-1β white adipose tissue adipocytes (Neves et al., 2016).

WAT is able to secrete more than 100 pro- and anti-inflammatory cytokines and adipokines (Batista et al., 2013, Batista et al., 2012). Therefore, WAT is largely contributing for the progression of cachexia as already shown by previous animal and human studies (Argilés et al., 2014, Machado et al., 2004, Kazantzis and Seelaender, 2005). However, less is known about the dynamics of the adipose tissue immune cell infiltration in cachexia.

1.4.2 ADIPOSE TISSUE DEPLETION IN CANCER CACHEXIA

DEXA, bio impedance and computed tomography scan analysis in different studies with cachectic cancer patients demonstrated that the loss of fat mass does not appear necessarily together with a concurrent loss of lean mass (Ebadi and Mazurak, 2014). Fat depletion in cancer cachexia gains importance as it is associated with shorter survival times (Murphy et al., 2010, Fouladiun et al., 2005). In animal and human studies it was shown that the loss of fat mass was accompanied with reduced adipocyte size indicating a decreased lipid storage capacity (Agustsson et al., 2007, Rydén et al., 2008, Dahlman et al., 2010, Mracek et al., 2011). The loss of adipose tissue is not only the result of a lower food intake due to more anorexia among cachectic cancer patients but the result of the transition from fat storing to futile cycling and browning due to increased lipolysis and browning induced thermogenesis as well as and lower lipid uptake, liponeogenesis or adipogenesis (figure 5) (Ebadi and Mazurak, 2014, Tsoli et al., 2016).

Cytokines released by infiltrating T-helper 1-cells (Th1) increase catecholamine levels that lead to a higher expression lipolytic enzymes such as HSL and ATGL (Schcolnik-Cabrera et al., 2017). In several cancers such as CRC, pancreatic, ovarian, stomach and esophageal cancer, HSL mRNA expression was associated with higher serum free fatty acids (FFA) (Thompson et al., 1993, Balaban et al., 2015). TNF-α is facilitating the access to the lipid droplets for lipases by suppressing the expression of perilipins (Cawthorn and Sethi, 2008). Circulating lipolytic factors namely Zink-α2-glycoprotein (ZAG) and tumor derived lipid mobilization factor (LMF) have a similar amino

acid sequence and are elevated in cancer cachexia (Kong et al., 2010, Sanders and Tisdale, 2004). Fatty acid oxidation inhibits the re-esterification of FFA (Zuijdgeest-van Leeuwen et al., 2000). Reasons for the higher oxidation rate are the upregulation of genes involved in browning such as uncoupling proteins (UCPs) and PGC-1α – a key activator of mitochondrial biogenesis (Petruzzelli et al., 2014, Kir et al., 2014). In cancer cachexia, these browning genes and also tumor derived PTHRP and IL-6 are associated with the browning tradition of adipocytes which then do thermogenesis (Petruzzelli et al., 2014, Kir et al., 2014).

Lipoproteinlipase (LPL) enables FA uptake into the cell by hydrolysis of very low density lipoprotein (VLDL) and chylomicrons (Thompson et al., 2010). In animal cancer and cancer cachexia models LPL activity was reduced and in humans lower LPL mRNA levels were observed in adipose tissue proximal to the tumor (López-Soriano et al., 1996, Lanza-Jacoby et al., 1984, López-Soriano et al., 1997, Lopez-Soriano et al., 1995). Adipocyte differentiation is impaired by TNF-α that suppresses the expression of adipogenic transcription factors such as CCAAT/enhancer binding protein α (C/EBPα) and peroxisome proliferator-activated receptor gamma (PPAR-γ) (Cawthorn et al., 2007).

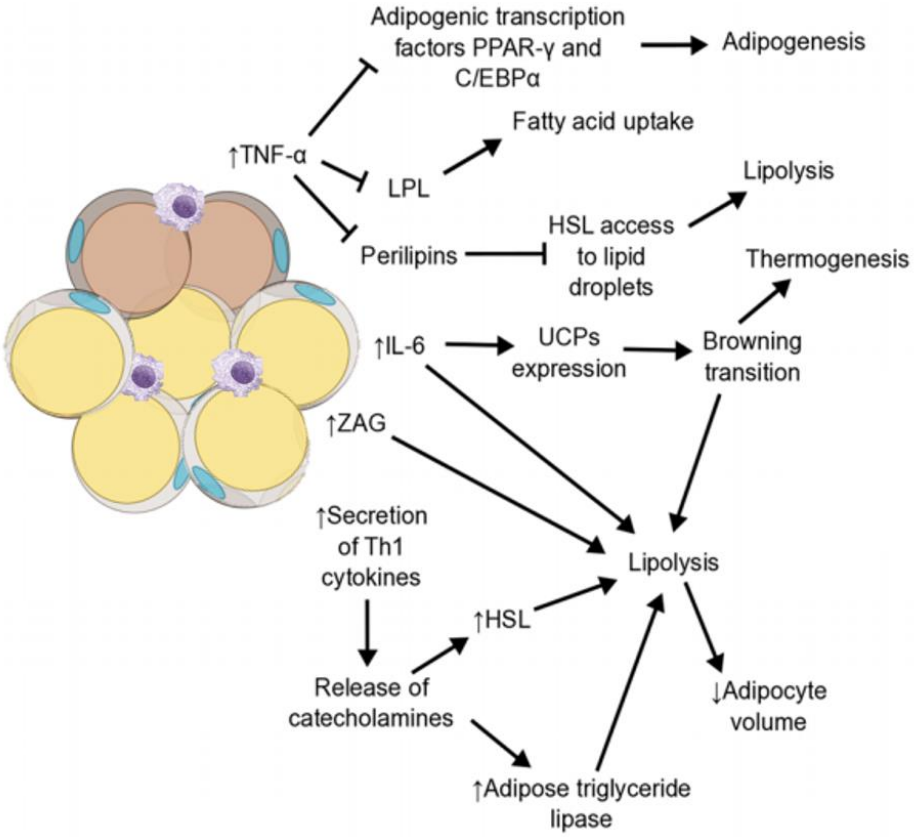


Figure 5: Mechanisms of adipose tissue depletion after Alejandro et al. Adipose tissue wasting is the effect of enhanced lipolysis and browning transition but also lower adipogenenis and lipid uptake via LPL often promoted by pro-inflammatory cytokines. Abbreviations: TNF-α: tumor necrosis factor α; PPAR-γ: peroxisome proliferator-activated receptor-gamma; C/EBP: CCAAT/enhancer-binding protein; LPL: lipoprotein lipase; HSL: hormone-sensitive lipase; IL-6: interleukin 6; UCPs: uncoupling proteins; ZAG: zinc-α2-glycoprotein.

1.5 FATTY ACIDS AND LIPID MEDIATORS

Fatty acids and lipid mediators belong to a group of many different bioactive compounds which fulfill a large spectrum of specific immunomodulatory functions. Therefore understanding the mechanisms of FA and LM and their formation is of great interest in the context of cancer and cancer cachexia.

1.5.1 METABOLISM OF FATTY ACIDS AND LIPID MEDIATORS

Under physiological conditions, liponeogenesis occurs in adipose tissue, liver, kidney and lactating breast tissue catalyzed by the fatty acid synthase (FASN) (Peck and Schulze, 2016). In neoplastic tissue, FASN expression was shown to be augmented (Ogino et al., 2007). A product of the de novo synthesis of lipids is palmitic acid (C16:0) which accounts for 21-23% of WAT fatty acids (Garaulet et al., 2001). It is the second most frequent FA in WAT and in plasma (Abdelmagid et al., 2015).

The synthesis of the different FA species, their elongation and desaturation is highly regulated as each step is catalyzed by a specialized FA modifying enzyme (Murphy et al., 2010, Zhang et al., 2016). Palmitic acid can be elongated to stearic acid (C18:0) by the action of elongase 6. Then palmitic and stearic acid can be desaturated by stearoyl coenzyme A desaturase (SCD) which is essential for the desaturation of these saturated fatty acids (SFAs) to monounsaturated fatty acids (MUFAs) palmitoleate (C16:1) and oleate (C18:1) (Jump, 2009). Oleate is the most frequent FA in WAT (44-48%) and the third most in plasma, while palmitoleate contents are much lower (Garaulet et al., 2001, Abdelmagid et al., 2015).

The generation of long chain n3 and n6 polyunsaturated fatty acids (PUFAs), is catalyzed by elongases namely elongase 2 or 5 (ELOVL2, ELOVL5) and fatty acid desaturases 1 or 2 (FADS1, FADS2) (Abdelmagid et al., 2015, Flowers and Ntambi, 2008, Tosi et al., 2014). Long chain PUFAs derive from the dietary FA linoleic acid (C18:2n6) and α-linolenic acid (C18:3n3) (Howard et al., 2014, Leonard et al., 2004). C18:2n6 is the main plasma fatty acid and in WAT it accounts for 15% of all FA (figure 6).

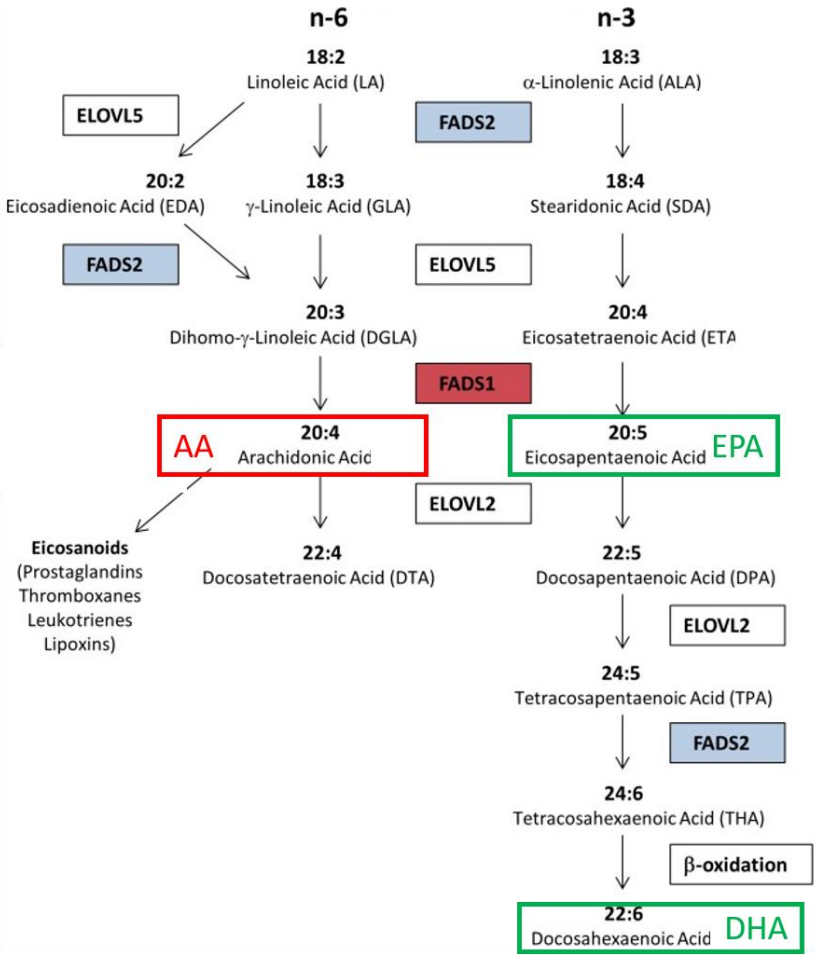


Figure 6: Elongation and desaturation of long chain poly unsaturated fatty acids after Howard et al. 2014.

Long polyunsaturated fatty acids such as n-3 FA eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are precursors for the generation of inflammation resolving lipid mediators (LM), namely resolvins, protectins and maresins, first described by Serhan and colleagues (figure 7) (Serhan and Chiang, 2013). In contrast, n-6 PUFAs, especially arachidonic acid (AA), are mainly precursors of pro-inflammatory bioactive lipid compounds including prostaglandins, leukotrienes, prostacyclins or thromboxanes (Needleman et al., 1986), though AA can be converted to anti-inflammatory lipoxins (Serhan and Chiang, 2002). The precursors for LM are not only deriving from dietary FA but also from membrane lipids shed by phospholipase A2.

AA, EPA and DHA can be oxygenized by cyclooxygenases and lipoxigenases. AA can be converted by the constitutive and inducible cyclooxygenase (COX-1 and COX-

2) to prostaglandins. When COX-2 is acetylated by aspirin the enzyme catalyzes the generation of resolvin E1 and E2 from EPA. Arachidonic acid lipoxigenase 15 (ALOX15) is necessary for the synthesis of 15S-HETE and 17S-HDHA. 15S-HETE is converted by ALOX5 to lipoxins while 17S-HDHA is a precursor of resolvins and protectins, all involved in inflammation resolution. In combination with its activation protein ALOX5AP (FLAP), ALOX5 also catalyzes the synthesis of pro-inflammatory leukotriens from arachidonic acid (O'Connor et al., 2014, Titos and Clària, 2013).

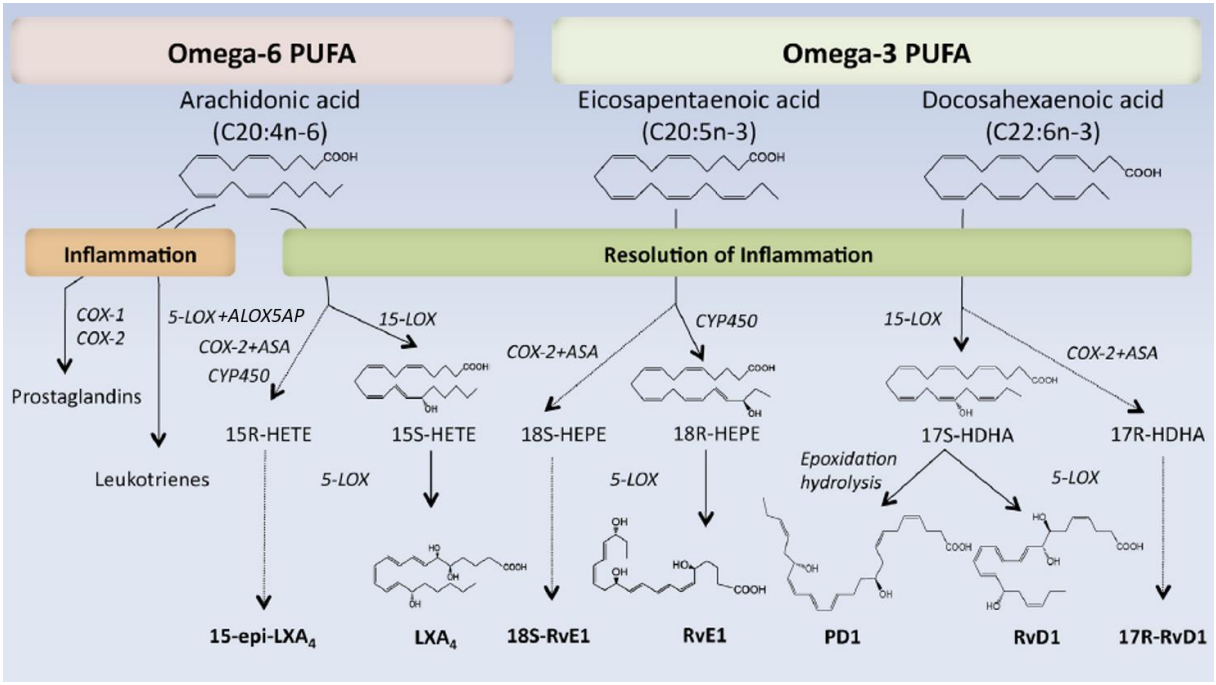


Figure 7: Lipid mediator formation after Titos et al. 2013.

1.5.2 INFLAMMATORY PROPERTY OF FATTY ACIDS AND LIPID MEDIATORS

Fatty acids and LM modulate immune responses via binding to specific receptors. Palmitic acid (C16:0) activates toll-like-receptor 2 (TLR2) that leads to NALP3 upregulation and inflammation mediated increase in IL-1β expression in human monocytes (Snodgrass et al., 2013). Other SFAs such as stearic acid (C18:0) act as ligands for the Toll-like-receptor 4 (TLR4) activating the NFκB pathway and expression of inflammatory factors such as IL-1β, IL-6, TNF-α or IFN-γ in murine macrophages (Rocha et al., 2016, Wang et al., 2016).

N6 PUFAs, especially AA are precursors of pro-inflammatory bioactive lipid compounds including prostaglandins, leukotrienes, prostacyclins or thromboxanes (Lu et al., 2008a). Prostaglandin E (PGE_2) and leucotriens promote immune cell infiltration and PGE_2 is also stimulating T-effector cell differentiation and tumor cell growth (Kawamori et al., 2006, Kawamori et al., 2009). In contrast, PUFAs can inhibit inflammatory pathways. C18:2n6 was shown to impair the TLR4 activation in obesity (Kremmyda et al., 2011). In terms of anti-inflammatory properties, n3 PUFAs are most intensively studied. The n3 PUFAs can inhibit TLR4 action by the association with GPR120, the internalization of the complex by the cell suppresses players of the TLR4 pathway (Oh et al., 2010).

Lipoxins reduce immune cell migration and infiltration into tissues and also inhibit cytokine production of T-cells and dendritic cells. Resolvins improve clinical signs of inflammation (Makriyannis and Nikas, 2011). E-resolvins act via the chemokine like receptor 1 (CMKLR1), these LM suppress platelet aggregation as well as the platelet-leucocyte adhesion (Serhan, 2009) and immune cell migration, while resolvins of the D-series inhibit TNF-α secretion by blocking the NFκB-pathway.

1.5.3 THE IMPACT OF FATTY ACIDS AND LIPID MEDIATORS IN CANCER

Cancer cachexia accompanied by quantitative changes in plasma TAGs and FFAs, but also associated with qualitative alterations of the fatty acid profile. Murphy et al. evaluated the FA profile in gastrointestinal cancer patients and found a correlation between the time to death of the patient and the plasma content of omega(n)-3 FA such as ALA C18:3n-3, EPA C20:5n-3 or DHA C22:6n-3 and n-6 FA e.g. LA C18:2n-6 or AA C20:4n-6 (Murphy et al., 2010). These changes in plasma FA profile during cachexia may be related to the release or retention of these fatty acids by various tissues. C18:2n6 can be metabolized by ALOX15 to 13-hydroxyoctadecadienoic acid (13-HODE). 13-HODE has anti-tumorigenic effects and it was shown that high linoleic acid concentrations increase apoptosis of tumor cells (Lu et al., 2010a, Lu et al., 2010b). 15-HETE – an ALOX15 product does also have protective properties by inhibiting 5- and 12-HETE (Vanderhoek et al., 1980, Bailey et al., 1982), which promotes colon cancer metastasis (Stadler et al., 2017). ALOX15 is inversely associated with CRC as lower ALOX15

expression levels were found in adenomas of patients with sporadic polyps (Heslin et al., 2005).

In contrast, higher COX-2 expression in colon tumor tissue was related to aggressive CRC and low survival (Wang and Dubois, 2010, Rahman et al., 2012). Increased COX-2 expression results in higher PGE_2 levels, which was demonstrated to be pro-carcinogenic (Rigas et al., 1993). Studies investigating the impact of n3 PUFAs (EPA, DHA) have shown beneficial effects on attenuating cancer cachexia (Laviano et al., 2013). Two recent literature analyses investigated the benefits of n3 FA supplementation in cancer and concluded that n3 FA consistently reduced inflammation, cancer cell proliferation and cancer cell survival, angiogenesis and metastasis (Pappalardo et al., 2015, Eltweri et al., 2016). Nutritional status and body composition improved in patients due to n3 treatment. However, most studies focus on circulating FA and LM or the expression of converting enzymes in tumor tissue but little is known about these mechanisms in the adipose tissue.

1.6 HYPOTHESIS AND JUSTIFICATION

In cachectic patients, the endogenous inflammation resolving capacity seems to be impaired, the fatty acid profile altered, and therefore, the continuous release of inflammatory mediators become harmful to the tissues as described above. These changes may trigger and support wasting (Wang and Dubois, 2010, Eltweri et al., 2016, Machado et al., 2004). Thus elucidating the ability of fatty acids and lipid mediators to decrease or increase inflammation in the adipose tissue and adipose-related immune cells could provide important hints for further therapies in cancer cachexia. On the other side, investigations regarding the endogenous production of pro-resolving or inflammatory lipid mediators in cachexia are rare and thus we aim to contribute to a better understanding of sustained inflammation in the scenario.

1.7 OBJECTIVES

1.7.1.1 CHARACTERIZATION OF THE SYSTEMIC AND LOCAL WHITE ADIPOSE TISSUE IN CONTROL, WSC AND CC

To investigate, how the expression of pro-inflammatory markers (Cytokines, PGE_2), fatty acids and pro-resolving compounds differ between controls, cachectic and non-cachectic patients. These factors will be assessed in plasma and adipose tissue of controls, cachectic and non-cachectic patients.

1.7.2 INVESTIGATION OF ENZYMATIC FA MODIFICATION DUE TO INFLAMMATORY STIMULI IN CACHEXIA, AS LITTLE IS KNOWN ABOUT CHANGES IN FA ELONGATION AND DESATURATION IN CACHEXIA.

Many different enzymes are involved in PUFA- or LM-formation such as Elongase 2, 5 or 6, the desaturases FADS1 and FADS2 (Zhang et al., 2016, Seegmiller, 2014), (Figure 8) as well as cyclooxygenases and lipoxygenases. The expression and hence the activity of these enzymes may be altered either by inflammatory factors or by fatty acids themselves and influencing the balance between resolving and pro-inflammatory lipid compounds.

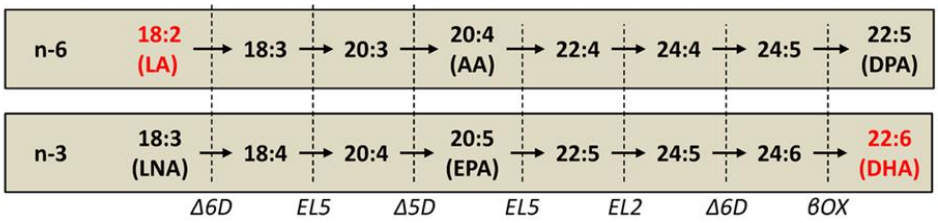


Figure 8: Unsaturated fatty acid metabolism in the n3 and n6 Pathways. LA, linoleate; AA, arachidonate; DPA, docosapentaenoate; LNA, alpha-linolenate; EPA, eicosapentaenoate; DHA, docosapentaenoate; Δ6D, fatty acid desaturase 2, EL5, elongase 5; Δ5D, fatty acid desaturase 1; EL2, elongase 2; βOX, β-oxidation.

1.7.3 EFFECTS OF AN ALTERED FA PROFILE AND INCREASED INFLAMMATORY MARKERS (AS FOUND *IN VIVO* IN CACHECTIC PATIENTS WITH GI TUMORS) ON IMMUNE CELLS AND ADIPOCYTES.

Human derived macrophages and murine adipocytes will be exposed *in vitro* to fatty acids as and cytokines found in patients *in vivo.* The in vitro studies will allow revealing potential molecular mechanisms that enhance the inflammatory response of macrophages and adipocytes. Macrophages and adipocytes express many enzymes converting FA to bioactive lipid mediators and carry receptors that are activated by FA or cytokines. The present study will investigate, how the synthesis of FA and LM might be affected in resident/infiltrating immune cells and adipocytes in the pro-inflammatory environment of cancer cachexia and how this in turn may contribute in the inflammatory status.

2 MATERIALS AND METHODS

2.1 SELECTION OF PATIENTS

212 patients were recruited at the University Hospital of the University of São Paulo and the Santa Casa hospital of Sao Paulo. The selection process was advised by physicians involved in the project. Recruited patients underwent either a treatment of hernia, cholelithiasis, cholecystitis, intestinal fistula and closure of enterostomy (Control n=65) or an excision of gastro-intestinal tumors (weight stable cancer group WSC n=59 and cachectic cancer group CC n=88).Criteria for gastro-intestinal cancer will be ratified by the INCA, TNM classification of tumors, 2004 (esophagus, stomach, small intestine, colon and rectum).

2.1.1 ETHICS COMMITTEE APPROVAL

This project (FAPESP 2013/25207-7) was embedded in a larger project (FAPESP 2012/50079-0). It was approved by the local ethics committees of ICB I: 1.407.862/CAAE 28268414.4.0000.5467; University Hospital HU-USP: 1.424.462/CAAE 28268414.4.3000.0076 and Irmandade da Santa Casa de Misericórdia de São Paulo Hospital: 1.428.350/CAAE 28268414.4.3001.5479. The project is registered on the national ethics platform “Plataforma Brasil”. After selection of potential participants, patients signed a consent form in which they agree to participate in the study. Anthropometric measurements (weight, height) will be followed by interviews of the patients, adopting two questionnaires (Quality of life EORTCQLC-C30 and Anorexia - FAACT-ESPEN (The Functional Assessment of Anorexia/Cachexia Therapy) (Laviano et al., 2011).

2.1.2 BLOOD COLLECTION AND ANALYSIS OF BIOCHEMICAL PARAMETERS

Approximately 10 ml of blood were collected as part of the process of venous access for anesthesia during surgery. The collected blood is centrifuged, serum and plasma collected and frozen at -80 ° C for further analysis. Serum levels of hemoglobin, albumin, C-reactive protein (CRP), triacylglycerides, cholesterol, high density lipoprotein cholesterol (HDL), low density lipoprotein cholesterol (LDL), were measured using the Labtest-kits and Labmax 240 system (hemoglobin: No. 43, albumin: No. 19, CRP: No. 335, triacylglycerides: No. 87, cholesterol: No. 76, HDL: No.13, LDL: No. 146) according to the manufacturer's recommendations.

2.1.3 CLASSIFICATION AND GROUP DIVISION

The exclusion criteria were anti-cancer or continuing anti-inflammatory treatment, liver failure, renal failure, AIDS, inflammatory bowel diseases and autoimmune disorders, blood transfusion 72 hours prior surgery, all diagnosed by doctors. Patients undergoing hernia treatment with serum C-reactive protein > 10µg/ml as indicator for pathological inflammation (McMillan, 2008) do not represent appropriate controls and were excluded from the study. The “Control” includes patients in the treatment of hernia that accomplish the inclusion criteria and did not exhibit any exclusion criteria. Patients in cancer treatment accomplishing the inclusion criteria and do not exhibit any exclusion criteria were divided into two groups based on the data received in the questionnaire, anthropometric measurements and biochemical parameters. The group “CC cachectic cancer” consist of patients with unintentional weight loss > 5% over the last 6 months, declared fatigue and increased concentration of the inflammatory marker C-reactive protein > 5µg/ml serum (Evans et al., 2008). The group “WSC weight stable cancer” is composed of patients being treated for cancer without weight loss of > 5% over the last 6 months and without severe systemic inflammation.

2.2 COLLECTION OF WHITE ADIPOSE TISSUE

During the surgical procedure approximately 1g of subcutaneous (SAT) and visceral adipose tissue (VAT) is withdrawn. Samples used for total RNA or protein extraction were transferred to cryo tubes and snap frozen in liquid nitrogen or dry ice, samples for explant culture or adipocyte isolation were slightly cooled in PBS containing 1% Penicillin/Streptomycin. Cryo tubes were stored at -80°C for further analysis.

2.2.1 ISOLATION OF ADIPOCYTES

Isolation and cultivation of adipocytes, pre-adipocytes and stromal vascular cells will be performed as described before (Hauner et al., 1995). The isolation of stromal-vascular cells will be performed with tissue samples (1g) from the individual donors. The samples will be kept at 4°C in PBS containing 1% Penicillin/Streptomycin (Sigma Chemical Co., Munich, Germany), pH 7.4, and immediately transferred to the laboratory. The samples are repeatedly rinsed in PBS to remove any blood. Fibrous material and blood vessels will be carefully dissected and discarded. The remaining tissue was cut into small pieces (10-20 mg) and digested in Dulbeccos Modified Eagle Medium (DMEM)-High Glucose medium (Gibco, Life-technologies) containing 1.5 mg/ml collagenase (type I; Worthington Biochemical Corp., Freehold, NJ). The ratio between adipose tissue mass to incubation solution is 1 g/4 ml. The tissue pieces are digested for 45 min at 37°C under intermittent shaking. The dispersed tissue is filtered through a nylon mesh (pore size 250 Mm) and the reaction was stopped by adding 5ml DMEM medium + 10% Fetal Calf Serum. Cell suspension was gently mixed and centrifuged for 10 min at 200 g. The floating mature adipocytes were transferred in a 1.5 ml reaction tube and washed twice with PBS (4°C, 5min, 3500 rpm). After the removal of the PBS, adipocytes were stored at -80°C for protein extraction.

2.2.2 EX-VIVO CULTURE OF ADIPOSE TISSUE EXPLANTS

White adipose tissue was kept in PBS after surgery, after the removal of connective and vessels, the tissue was chopped into approximately 40-60 mg pieces and transferred into 12-well cell culture plates containing 500 µl DMEM/F12 medium supplemented with 10% fetal bovine serum, 1% non-essential amino acids, 1% Penicillin/Streptomycin and 2% fungizon. To investigate the impact of TNF-α on adipose tissue ex-vivo, the explants were incubated 24 h with or without 50ng/ml TNF-α in culture medium at 37°C, 5% CO2. After 24 hours the tissue segments were rinsed in cold PBS, the samples of two wells were pooled and frozen at -80°C. To measure cytokines in the culture medium the supernatant was collected and centrifuged at 3500 rpm, 4°C to remove cell debris and stored at -80°C.

2.3 CELL CULTURE

2.3.1 THP AND U937 CELL CULTURE

The human monocytic cell lines THP-1 and U937 will be cultivated in medium RPMI1640 with 10% heat-inactivated FCS and 1% antibiotics and seeded in 35-mm diameter culture plates with 1 x 106 cells per plate (Sarstedt). Monocytes are differentiated into macrophages by addition of 100ng/ml PMA for 24h. After removing the medium, macrophages are washed with RPMI1640 and incubated in serum-starved RPMI1640 without PMA for 24h. For cell-experiments and preparation of supernatants macrophages were stimulated for another 24h with cytokines namely IL-6 100ng/ml (Miltony), TNF-α 100ng/ml, CXCL10 50ng/ml, IL-8 50ng/ml (Peprotec), Palmitate 100nM (Sigma) and/or 100ng/ml LPS (Sigma). After 24h culture medium was removed, cells were washed twice with PBS and shock-frozen in liquid nitrogen and stored at -70°C for further analysis.

2.3.2 3T3-L1 CULTURE

3T3-L1 fibroblasts are passaged in high-glucose Dulbecco modified Eagle medium (DMEM) plus 10% fetal calf serum (biochrome) and 1mM l-glutamine. 3T3-L1 fibroblasts will be differentiated into adipocytes after they reached confluency. 3x104 cells were plated on a 12-well plate (falcon, Themo Fisher) and after 24 hours the culture medium was changed. After another 48 hours differentiation was initiated by the addition of differentiation medium (high-glucose DMEM containing 10% FCS, 1mM l-glutamine, 300µM isobutylmethylxanthine (Sigma), 1µM dexamethasone, and 1µg of insulin per ml). After 2 days, the 3T3-L1 cells are transferred to adipocyte growth medium (high-glucose DMEM plus 10% FCS, 1mM l-glutamine, and 1µg of insulin per ml) and refed every 2 days for about a week (Reusch et al., 2000). Cells were incubated with experimental medium of 24h, washed twice with PBS and frozen at -80°C.

2.4 MOLECULAR BIOLOGICAL METHODS

2.4.1 PROTEIN EXTRACTION

Total protein was extracted out of 300 mg tissue, 100-200 mg explants or ~200 µl isolated adipocytes. Samples were mixed with RIPA buffer containing protease and phosphatase inhibitor (cOmplete and phosstop, ROCHE) and homogenized (Biobase homogenizer BK-HG169). 500ml RIPA buffer contained 9.2g NaCl, 0.61g Tris(hydroxymethyl)aminomethane, 1.85g EDTA (all LabSynth) and 5ml Triton X-100 (Amresco). The homogenized samples were then centrifuges at 4°C for 30min (12000rpm). Supernatant was transferred in a fresh tube and stored at -80°C.

For the assessment of the protein concentration, lysates were diluted in aqua dest 1:10 (SAT, adipocytes, VAT explants) or 1:5 (VAT). 10µl of the dilution was mixed with 190µl Bradford reagent (Biorad) in a 96 well plate (Greiner), incubated for 5 min at 37°C and protein concentration measured photometrically (595nm) using a microplate reader (Biotek, Synergy H1). 3T3-adipocytes (3 wells pooled) were lyzed in 300µl of lysis buffer (pH 8; 10mM Tris-HCL, 1mM EDTA, 0.5% SDS, all Labsynth) sonicated, centrifuged (10.000 x g, 15 min, 4°C) and supernatants were stored at -20°C.

2.4.2 DETERMINATION OF CYTOKINES IN PLASMA, ADIPOSE TISSUE, ADIPOCYTES AND CULTURE MEDIUM

The assessment of cytokines in plasma, SAT, VAT, adipocytes, supernatant, VAT explants was carried out according the manufacture's instructions of the HCYTMAG-60K-PX30 human cytokine panel. Adipose tissue lysates were used as 1/5 (SAT) or 1/10 dilution (VAT), whereas plasma, supernatant, adipocyte lysates and VAT explant lysates were not diluted. The components of the reaction mixture were collocated into the well plate. First a distinct amount of capture antibodies immobilized on magnetic beads and the sample were added and incubated for 2 hours (figure 9A). Each anti-body species is immobilized on a set of magnetic microspheres that have a specific color shade (figure 9B). The antibodies bind the proteins of interest, after washing steps; the biotinylated detection antibodies are added to the beads (1 hour). Unbounded biotinylated antibodies are washed away and fluorophore (phycoerithrin) labelled streptavidin will bind to biotin (30 min incubation) (figure 9C).

In the Luminex Multiplex xMap Analyser (MAGPIX, Life Technologies) a magnet holds the paramagnetic beads in a monolayer and two spectrally distinct Light Emitting Diodes (LEDs) illuminate the beads. One LED identifies the analyte by the specific bead colour shade and the second LED determines the magnitude of the PE-derived signal. This magnitude is given in median fluorescence intensity (MFI) which is proportional to the protein amount in the sample (figure 9D). Hence, by a serial dilution of a standard mix, the cytokine concentration in the sample can be calculated with the Analyst 5.1 Software (figure 9E). The cytokine contents was normalized to total protein concentration.

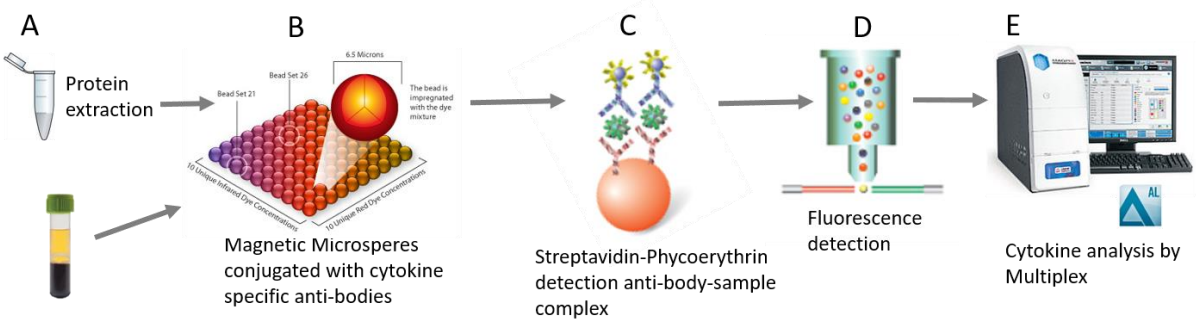


Figure 9: Illustration of Assay principle adapted from R&D System Products.

2.4.3 WESTERN BLOT ANALYSIS

The protein lysates (50 µg protein) was mixed with Lämmli-buffer containing mecraptoethanol and dithiothreitol (15 mg/100µl Lämmli-buffer; sigma) and heated up for 5 min at 95°C. Proteins are separated 1-2 hours (100V) by molecular weight using SDS-PAGE (Biorad Mini-PROTEAN Tetra Cell System). Gradient gels (Mini-PROTEAN TGX precast Gels; Biorad) were used for the analysis of FASN, ALOX15 and FADS1, while for SCD 10% gels were prepared. By Western Blotting proteins were transferred to a PVDF-membrane which was activated for 10s in methanol. The membrane was incubated for 1 hour in blocking solution and washed (3x 10min with TBS-T). Then the membranes were incubated overnight with primary antibodies against the target proteins, washed and incubated with horse-reddish-peroxidase (HRP) labeled secondary antibodies. After a final washing wash the membranes were incubated with HRP substrate (SuperSignal™ West Pico PLUS Chemiluminescent Substrate; Thermofisher) for 5min and developed with the LI-COR C-DiGit Chemiluminescence Western Blot Scanner.

In a semi-quantitative manner, protein content was assessed by measuring the band intensity of the target (BI_T) and the endogenous control (BI_{EC}) protein or the Fast-green lane (sigma) using free ImageJ software. Relative protein contents were calculated as following: At first the background was subtracted from the band intensity of T and EC. Then the target protein was normalized to the endogenous control or fastgreen ($BI_{Tnorm}=BI_T/ BI_{EC}$). The mean of the BI_{Tnorm}-values of the Control group ($BI_{TControl}$) was set 100% and the protein content in each individual sample was expressed as relative expression compared to that mean value (relative protein expression (%) = $BI_{Tnorm}*100/ BI_{TControl}$).

Table 1: SDS-PAGE gel composition.

	Resolving gel 10% (10ml)	**Stacking gel (10ml)**
Water	4.1ml	6.1ml
Acrylamide/bis 30% 37.5:1 (Bio-Rad)	3.3ml	1.3ml
Tris–HCl 1.5 M, pH 8.8	2.5ml	2.5ml
SDS, 10% (Biorad)	100µl	100µl
N,N,N′,N′-tetramethylethylene-diamine (TEMED) (Bio-Rad)	10µl	10µl
Ammonium persulfate, 10% (Biorad)	32µl	100µl

2.4.3.1 WESTERN BLOTTING BUFFERS AND ANTIBODIES

Unless otherwise stated all reagents were sourced from Labsynth, Brazil.

Lämmli buffer 5X pH6.8 (10ml): 1.75ml 0.5M Tris-HCL, 4.5ml glycerol, 2ml SDS (0.25g/ml Tris-HCL; Biorad), 0.5ml 0.25% Bromophenol blue in H_20 (Sigma), 1.25ml B-mercaptoethanol (Sigma)

Running buffer (Tris-Glycine/SDS) pH8.3: 25 mM Tris base, 190 mM glycine, 0.1% SDS (Biorad)

Transfer buffer semi dry: 48 mM Tris base, 39 mM glycine, 20% methanol (Sigma), 0.04% SDS

TBS-T pH7.6: 20mM Tris base, 150mM NaCl, 0.1% Tween 20 (Amresco)

Blocking solution: 3% BSA (Sigma) in TBS-T

Table 2: Antibodies used in Western-Blot experiments.

Target	No.	Company
Anti-FASN (mouse)	sc-48357	Santa Cruz
Anti-SCD (mouse)	sc-58420	Santa Cruz
Anti-FADS1 (goat)	VPA00098	Biorad
Anti-ALOX15 1 (mouse)	ab80221	Abcam
Anti-GAPDH (mouse)	sc-47724	Santa Cruz
Anti-beta-TUBULIN (rabbit)	ab15568	Abcam
Anti-beta-ACTIN (mouse)	sc-47778	Santa Cruz
Anti-mouse IgGκ BP-HRP	sc-516102	Santa Cruz
Mouse anti-goat IgG-HRP	sc-2354	Santa Cruz
Mouse anti-rabbit IgG-HRP	sc-2357	Santa Cruz

2.4.4 TOTAL RNA ISOLATION

RNA will be isolated from macrophages using the peqGold Total RNA Kit. cDNA was synthesized from 600 - 1500ng purified RNA with 500ng Oligo(dT) using 200U of RevertAid™ M-MuLV reverse transcriptase according to the instruction of the provider. For the extraction of total tissue RNA, Trizol (Quiagen) was added to the sample (300mg SAT, VAT or 150-200mg VAT explants) which were then homogenized (Biobase homogenizer). Homogenates were kept for 5 min at room temperature (RT). 200µl chilled chloroform (Sigma) was added to the samples, mixed gently 30s with isopropanol (Labsynth), incubated 10 min at RT and centrifuged (10min, 4°C, 10600rpm). Supernatants were discarded and pellets washed twice with 1ml 95% ethanol (Labsynth), 5min, 4°C, 7500 rpm. The pellets dried at RT and were dissolved in 20-30µl inactive DEPC water (500µl diethylpyrocarbonate (Sigma) in 500ml aqua dest). RNA concentration was quantified by pipetting in 2µl sample on a micro-volume-plate (Biotek, take3-plate) and measuring the absorbance at 260/280nm (Synergy H1 Hybrid Multi-Mode reader, Biotek).

2.4.5 REAL TIME RT-QPCR ANALYSIS

Real-time PCR for the quantification of each transcript was carried out in triplicates in a reaction mixture of 2x qPCR Sybr Green Polymerase Mix (cells: Sybr Green Master Mix, Fermentas; tissue: Fast Sybr Green Master Mix, Applied Biosystems), forward and reverse oligonucleotides, and cDNA (20ng cells, 10ng tissue) in a total volume of 10µl. qPCR was performed with an initial enzyme activation step at 95°C for 10min, followed by fifty cycles of denaturation at 95°C for 20sec, annealing at 57-60°C for 30 sec and extension at 72°C for 20 sec with a subsequent melt curve analysis in a q-RT-PCR-Thermocycler (cells: CFX96™ Thermal Cycler, Biorad; tissue: QuantStudio 12k Flex Real-Time PCR System (Thermo Fisher Scientific). The expression level was calculated as an n-fold induction of the gene of interest (table 2) in treated versus control (Ctrl) cells normalized to a reference gene (3T3-L1: hprt; THP-1: β-ACTIN; SAT and VAT: RPL27, 18S; VAT explants: 18S). For cell culture experiments, the calculation is based on the differences in the threshold cycles between control (Ctrl) and treated (treat) groups according to the formula: fold induction = $2^{(ctrl\text{-}treat)int} / 2^{(ctrl\text{-}treat)ref}$. Relative

mRNA expression in adipose tissue and explants was calculated using the $2^{(-ddCT)}$-method (J and D, 2001). The CT-value of one or the mean of both reference genes was subtracted from the gene of interest, generating the delta-CT value (dCT). The mean of the Control group dCTs was then subtracted from the individual dCTs, resulting in delta-delta-CT values (ddCT). The gene expression is finally given as $2^{(-ddCT)}$.

Table 3: List of Primer-sequences

Gene	Sense	Antisense	
ALOX15 (human)	GGGCAAGGAGACAGAACTCAA	AGCGGTAACAAGGGAACCTG	NM_001140.3
ALOX5 (human)	CAAAGCGATGGAGAACCTGT	GTTGCAGCCATTCAGGAACT	NM_000698.3
ALOX5AP (human)	TTGCCCATAAAGTGGAGCAC	TTCTGGTTGGCAGTGTAGACC	NM_001204406.1
β-Actin (human)	CCCCAAGGCCAACCGCGAGAAGATG	AGGTCCCGGCCAGCCAGGTCCAG	NM_001101
CMKLR1 (human)	CAGAAAGAAGCCAGGGTGTG	ATGGGCTTCCCTCTGGTCT	NM_001142343.1
COX-2 (human)	TGTGCCTGATGATTGCCCGACTCC	TGTTGTGTTCCCGCAGCCAGATTG	NM_000963
CXCL10 (human)	CCATTCTGATTTGCTGCCTTA	TTCTTGATGGCCTTCGATTC	NM_001565.3
Cxcl10 (mouse)	ATGACGGGCCAGTGAGAATG	GAGGCTCTCTGCTGTCCATC	NM_021274
ELOVL5 (human)	TGCTAGGCCCTCGAGATACT	AGCAGTGTGAGTCCAAGGTT	NM_001301856.1
Elovl5 (mouse)	TCATTGTATGGCTGGGACCA	AGAGGACGCGGATGATCTTC	NM_134255.3
FADS1 (human)	TGTGGAGCTTGGGAAACAGA	AATCATCCAGGCCAAGTCCA	NM_013402.4
Fads1 (mouse)	GCCTGAGCCTGAACTGTACT	ACCCACCAAGAATAAAGCGC	NM_146094.2
FADS2 (human)	CGCTGCTCATCCCCATGTAT	CGGGGCGATCTTGTGTAAGT	NM_004265.3
Fads2 (mouse)	CATGACTATGGCCACCTTTC	GTCCTTGTGGAAGATGTTGG	NM_019699
Hprt (mouse)	TCCCAGCGTCGTGATTAGC	CCAGCAGGTCAGCAAAGAAC	NM_013556.2
IL-6 (human)	CAGCCCTGAGAAAGGAGACAT	AGCCATCTTTGGAAGGTTCA	NM_000600.3
IL-8 (human)	AGCTCTGTGTGAAGGTGAT	TTTGGGGTGGAAAGGTTTG	NM_000584.3

Mip2 (mouse)	TGAGTGTGACGCCCCCAGGAC	TCAGACAGCGAGGCACATCAGGTA	NM_009140.2
PTGES (human)	ACAGCCTGGTGATGAGCA	AGGCCTTCTTCCGCAGC	NM_004878.4
RPL-27 (human)	CCGAAATGGGCAAGTTCAT	CCATCATCAATGTTCTTCACGA	NM 000988.3
SCD (human)	TCTCTGCTACACTTGGGAGC	GCCCATTCATAGACATCATTCTG	NM_005063.4
Scd-1 (mouse)	GCGTTCCAGAATGACGTGTA	GCCGGGCTTGTAGTACCTC	NM_009127.4
TNF-α (human)	TTCCTCAGCCTCTTCTCCT	GAGGGTTTGCTACAACATGG	NM_000594.3
18S (human)	CCTGCGGCTTAATTTGACTC	ATGCCAGAGTCTCGTTCGTT	NR_003286.2

2.5 FATTY ACID AND LIPID MEDIATOR ANALYSIS

2.5.1 IDENTIFICATION OF FATTY ACIDS BY GAS CHROMATOGRAPHY AND ANALYSIS BY MASS SPECTROMETRY (GC-MS)

Lipids from 90-110 mg adipose tissue were extracted using 2:1 Chloroform/Methanol (Merck) after Folch et al., 1957 (FOLCH et al., 1957). Fatty acid methyl esters were generated using anhydrous methanol/sulfuric acid. Methyl esters were then separated on a DB-23 column [(50% cyanopropyl) methyl polysiloxane 0.25 mm thick film, 0.250 mm; 60 m], with an injection inlet at 220°C, 250°C at the interface between the column and the mass spectrometer and helium carrier gas (99.999%) on a GC-MS (Shimadzu QP5050 model). Experiment conditions were programmed as follows: 150°C hold for 2 min post-injection (in the first 9 minutes the initial values have not been measured because the solvent may saturate the mass spectrometer), then 150-200°C at a rate of 10°C/min; 200-230°C at 1.3°C/min; 230-250°C at 10°C/min. The fatty acids were identified by comparison with retention times of authentic standards and by mass spectra as described by Ramos and Colquhoun, 2003 (Ramos and Colquhoun, 2003).

2.5.2 ANALYSIS OF PGE_2 IN VAT AND RESOLVIN D1 IN PLASMA

Concentrations of the lipid mediators Resolvin D1, PGE_2) were evaluated with ELISAs based on competitive enzyme-linked-immunosorbent-assay-technique. Two Assay Kits were used as described in the manufactures' instructions (PGE_2 Express ELISA No. 500141, Resolvin D1 ELISA No. 500380; Cayman Chemicals, USA). The Microtiter plates were pre-coated with a specific antibody directed against the lipid mediator. Enzyme-coupled lipid mediator conjugates (Acetylcholine esterase) compete with the lipid mediators in the sample for free antibody binding sites (Figure 10). If binding sites are blocked with lipid mediator of the sample, the enzyme-coupled conjugate cannot bind and the applied substrate will not be converted to a colored complex. Thus, a high concentration of the observed lipid mediator in the sample is negatively correlated with the production of the colored complex. The intensity of color was measured spectrophotometrically at 412nm in a microplate reader after 110 minutes. The concentration in each sample was interpolated from the standard curve, PGE_2 concentration were normalized to tissue weight.

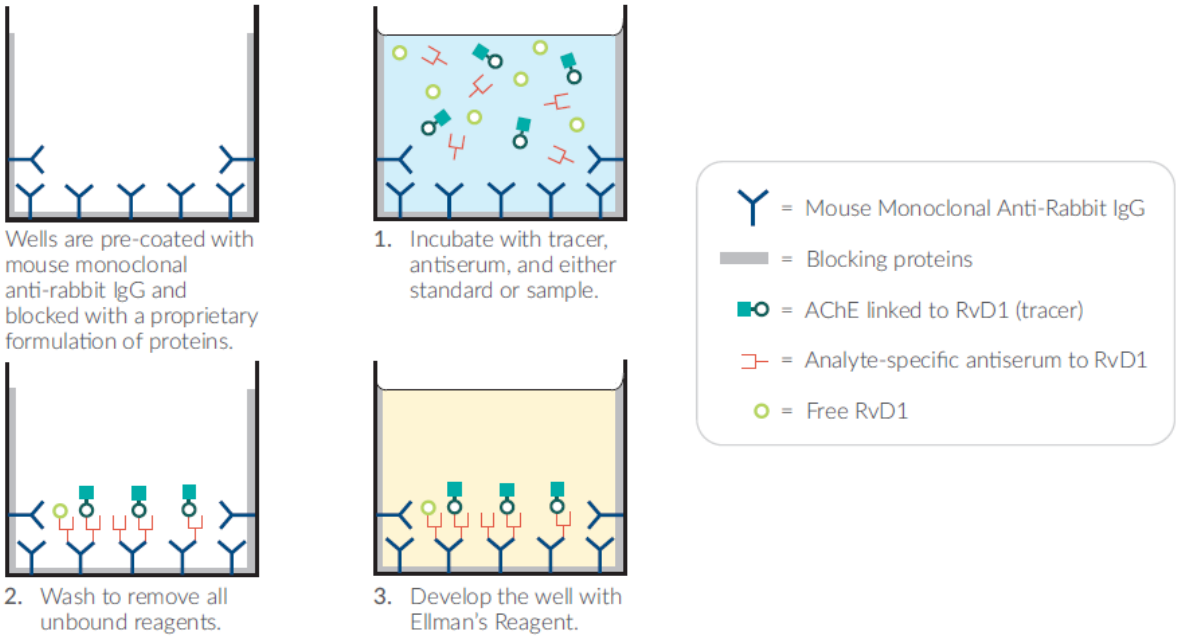


Figure 10: Illustration of assay principle.

The sample preparation for the PGE_2-ELISA was adapted from Massey et al.(Massey and Nicolaou, 2013). In brief, Solid phase extraction (SPE) columns (C18-E columns (55um, 70A, 3ml), Strata) were activated by washing them with 10ml methanol (Sigma) followed by 10ml water. Approximately 100mg frozen VAT tissue was placed in a 2 ml glass tissue grinder (Thermo Fisher) and 1ml ice cold 15% methanol in water (v/v).

Tissue grinding was carried out on ice and the homogenate was transferred into a glass vial. Grinder and pestle were washed with 3x1ml ice cold 15% methanol and the washes transferred into the sample vial. Samples were incubated in the dark 15 min on ice before sample vials were centrifuged for 10 min at 5000rpm, 4°C. The supernatant was transferred to e new vial and acidified to pH 3.0 with ~30 drops of 0.1M HCL and then loaded onto the activated SPE columns. SPE was carried out using the SPE 12-Position Vacuum Manifold Set (Phenomenex) under light protection. Columns were then washed with 10ml 15% methanol in water (v/v), 10ml water and 5ml Hexan (LabSynth). The prostaglandins were eluted with 6 ml methylformate (Merck) into a fresh vial and the solvent was evaporated under nitrogen steam. Samples were dissolved in 130µl EIA buffer, and stored at 80°C until the analysis. Samples were measured in duplicates using a sample volume of 50µl. For the Resolvin D1 ELISA plasma samples were defrosted on ice, centrifuged for 5min at 3500rpm, 4°C and analyzed in duplicates of 50µl.

2.6 DATA ANALYSIS

All statistical data analysis´were carried out using Prism graphpad software version 7. Data following normal distribution are expresses as mean ± standard error and non-parametric data as median [minimum; maximum]. Multiple comparisons were performed using one-way ANOVA (parametric data), the Kruskal-Wallis test (non-parametric data), Repeated measures ANOVA (paired parametric data) and Friedman test (paired non-parametric data). Tukey´s or Holm-Sidak posttests were applied. Two data sets were compared using Wilcoxon matched-pairs signed rank test for non-parametric paired data or paired t-test for parametric data. Significance was determined as $p < 0.05$.

3 RESULTS

3.1 CHARACTERIZATION OF PATIENTS AND BIOCHEMICAL PARAMETERS

The clinical characteristics of the study groups are shown in the table 4. Both cancer groups (WSC, CC) had a higher mean age than the control group (62.7, 59.9 and 54.4 years respectively, $p<0.002$). Main reason for surgery in the Control group was hernia removal (64.4%), in the cancer groups, colon cancer was most common (WSC=55.9%, CC=60.2%) followed by stomach carcinoma (WSC=30.5%, CC=30.7%). The majority (70.3%) of the CC patients presented advanced cancer compared to about half of the WSC patients (48.6%). However, the prevalence of cachexia did not depend on tumor progression ($chi^2=0.07$). Patient self-reported body mass and calculated BMI 6 months before surgery showed no statistical difference between groups. At time of surgery the median body mass of CC (62kg) was significantly lower than Control and WSC (Control=72.1kg, WSC=72kg, $p<0.0001$) reflecting a striking typical weight loss of 15.0%. The median body mass index BMI (kg/m^2) of CC (22.7) was significantly lower than the BMI of the other groups (Control=26.5, WSC=27.2, $p<0.0001$). The WSC and CC groups had significantly lower ratings of quality of life compared to the Control group (WSC=66.7 and CC=66.7 vs Control=83.3). The final score of the FAACT-ESPEN-questionnaire applied to assess the degree of anorexia revealed that CC showed significantly lower anorexia score (meaning more anorexia) than the other groups (CC=30 vs Control=39 and WSC=37).

Table 4: Descriptive data at 6 months pre and at surgery

	Group			
Variable	Control	WSC	CC	p-value
n	65	59	88	
Female/Male	22/43	28/31	43/45	
Mean age (years)	54.4 ± 1.9	62.7 ± 1.5*	59.9 ± 1.5*	ANOVA 0.002
Height (m)	1.65 [1.45-1.85]	1.64 [1.47-1.86]	1.66 [0.94-1.87]	0.791
Diagnosis; tumor localization (%)	Cholelithiasis: 8 (12.3%) Hernia: 42 (64.6%) Cholecystitis: 2 (3.1%) Intestinal fistula: 4 (6.2%) Closure of enterostomy: 9 (13.8%)	Colon: 33 (55.9%) Rectum: 7 (11.9%) Small intestine: 1 (1.7%) Stomach: 18 (30.5%)	Colon: 53 (60.2%) Rectum: 6 (6.8%) Esophagus: 1 (1.1%) Stomach: 27 (30.7%) Pancreas: 1 (1.1%)	
Tumor staging (advanced cancer: stages III and IV)		I: 7 (20%) II: 11 (31.4%) III: 12 (34.3%) IV: 5 (14.3%) III & IV: 17 (48.6%) not reported 25	I: 3 (5.6%) II: 13 (24.1%) III: 20 (37%) IV: 18 (33.3%) III & IV: 38 (70.3%) not reported 34	Chi^2 =0.07
<u>6 mth pre-surgery</u>				
Bodyweight	72.3 [48.4-106]	73.0 [46.5-139]	73.0 [43-145]	0.768
BMI	26.5 [19.6-35.3]	27.4 [19.1-61.0]	27.4 [16.8-50.4]	0.580
<u>At surgery</u>				
Bodyweight	72.1 [45-106]	72.0 [46.5-139]*	62.0 [35-108]*#	<0.0001
BMI	26.5 [19.2-35.3]	27.2 [19.1-61.0]*	22.7 [13.7-44.7]*#	<0.0001
%weight loss	0.0 [0-10]	0.0 [0-9.1]*	15.0 [4.0-46.0]*#	<0.0001
Quality of life – EORTC QLC-C30	83.3 [50-100]	66.7 [33.3-100]*	66.7 [16.7-100]*	<0.0001
Anorexia Score – FAACT-ESPEN	39.0 [31-47]	37.0 [19-43]*	30.0 [16-43]*#	<0.0001

* Significantly different to control, # significantly different to WSC, Square brackets contain min-max values, statistical tests are Kruskal-Wallis and Dunn´s posttest unless otherwise stated

Cancer cachexia is accompanied by systemic inflammation and metabolic changes. Therefore, Evans et al. and other groups suggested the consideration of systemic blood parameters such as haemoglobin, CRP and albumin for the diagnosis of cachexia (Evans et al., 2008, Muscaritoli et al., 2011). The concentration of CRP, an important systemic inflammation marker, was five times higher in CC compared to Control and two times higher than the non-cachectic cancer patients (CC =11.06mg/l vs Control=1.65mg/l and WSC=4.6mg/l, $p<0.0001$, table 2). In contrast to CRP, levels of both albumin and hemoglobin were significantly lower in CC compared to Control and WSC. The CC hemoglobin serum levels median value was 11.25 g/dl, which is below the 12.5 g/dl threshold for anemia. The CC median albumin concentration of 3.47g/dl was in the healthy physiological range (>3.2g/dl), even though significantly lower than the Control

and WSC groups. When evaluating individual serum albumin concentrations in CC, it was found that 43 of 70 patients had concentrations above 3.2 g/dl. Therefore, to understand these contradictory results, the albumin values were corrected to reflect a ratio with CRP. The CRP/albumin ratio was significantly higher in the CC group (CC=0.31 vs Control=0.03 and WSC=0.14, p<0.0001), suggesting that this maybe more diagnostically conclusive than just the albumin concentration.

Lipid metabolism was assessed through the measurement of triacylglycerides (TAG), total cholesterol and HDL and LDL lipoproteins. Lower serum TAG levels were observed in CC when compared to Control (CC=108 mg/dl vs Control=158 mg/dl, p=0.03). No significant difference in TAG between CC and WSC was observed. Although CC HDL was the lower than other groups and the overall Kruskal-Wallis test was significant, post-hoc analysis revealed no differences between the groups. Likewise, while not statistically significant, total cholesterol and LDL fractions were decreased in the serum of cachectic patients (table 5).

Table 5: Systemic blood parameters

Variable	Group									p
	Control			WSC			CC			
	median	[range]	n	median	[range]	n	median	[range]	n	
Hemoglobin (g/dl)	14.75	[8.9-16.9]	36	13.1*	[7.3-18.1]	40	11.25*#	[4.5-15.7]	50	<0.0001
CRP (mg/l)	1.65	[0.1-14.3]	61	4.6*	[0.14-16.9]	55	11.06*#	[0.2-16.8]	69	<0.0001
Albumin (g/dl)	4.18	[0.42-6.81]	61	4.05	[1.43-6.29]	53	3.47*#	[1.26-5.41]	69	0.0001
CRP/Albumin	0.03	[0.0-3.38]	43	0.14*	[0.0-0.94]	34	0.31*#	[0-1.33]	44	<0.0001
TAG (mg/dl)	158.00	[4.0-763]	58	135.00	[51-380]	50	108.00*	[22-331]	69	0.03
Total Cholesterol (mg/dl)	193.00	[11.0-446]	59	173.00	[87-318]	49	156.00	[42-347]	69	0.08
HDL (mg/dl)	37.50	[6-107]	56	38.00	[19-75]	48	33.00	[6-123]	67	0.04
LDL (mg/dl)	109.50	[12-253]	56	97.00	[44-191]	46	76.00	[0-197]	65	0.10

* Significantly different to control, # = significantly different to WSC, Square brackets contain min - max values, statistical tests are Kruskal-Wallis unless otherwise stated. CPR=C-reactive protein, TAG=triacyglycerides, HDL=high density lipoprotein, LDL=low density lipoprotein.

Cancer patients, mostly colon and stomach cancer, were divided into WSC and CC group and though not statistically significant, the majority of the CC group suffered from advanced cancer. Patients classified as cachectic presented a profound weight loss over the previous 6 months, a lower bodyweight and BMI accompanied with reduced quality of life and more anorexia than WSC. Systemic inflammation was confirmed by higher CRP levels at the same time, patients presented lower hemoglobin, albumin and lipid levels.

3.2 SYSTEMIC AND LOCAL ADIPOSE TISSUE INFLAMMATION

The pro-inflammatory status was further characterized by measurements cytokines namely TNF-α, IL-6, (figure 11A-C). In the plasma (figure 11A), the concentrations of the pro-inflammatory cytokines TNF-α and IL-6 were higher in CC than in the Control or WSC group. Notably, for TNF-α, IL-6 and IL-8 (see next paragraph) both the spread above the median as well as the number of patients displaying up to 160-fold higher cytokine plasma levels was larger in CC than in any other group (figure 11A, figure 12A). In contrast to circulating cytokine levels, there was not the expected pattern of TNF-α and IL-6 levels in SAT or VAT in the CC group.

Unexpectedly, the concentrations of these cytokines present in SAT of the WSC group were significantly lower than in Control (figure 11A). However, it is noteworthy to mention that the total SAT-lysate protein concentration was higher in WSC and CC when compared to Control. When the cytokine concentration was not normalized to total protein, no differences in TNF-α and IL-6 were discriminated between the groups in SAT. While in VAT, TNF-α as well as IL-6 contents were higher without the normalization to total protein (appendix B). Thus, correcting for protein content might obscure the increase of cytokine levels in CC adipose tissue as a result of the massive loss of triglycerides.

In VAT, cytokine levels were notably higher than in SAT across all groups. The mean TNF-α concentration was about 13-fold higher (SAT 0.18 pg/mg protein, VAT 2.45 pg/mg protein) while IL-6 concentration was about 113-fold higher (SAT=1.97 pg/mg protein, VAT=223.8 pg/mg protein; figure 11A). In lysates of isolated subcutaneous

adipocytes (figure 11B) and also when tested the cytokine release by subcutaneous adipose tissue explants after 24h cultivation (figure 11C) there were no differences in TNF-α or IL-6 content between the groups.

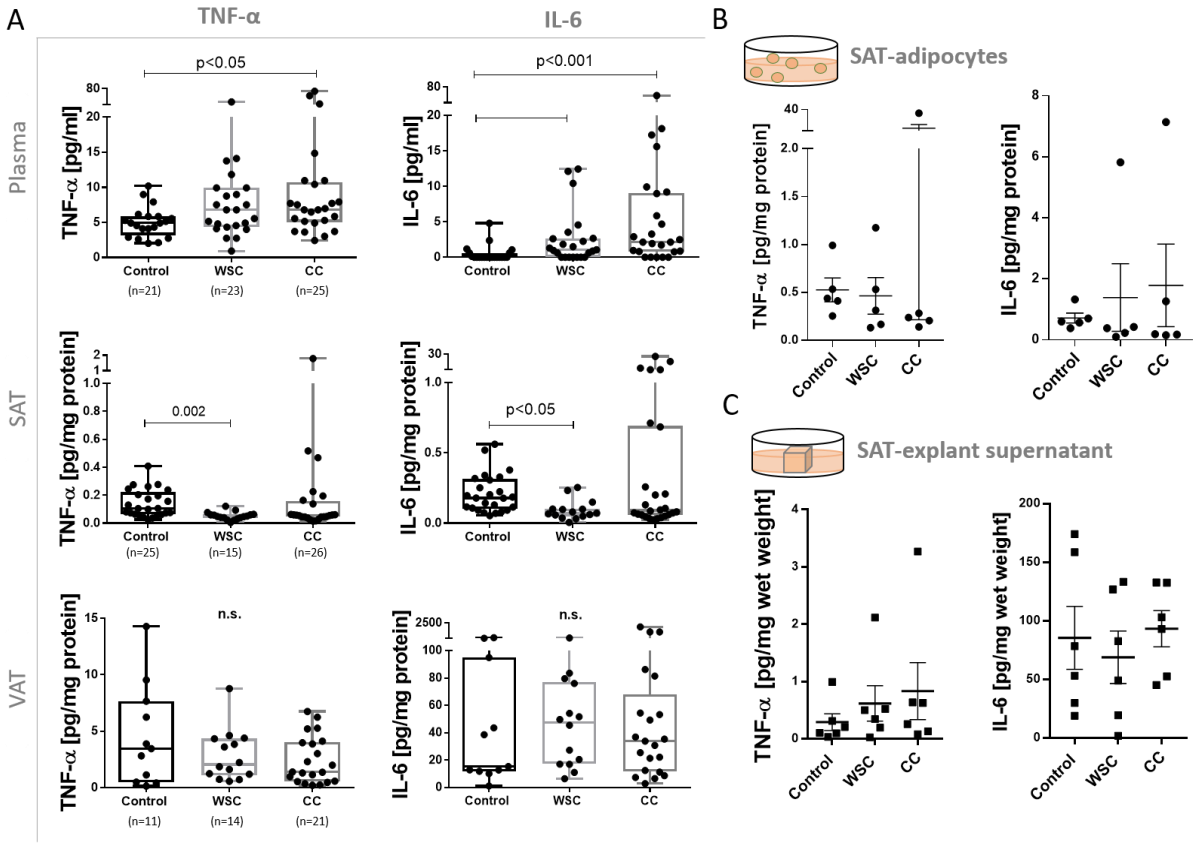


Figure 11: Distribution of pro-inflammatory cytokines in plasma, SAT, VAT, SAT adipocytes and supernatant of SAT explants. Cytokine concentration of plasma samples (A), supernatant of subcutaneous adipose tissue explants (C) and lysates of adipose tissue and adipocytes (B). SAT=subcutaneous adipose tissue, VAT=visceral adipose tissue. Results expressed as median [min-max], p=significance of Kruskal-Wallis test. *significant difference vs Control, # significant difference vs WSC. pg/mg protein=cytokine concentration in pg per mg total protein.

In contrast to the pro-inflammatory cytokines, the chemotactic IL-8 and the chemoattractant CXCL10 (figure 12A-C) showed a different distribution pattern in SAT and VAT. The concentrations of these chemokines were significantly higher in both SAT and VAT in CC compared to Control patients (figure 12A) with the exception of IL-8 in SAT. Importantly, CXCL10 median levels in VAT were about 8.4-fold higher (CC SAT=23.35 pg/mg protein, CC VAT=196.9 pg/mg protein). However, CXCL10 plasma

concentrations were not different between the groups, while the IL-8 plasma concentration was significantly elevated in CC patients compared with Control and WSC group (figure 12A). Chemokine levels in adipocytes and those secreted by adipose tissue explants were not distinctive among the study groups (figure 12B, 12C). Across the entire study population, VAT chemokine concentrations correlated positively with plasma contents (figure 13). These trends, while observed within groups, only achieved statistical significance for IL-8 in CC and CXCL10 in the Control group (figure 13).

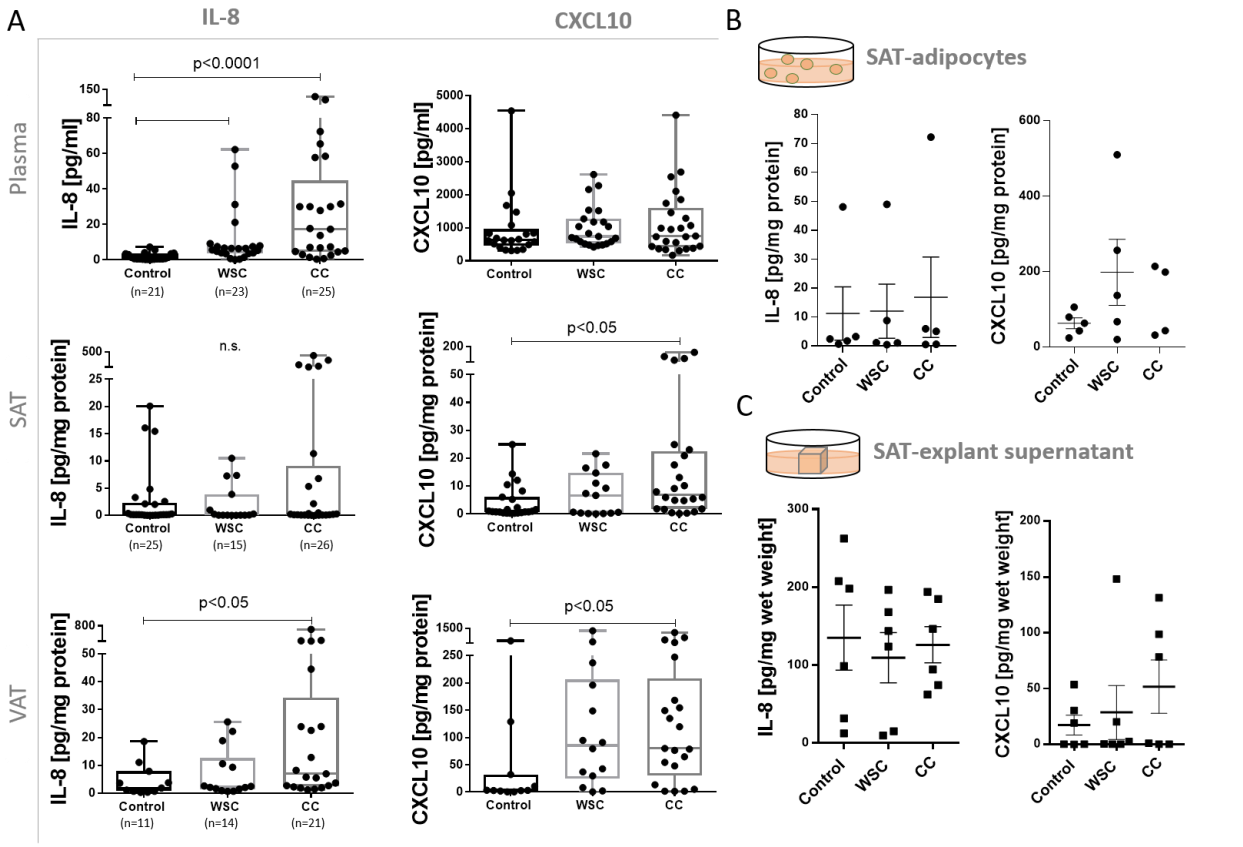
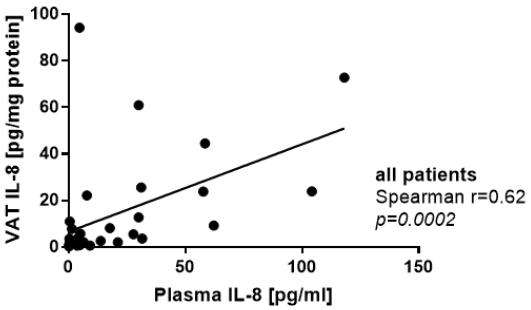


Figure 12: Distribution of chemokines in plasma, SAT, VAT, SAT adipocytes and supernatant of SAT explants. Chemokine concentration of plasma samples (A), supernatant of subcutaneous adipose tissue explants (C) and lysates of adipose tissue and adipocytes (B). SAT=subcutaneous adipose tissue, VAT=visceral adipose tissue. Results expressed as median [min-max], p=significance of Kruskal-Wallis test. *significant difference vs Control, # significant difference vs WSC. pg/mg protein=cytokine concentration in pg per mg total protein.

A

IL-8
VAT<>Plasma

	all patients n=32	Control n=7	WSC n=10	CC n=15
Spearman r	**0.62**	0.36	0.43	**0.64**
p-value	*0.0002*	*0.44*	*0.21*	*0.013*



B

CXCL10
VAT<>Plasma

	all patients n=32	Control n=7	WSC n=10	CC n=15
Spearman r	**0.65**	**0.93**	**0.6**	**0.47**
p-value	*<0.0001*	*0.007*	*0.07*	*0.08*

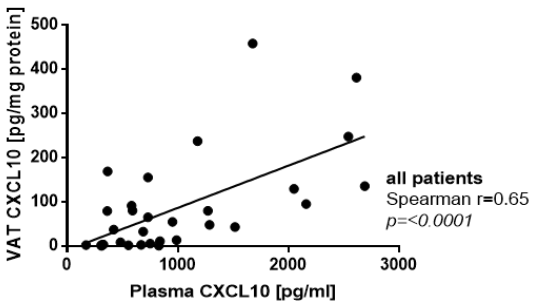


Figure 13: Higher chemokine concentrations in plasma correlate with higher chemokine levels in VAT. (A) Spearman correlation of VAT IL-8 concentrations with plasma IL-8 concentration for all groups and exemplary graph of the total group. (B) Spearman correlation if VAT CXCL10 concentrations with CXCL10 plasma contents.

Inflammation patterns were different between groups and tissues. In plasma of cachectic patients, contents of pro-inflammatory cytokines were higher while this was not reflected by the cytokine concentrations in the adipose tissue. The concentration of chemotactic factors was higher in both the circulation and the visceral adipose tissue in CC. No differences were found in adipocytes and SAT-explants.

3.3 FATTY ACID PROFILE IN SUBCUTANEOUS ADIPOSE TISSUE

In a previous study, it was shown that the plasma fatty acid profile of cachectic patients was different from those of Control and WSC (see Appendix A). Specifically, the percentage of palmitic acid (C16:0) was higher in CC and WSC compared to Control and stearic acid (C18:0) levels were higher in CC compared to Control. In both cancer groups the percentage of the monounsaturated fatty acids (MUFA) palmitoleic acid (C16:1) and oleic acid (C18:1n9) was significantly higher than in Control (Appendix A). In contrast, PUFA contents, namely LA C18:2n6 and ALA C18:3n3, were lower in all patients with

cancer in comparison to Control. In cachexic patients, levels of dihomo gamma linoleic acid (DGLA, C20:3n6) and eicosapentaenoic acid (DPA, C22:5n3) were lower compared to WSC. In the present data, only slight differences in the fatty acid profile in SAT were observed between the patient groups. Saturated fatty acid and MUFA percentages were not different according to cancer or cancer cachexia status (table 6) whereas plasma level variations were observed for PUFA, MUFA and saturated fatty acids for Control, WSC and CC (appendix A). Compared to γ-linolenic acid (GLA, C18:3n6) in SAT being lower for CC than Control, plasma levels were not detectable in any group. The content of α-linolenic acid (ALA, C18:3n3), the only detected n3 PUFA was also lower in the cachectic group, however this result was not significant (table 3). DPA, important for the synthesis of anti-inflammatory lipid mediators was not detected in SAT. Arachidonic acid (AA, C20:4n6), an important precursor for mostly pro-inflammatory lipid mediators, was not different among groups, neither in plasma (Appendix A) nor in SAT.

Table 6: Fatty acid profile in subcutaneous adipose tissue

Fatty acid (%)		Control n=13		WSC n=10		CC n=13		*p*
		median mean	range] ±SEM	median mean	range] ±SEM	median mean	range] ±SEM	
C12:0	Lauric acid	**0.28**	[0.12-0.94]	**0.23**	[0.0-0.61]	**0.21**	[0.0-0.99]	*0.55*
C14:0	Myristic acid	**3.07**	±0.21	**2.99**	±0.32	**2.81**	±0.28	*0.77*
C15:0	Pentadecanoic acid	**0.37**	±0.04	**0.45**	±0.06	**0.33**	±0.04	*0.21*
C16:0	Palmitic acid	**20.10**	±0.71	**20.20**	±1.25	**20.29**	±0.88	*0.99*
C16:1	Palmitoleic acid	**5.63**	±0.42	**5.52**	±1.06	**4.34**	±0.76	*0.39*
C17:0	Heptadecanoic acid	**0.33**	±0.04	**0.32**	±0.03	**0.38**	±0.02	*0.46*
C18:0	Stearic acid	**0.00**	[0.0-6.80]	**0.00**	[0.0-5.70]	**0.00**	[0.0-9.74]	*0.94*
C18:1	Oleic acid	**41.99**	[33.42-48.01]	**42.10**	[27.74-53.12]	**42.57**	[36.21-50.75]	*0.42*
C20:0	Arachidic acid	**0.22**	[0.0-0.86]	**0.27**	[0.02-0.75]	**0.23**	[0.11-0.62]	*0.67*
C20:1	Eicosenoic acid	**0.70**	[0.0-1.14]	**0.82**	[0.28-1.52]	**0.73**	[0.24-1.77]	*0.58*
C18:2n6	Linoleic acid	**20.00**	±1.01	**19.49**	±1.24	**19.31**	±0.59	*0.86*
C18:3n6	**γ-linolenic acid**	**0.01**	**[0.0-1.65]**	**0.00**	**[0.0-3.53]**	**0.00***	**[0.0-0.16]**	***0.04***
C20:2n6	Eicosadienoic acid	**0.23**	±0.04	**0.21**	±0.05	**0.29**	±0.05	*0.47*
C20:3n6	Dihomo-γ-linolenic acid	**0.28**	[0.0-0.64]	**0.27**	[0.06-0.55]	**0.16**	[0.0-0.60]	*0.99*
C20:4n6	Arachidonic acid	**0.55**	±0.07	**0.47**	±0.06	**0.48**	±0.06	*0.64*
C18:3n3	**α-linolenic acid**	**1.00**	**±0.12**	**0.95**	**±0.15**	**0.68**	**±0.06**	***0.09***

Fatty acid data expressed as % of all identified fatty acids. One-way ANOVA significance test was applied for parametric data (mean±SEM), and Kruskal-Wallis test for non-parametric data (median [min-max]), *significant difference vs Control.

When considering groups of fatty acids (saturated fatty acids, MUFA, n6-PUFA, n3-PUFA), there were no differences in SAT (figure 14A) between groups whereas there were observed plasma level variation for PUFA, MUFA and saturated fatty acids for Control, WSC and CC (appendix A). However, the balance between n6 and n3 PUFAs in both plasma and SAT shifted to n6 demonstrated by higher n6/n3 ratios in CC compared to Control (figure 14B). Although the percentage of C16:1 and C16:0 was not distinctive in the cachexia group (table 6), the ratio of the fatty acids tends to be lower in CC compared to Control (figure 14C).

The synthesis of C20:4n6 from C18:2n6 involves three steps. C18:2n6 is either desaturated to γ-linoleic acid C18:3n6 or elongated to C20:2n6 and figure 4D shows that there was no difference between the study groups in the relation of precursor and the elongated product. In the intermediate step C20:2n6 is converted to C20:3n6 and the ratio in CC is lower than in WSC (figure 14E), this result may be a hint for a shift to the elongation of C18:2n6 rather than desaturation. However no difference in the levels of the intermediate C20:3n6 to the final product C20:4n6 was found (figure 14F).

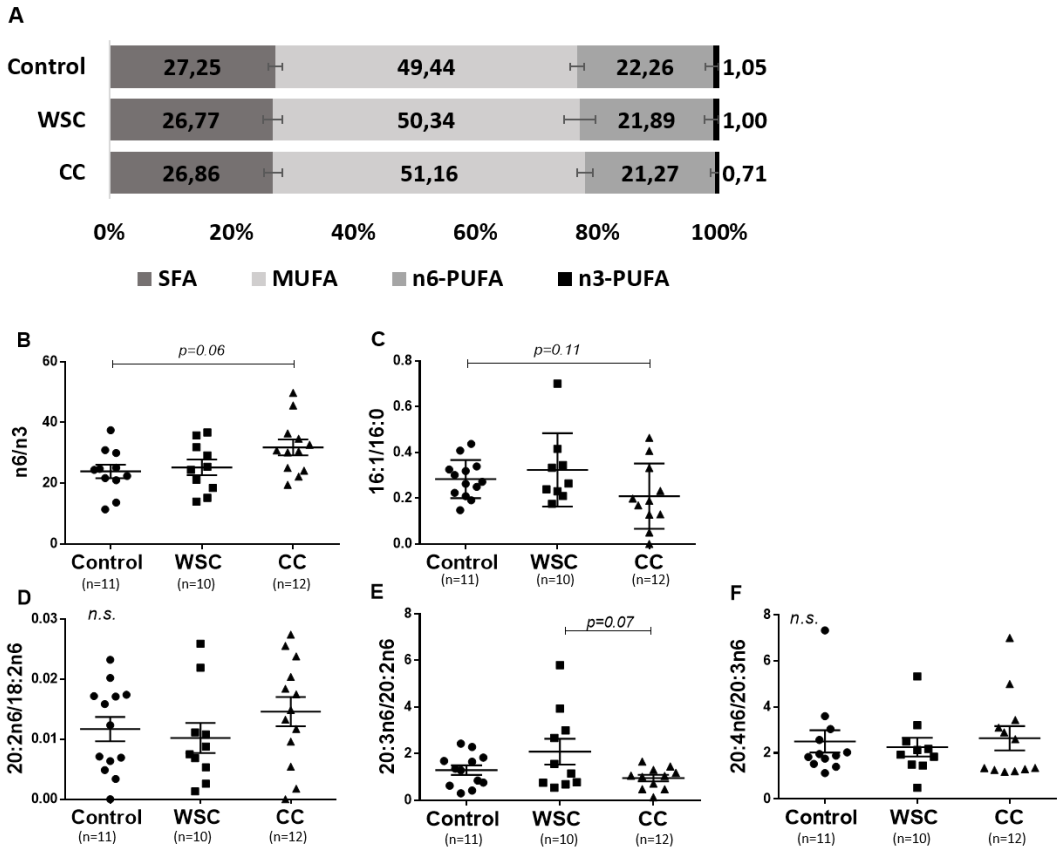


Figure 14: Fatty acid groups and ratios of n6-fatty acids. Data expressed as mean±SEM Statistical analysis One-way ANOVA.

Previously observed changes in the plasma fatty acid profile of cachectic patients could not be seen in SAT. Only slight changes in FAT fatty acid profile such as lower percentages of C18:3n6 and C18:3n3 were found in CC. In both plasma and SAT CC group presented higher n6/n3 ratios. A lower C16:1/C16:0 ratio in CC points to altered palmitate metabolism and the results further suggest changes in the AA synthesis pathway in this group.

3.4 SYNTHESIS OF SATURATED FATTY ACIDS

Fatty acid synthase (FASN) is one of the catalytic enzymes that synthesize saturated fatty acids (SFA) such as C12:0, C14:0 and C16:0. In SAT, neither the FASN mRNA expression (figure 5A) nor the protein expression (figure 15B) was different between groups. However, in VAT, FASN mRNA expression was significantly lower in CC than compared to Control (figure 15C). Furthermore, the FASN protein levels in VAT of WSC and CC were lower than those observed in Control patients (WSC 46.2% ± 9.1%, CC 53.0% ± 15.2% vs Control 100.0% ± 12.2% p<0.01; figure 15D).

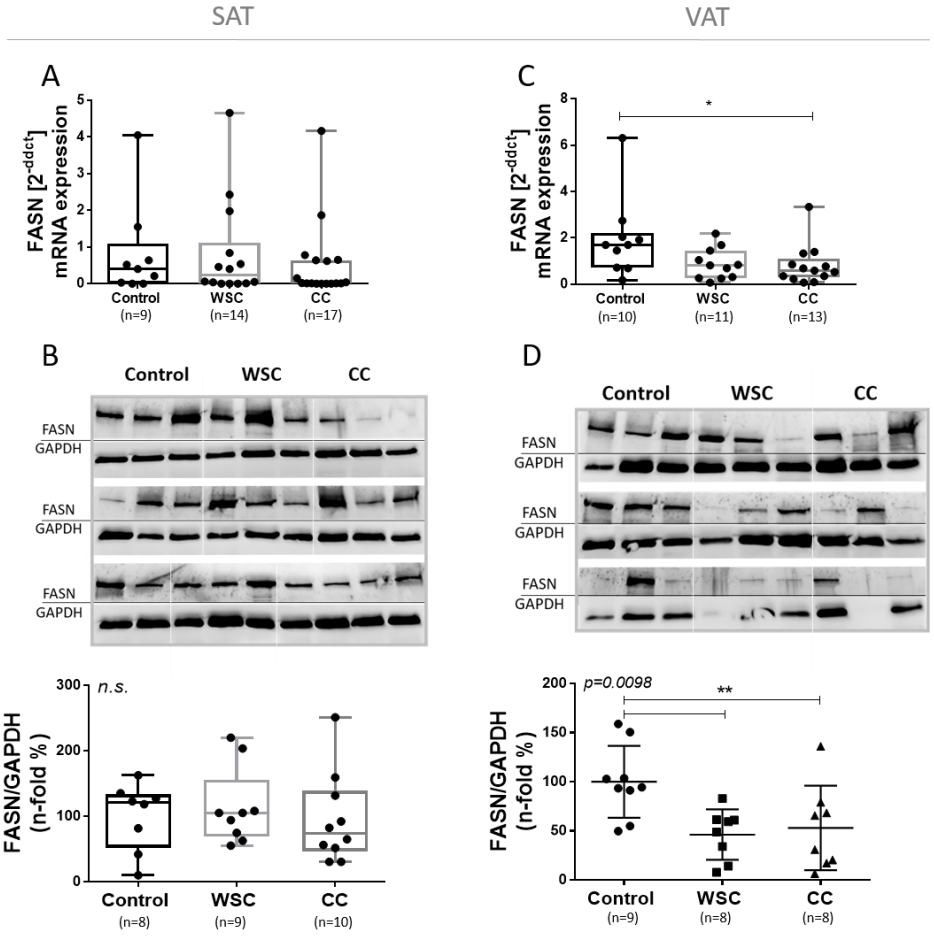


Figure 15: Fatty acid synthase mRNA and protein expression in SAT and VAT. (A, B) mRNA expression expressed as $2^{(-ddCT)}$, median [min-max]. Protein content expressed as n-fold (%) of Control band intensity normalized to GAPDH, (C) SAT median [min-max], (D) VAT mean±SEM. One-way ANOVA significance test was applied for parametric data and Kruskal-Wallis test for non-parametric data, *significant difference vs Control, *p<0.05, **p<0.01.

Lower FASN protein levels in visceral adipose tissue of cancer patients (WSC and CC) correlated negatively with CRP serum concentrations (figure 16A, 16C). This relationship was not observed in the Control group (figure 16A, 16B).

A

VAT FASN Protein (n-fold change) <> CRP

	all patients n=23	Control n=8	WSC n=8	CC n=7	WSC+CC n=15
Spearman r	**-0.74**	-0.31	**-0.88**	**-0.89**	**-0.76**
p-value	*<0.0001*	*0.46*	*0.0072*	*0.01*	*0.0015*

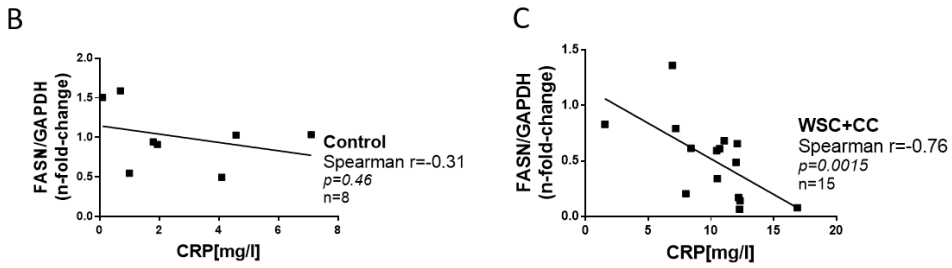


Figure 16: Negative correlation of fatty acid synthase and C-reactive protein. (A) Spearman correlation of VAT FASN relative protein content with CRP serum concentration [mg/l] for all groups (A) and exemplary graphs of Control (B) and cancer group WSC+CC (C).

Medium chain fatty acids are generated by the action of FASN (Zhu et al., 2014) and although levels of the pro-inflammatory palmitic acid C16:0 were higher in WSC and CC than in plasma of Control, the percentage myristic acid C14:0 which is another medium chain FA was lower in plasma of CC (appendix A). The data in table 7 showed that myristic acid C14:0 as well as lauric acid C12:0 were inversely associated with CRP and inflammatory cytokines. When considering the entire study group, the negative correlation of CRP levels and C14:0 was significant (n=36, r=-0.38, p=0.02). When broken down by group, a clear trend was observed in CC (n= 13, r=-0.54, p=0.06) and in cancer patients in general (WSC+CC n=23, r=-0.39, p=0.07) but not in the Control group.

Plasma concentrations of CCL2 and IL-6 had statistically significant negative correlations with C12:0 and C14:0 in CC and Control but not for WSC. When patient groups were combined, IL-6 concentrations in subcutaneous adipose tissue correlated negatively with saturated fatty acids (n=28, r=-0.4, p=0.04). Much of this relationship was explained by a strong correlation between IL-6 and SFA in CC (r=-0.73, p=0.02), while a

moderate negative correlation remained for the combined cancer group (WSC+CC r=-0.48, p=0.04).

Table 7: Negative correlation inflammatory markers and saturated fatty acids

		all patients	Control	WSC	CC	WSC+CC
		n=36	n=13	n=10	n=13	n=23
CRP <>C14:0	Spearman r	**-0.38**	0.12	-0.18	**-0.54**	**-0.39**
	p-value	*0.02*	*0.7*	*0.63*	*0.06*	*0.07*
		n=29	n=10	n=8	n=11	n=19
Plasma CCL2 <> C12:0	Spearman r	**-0.5**	**-0.53**	-0.42	**-0.62**	**-0.49**
	p-value	*0.005*	*0.12*	*0.3*	*0.04*	*0.03*
Plasma CCL2 <> C14:0	Spearman r	**-0.65**	**-0.64**	-0.62	**-0.65**	**-0.66**
	p-value	*0.0001*	*0.05*	*0.12*	*0.04*	*0.002*
Plasma IL-6 <> C12:0	Spearman r	**-0.69**	**-0.6**	**-0.87**	**-0.58**	**-0.7**
	p-value	*<0.0001*	*0.07*	*0.008*	*0.09*	*0.001*
Plasma IL-6 <> C14:0	Spearman r	**-0.65**	**-0.74**	-0.31	**-0.65**	**-0.62**
	p-value	*0.0002*	*0.02*	*0.46*	*0.04*	*0.006*
		n=28	n=10	n=9	n=9	n=18
SAT IL-6 <> SFA	Spearman r	**-0.4**	-0.28	-0.28	**-0.73**	**-0.48**
	p-value	*0.04*	*0.43*	*0.46*	*0.03*	*0.04*

Spearman correlation

The results concerning the synthesis of SFAs showed a decrease in FASN mRNA and protein levels in VAT of cancer patients. In all groups independent from cachexia, lower VAT-FASN-protein levels in VAT and SFA percentages in SAT were associated with higher concentrations of circulating inflammatory factors.

3.5 INFLAMMATION AND MUFA SYNTHESIS

The rate limiting step in the formation of MUFAs, namely palmitoleic acid C16:1 and oleic acid C18:1 from the saturated C16:0 and C18:0, is catalyzed by the stearoyl-CoA-desaturase (SCD). Since plasma levels of MUFA as well as their saturated precursors were higher in CC (appendix A) further analyses were performed on the SCD expression in adipose tissue. In SAT, the SCD mRNA expression was higher in CC than compared to Control (CC 6.61 [0.40-122] vs Control 0.64 [0.23-3.15], p=0.03, figure 17A), while in VAT, mRNA levels did not differ between the groups (figure 17C). However, while protein expression was not different among groups in both adipose tissue depots, large variance in SCD protein levels between individual samples was evident (figure 18C, D). Correlation analysis between mRNA expression and protein levels with the tested factors revealed no pattern.

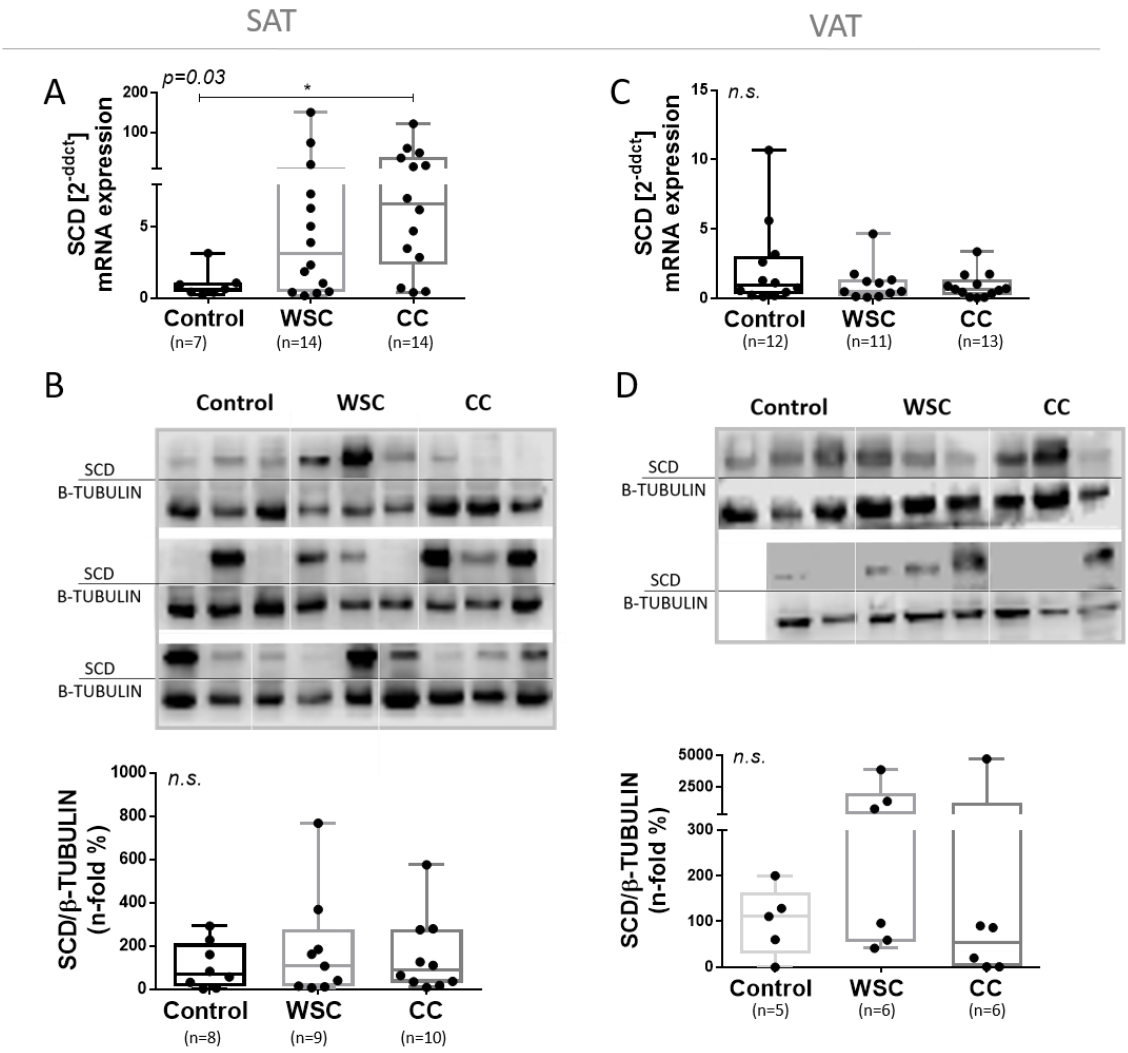


Figure 17: Stearoyl-CoA-desaturase mRNA and protein expression in SAT and VAT. (A, B) mRNA expression expressed as 2(-ddCT), median [min-max]. (C, D) Protein content expressed as n-fold (%) of Control band intensity normalized to β-Tubulin, median [min-max]. Kruskal-Wallis test was applied for non-parametric data, *significant difference vs Control.

In vitro, inflammatory cytokines were tested to see if they have an impact on SCD expression in two cell types present in adipose tissue: adipocytes and macrophages. In differentiated 3T3-L1 adipocytes, a 24hr incubation with TNF-α suppressed Scd-1 mRNA and protein expression (figure 18A) while TNF-α had no effect on SCD in THP-1 macrophages (figure 18B). In a next step, visceral adipose tissue explants of 7 patients (3 WSC, 4 CC) were incubated with TNF-α or without (Ctrl) ex vivo (figure 9 C, D). SCD mRNA expression was not influenced by TNF-α when combining the cancer groups nor

did individual groups demonstrate a homogenous pattern of SCD expression after TNF-α incubation (figure 18C). Immunoblotting showed that TNF-α had also no distinct effect on the SCD protein expression in WSC or CC (figure 18D).

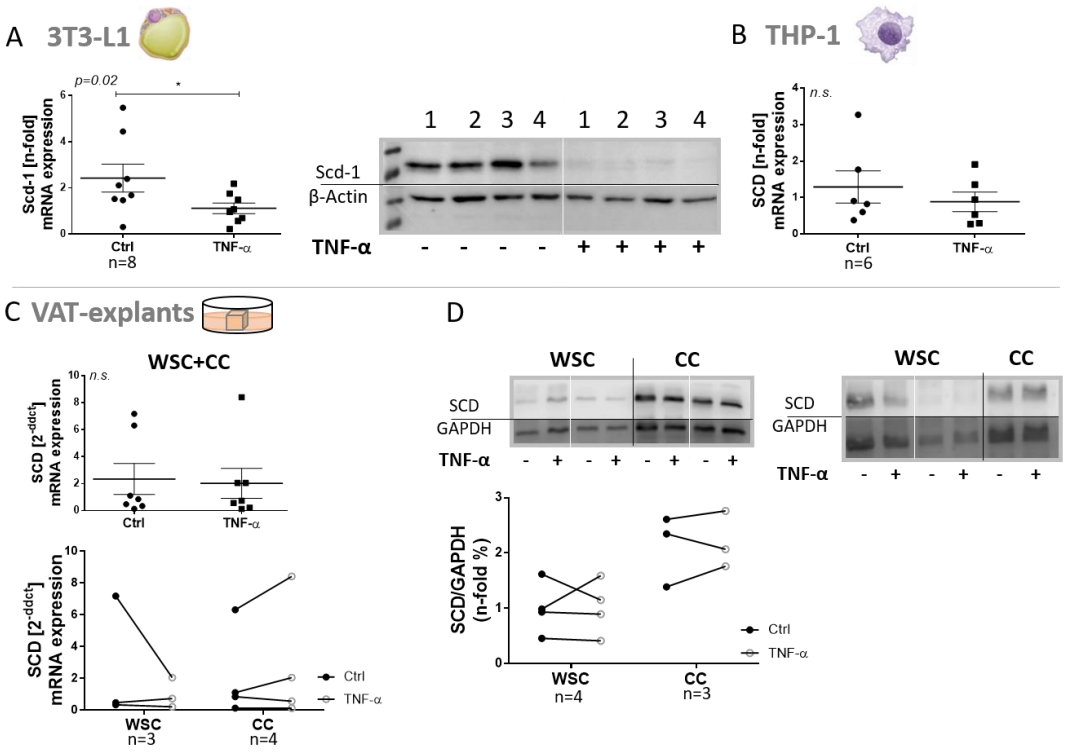


Figure 18: TNF-α down regulates SCD-1 in adipocytes but not in macrophages or adipose tissue explants. (A) SCD-1 gene expression and protein content of 3T3-L1 cells treated with 100 ng/ml TNF-α, relative mRNA expression was normalized to Hprt and protein to β-Actin (4 independent experiments). (B) SCD-1 expression (n-fold of $2^{(-ddCt)}$) in THP-1 cells. (C, upper graph) SCD expression in human visceral adipose tissue explants incubated with or without 100 ng/ml TNF-α from WSC and CC. (C, lower graph) SCD mRNA expression separated per group. (D) SCD immune blots of VAT explants incubated with TNF-α in 4 WSC and 3 CC patients normalized with GAPDH. *Significant difference by paired t-test (A, B, D) or Wilcoxon matched-pairs signed rank test (C).

Various correlations were observed between chemokines and products and substrates of SCD. A series of strong positive correlations were found between plasma IL-8 concentrations and C18:1 SAT levels in all groups except WSC (figure 19A). SAT IL-8 concentrations also correlated positively with C18:0 in the cancer group (WSC+CC r=0.51, p=0.03) explained by a strong correlation in CC (r=0.85, p=0.006) while no such individual relationship could be observed in WSC (figure 19A). Likewise, there was a positive correlation of CXCL10 plasma concentrations with MUFA in all groups but not in WSC. In contrast, when CXCL10 was correlated with palmitate, a substrate of SCD, a negative correlation was observed in WSC (r=-0.82, p=0.01) but not in CC (r=-0.17, p=0.70). The same pattern was found for the correlation of CXCL10 with SFA (figure 19A). Adipocytes and macrophages were then incubated with the two chemokines IL-8 (or its murine analog Mip-2) and CXCL10) in order to investigate if chemokines are capable to regulate SCD but neither IL-8 (figure 19B, C) nor CXCL10 (figure 19D, E) influenced SCD expression.

		all patients	Control	WSC	CC	WSC+CC
Plasma IL-8 <> 18:1		n=29	n=10	n=8	n=11	n=19
	Spearman r	**0.6**	**0.59**	0.47	**0.93**	**0.63**
	p-value	*0.001*	*0.08*	*0.25*	*0.0007*	*0.007*
SAT IL-8 <> 18:1		n=28	n=10	n=9	n=9	n=18
	Spearman r	0.29	-0.05	0.18	**0.85**	**0.51**
	p-value	*0.13*	*0.89*	*0.64*	*0.006*	*0.03*
SAT CXCL10 <> MUFA	*Spearman r*	**0.36**	**0.76**	0.03	**0.76**	**0.31**
	p-value	*0.07*	*0.04*	*0.94*	*0.04*	*0.02*
SAT CXCL10 <> 16:0	*Spearman r*	**-0.45**	-0.03	**-0.82**	-0.17	**-0.54**
	p-value	*0.02*	*0.95*	*0.01*	*0.7*	*0.03*
SAT CXCL10 <> SFA	*Spearman r*	**-0.44**	-0.05	**-0.63**	0	**-0.49**
	p-value	*0.03*	*0.93*	*0.08*	*>0.99*	*0.04*

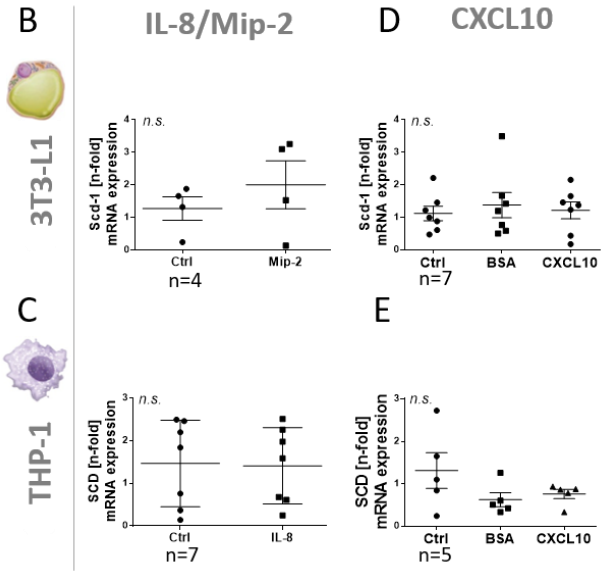


Figure 19: Positive correlation of chemokines and MUFA but negative correlation with SFA. (A) Spearman correlation of plasma and SAT IL-8 or SAT CXCL10 with MUFA and SFA for all groups. SCD-1 expression (n-fold of $2^{(-ddCt)}$) in 3T3 cells (B) and THP-1 cells (C) incubated with or without 50ng/ml IL-8/Mip-2. Impact of 50ng/ml CXCL10 on SCD-1 expression in 3T3 cells (D) and THP-1 cells (E). Data expressed as mean±SEM (B, D, E) or mean±SD (D). Significance was tested employing paired t-test (B, C) or Repeated measures one-way ANOVA (D, E).

In vitro 3T3-L1 and THP-1 cells were exposed to IL-6 and SCD substrate palmitate to test their potential impact on SCD regulation. In both adipocytes and macrophages, SCD expression was not altered by IL-6 (figure 20A, B), palmitate (figure 20C, D) or LPS that served as a positive control for that inflammatory pathway (figure 20E, F). However, a single case ex vivo exploratory test in a triplicate set of VAT explants from a CC patient showed that palmitate induced SCD up to 3-fold (figure 20G).

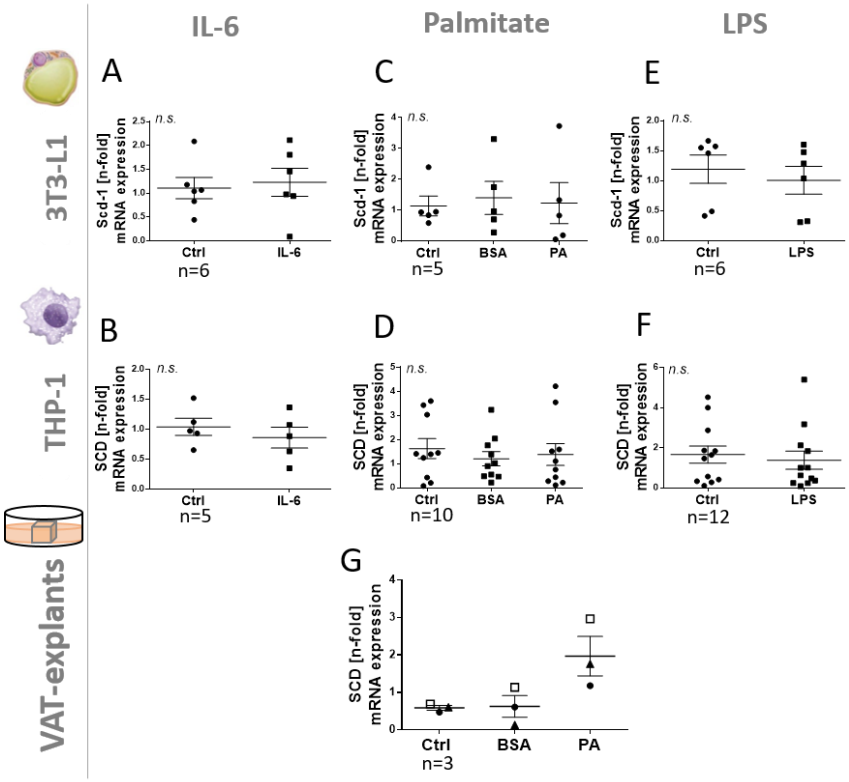


Figure 20: SCD is not regulated by IL-6, palmitate and LPS in 3T3 and THP cells. SCD-1 expression (n-fold of $2^{(-ddCt)}$) in 3T3 cells (A, C, D) and THP-1 cells (B, D, F) incubated with or without 100ng/ml IL-6 (A, B), 100nM palmitate (C, D, G) and 100ng/ml LPS (E, F). (G) SCD expression in VAT explants from one patient (triplicate). Data expressed as mean±SEM. Significance was tested employing paired t-test (A, B, E, F, G) or Repeated measures one-way ANOVA (C, D).

In summary, while SCD-1 apparently was induced on the mRNA but not on the protein level in SAT of CC patients, it was repressed in 3T3-L1 adipocytes by TNFα both on the mRNA and protein level. Other cytokines or chemokines had not impact on SCD-1 expression in vitro. However, like circulating cytokines, SAT chemokines were reversely associated with SFAs but positively related to MUFAs.

3.6 FATTY ACID ELONGATION AND DESATURATION IN THE INFLAMMATORY ENVIRONMENT IN CANCER CACHEXIA

It has previously been shown that both IL-6 and TNF-α can induce the production of pro-inflammatory eicosanoids in inflammatory cells (Harizi et al., 2008). These eicosanoids are derivatives of arachidonic acid that is produced from linoleic acid via intermediate forms, namely C18:3n6 or C20:2n6 and C20:3n6. In patients it was found that TNF-α plasma concentrations correlated with higher n6 PUFA levels in SAT (table 8). For linoleic acid C18:2n6, C20:2n6, C20:3n6 and C20:4n6 this relationship could be seen in total group, CC and WSC+CC, though not in WSC.

The strongest correlations of TNF-α with C20:3n6 were found in the Control group ($r=0.62$, $p=0.05$) and with C20:4n6 in CC ($r=0.61$, $p=0.04$). Total poly-n6 PUFA correlated with TNF-α in the cancer group (WSC+CC), explained by the correlation coefficient of $r=0.67$ in CC ($p=0.03$). CXCL10 was positively correlated with C20:3n6 in the whole group ($r=0.57$, $p=0.001$), CC ($r=0.64$, $p=0.04$) and WSC+CC ($r=0.66$, $p=0.002$) but not WSC. Higher CRP serum concentrations correlated moderately with n6/n3 ratios in the total group ($r=0.37$, $p=0.03$, table 8).

Table 8: Correlations between fatty acids and TNF-α, CXCL10 and CRP plasma concentrations

		all patients	Control	WSC	CC	WSC+CC
		n=29	n=10	n=8	n=11	n=19
TNF-α <> 18:2n6	Spearman r	0.27	-0.006	0.45	**0.66**	**0.66**
	p-value	*0.16*	*>0.99*	*0.27*	*0.03*	*0.003*
TNF-α <> 20:2n6	Spearman r	**0.44**	**0.62**	-0.2	**0.58**	**0.44**
	p-value	*0.02*	*0.06*	*0.63*	*0.07*	*0.07*
TNF-α <> 20:3n6	Spearman r	**0.52**	**0.62**	0.12	0.51	**0.55**
	p-value	*0.004*	*0.05*	*0.78*	*0.11*	*0.02*
TNF-α <> 20:4n6	*Spearman r*	**0.43**	0.49	-0.01	**0.61**	**0.49**
	p-value	*0.02*	*0.15*	*0.99*	*0.04*	*0.04*
TNF-α <> n6-PUFA	*Spearman r*	**0.32**	0.11	0.17	**0.67**	**0.67**
	p-value	*0.09*	*0.79*	*0.69*	*0.03*	*0.003*
CXCL10 <> 20:3n6	*Spearman r*	**0.57**	0.38	0.38	**0.64**	**0.66**
	p-value	*0.001*	*0.27*	*0.35*	*0.04*	*0.002*
		n=34	n=12	n=10	n=13	n=23
CRP <> n6/n3	Spearman r	**0.37**	**0.56**	0.14	0.43	**0.4**
	p-value	*0.03*	*0.08*	*0.7*	*0.14*	*0.06*

Spearman correlation

C20:4n6 is synthesized by the action of FADS2, ELOVL5 and FADS1 (Figure 6). It was therefore investigated if the expression of these fatty acid modifying enzymes changes due to systemic inflammation in cancer cachexia. In SAT, FADS2 mRNA expression was not different between groups (figure 21A) while VAT FADS2 tended to be lower in CC than in the other groups (Control 1.25 [0.03-16.55], WSC 0.36 [0.13-3.54], CC 0.26 [0.01-2.22], p=0.06, figure 21B). ELOVL5 mRNA levels did not differ among groups in both tissue depots (figure 21C, D). The lower FADS2 expression in VAT correlated in a negative manner with CRP serum concentrations in the cancer group (WSC+CC r=-0.49, p=0.03) due to a correlation in WSC (r=-0.65, p=0.04, figure 21E).

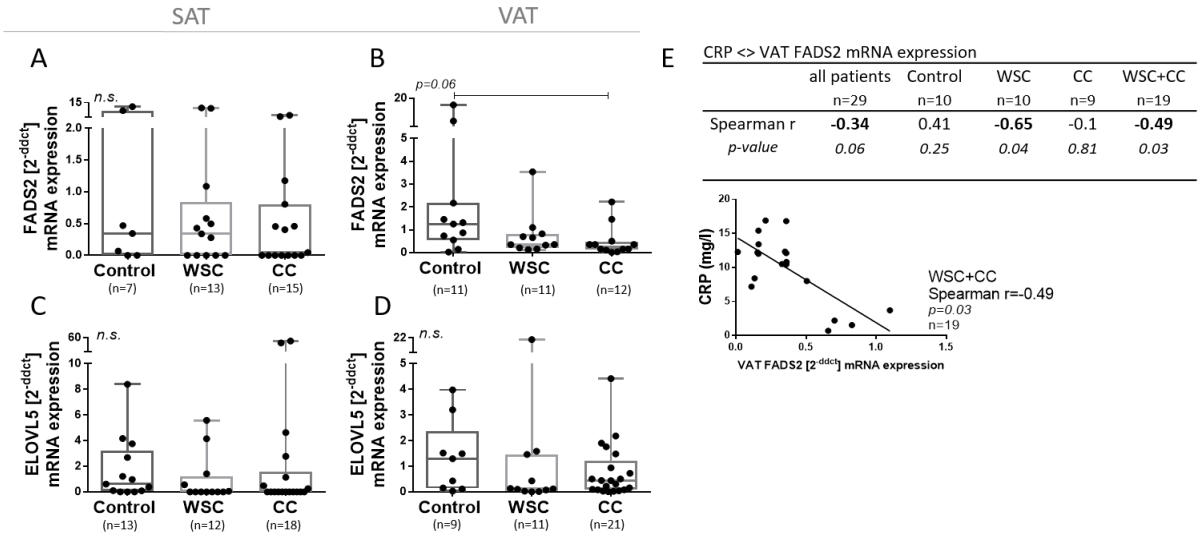


Figure 21: Lower FADS2 expression in VAT of cachectic patients and no differences in ELOVL5 gene expression in adipose tissue. mRNA expression of FADS2 (A, B) and ELOVL5 (C, D) in SAT (A, C) and VAT (B, D). Data expressed as $2^{(-ddCT)}$, median [min-max]. (E) Spearman correlation of serum CRP concentrations [mg/l] with VAT FADS2 expression for all groups and exemplary graphs the cancer group WSC+CC. Kruskal-Wallis test was applied for non-parametric data, *significant difference vs Control.

In 3T3-L1 cells but not in THP-1 cells, inflammation was negatively associated with FADS2 gene expression. It was shown that incubation with TNF-α suppressed FADS2 expression significantly (figure 22A, B). ELOVL5 mRNA expression in 3T3-L1 cells was not altered after TNF-α incubation while in THP-1 macrophages ELOVL5 was induced by the inflammatory stimulus (figure 22C, D). IL-6 had no impact on FADS2 gene expression in 3T3-L1 cells or THP-1 cells (figure 22E, F) and IL-6 had also no regulatory effect on ELOVL5 in 3T3-L1 cells (figure 22H). In THP-1 macrophages IL-6 increased ELOVL5 mRNA expression up to 1.4-fold (Ctrl 0.68±0.11, IL-6 0.97±0.18, $p<0.05$, figure 22I).

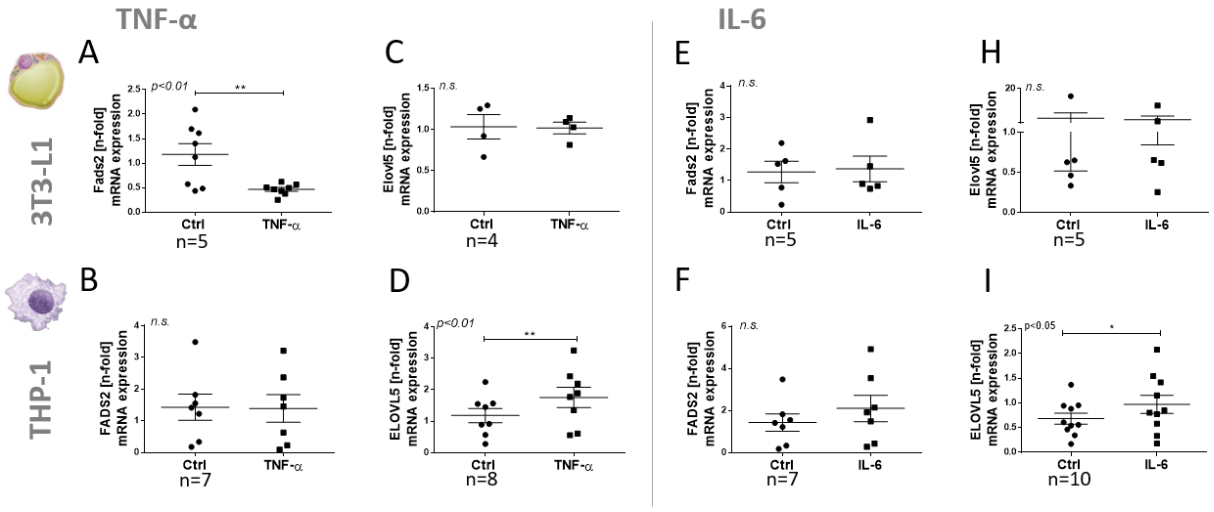


Figure 22: Pro-inflammatory cytokines suppress FADS2 expression in adipocytes and induce ELOVL5 in Macrophages. FADS2 (A, B, E, F) and ELOVL5 (C, D, H, I) expression (n-fold of $2^{(-ddCt)}$) in 3T3 cells (upper panel) and THP-1 cells (lower panel) incubated with or without 100ng/ml TNF-α (left) or IL-6 (right). Data expressed as mean±SEM (B, D, E) or mean±SD (D). Significance was tested employing paired t-test, *p<0.05, **p<0.01.

Table 9 shows that other inflammatory stimuli namely IL-8, CXCL10 and palmitate have no impact on FADS2 and ELOVL5 mRNA expression THP macrophages (upper section) or 3T3 adipocytes (lower section). Only LPS was able to induce ELOVL5 in (Ctrl 1.05±0.18, LPS 1.96±0.39, p=0.0095 table 9 upper section).

Table 9: Chemokines and palmitate have no impact on FADS2 and ELOVL5 expression

THP-1

	Ctrl		IL-8		n	p value
FADS2	1.46	±0.42	1.42	±0.40	7	0.90[a]
ELOVL5	0.78	[0.69-2.93]	0.98	[0.45-2.74]	7	0.69[b]

	Ctrl		LPS		n	p value
FADS2	1.43	±0.35	1.41	±0.45	10	0.97[a]
ELOVL5	**1.05**	**±0.18**	**1.96**	**±0.39**	**9**	**0.0095[a]**

	Ctrl		BSA		PA		n	p value
FADS2	1.45	[0.18-5.46]	1.12	[0.49-5.83]	1.26	[0.04-4.93]	8	0.97[c]
ELOVL5	1.19	[0.35-2.78]	1.33	[0.49-2.70]	1.19	[0.29-3.48]	12	0.87[d]

	Ctrl		BSA		CXCL10		n	p value
FADS2	1.24	±0.39	0.71	±0.16	0.71	±0.26	5	0.22[e]
ELOVL5	1.06	±0.18	0.9	±0.15	1	±0.11	7	0.43[e]

3T3-L1

	Ctrl		Mip-2		n	p value
Fads2	1.06	±0.20	1.34	±0.56	4	0.58[a]
Elovl5	1.04	±0.15	1.85	±0.68	4	0.26[a]

	Ctrl		BSA		CXCL10		n	p value
Fads2	1.38	±0.36	1.07	±0.25	0.98	±0.25	8	0.07[e]
Elovl5	1.26	±0.15	1.37	±0.24	1.42	±0.28	5	0.40[e]

	Ctrl		FCS		PA		LPS		n	p value
Fads2	1.36	±0.50	1.4	±0.61	1.82	±0.28	1.34	±0.2	4	0.42[e]
Elovl5	1.04	±0.15	0.88	±0.14	1.16	±0.09	1.05	±0.08	4	0.40[e]

FADS1 and ELOVL5 expression (n-fold of $2^{(-ddCt)}$) in THP-1 and 3T3 cells incubated with or without 50ng/ml IL-8/Mip-2, 50ng/ml CXCL10, 100nM palmitate and 100ng/ml LPS. (G) SCD expression in VAT explants from one patient (triplicate). Data expressed as mean±SEM (parametric data) or median [min-max] (non-parametric data). Significance was tested employing a=paired ttest, b=Wilcoxon matched-pairs signed rank test, c=friedman test, d=Kruskal-Wallis, e=repeated measures one-way ANOVA.

FADS1 mRNA expression was not different among groups in SAT and VAT (figure 23A, B) and also FADS1 protein levels were not affected by cancer or cancer cachexia in both adipose tissue depots (figure 23C, D).

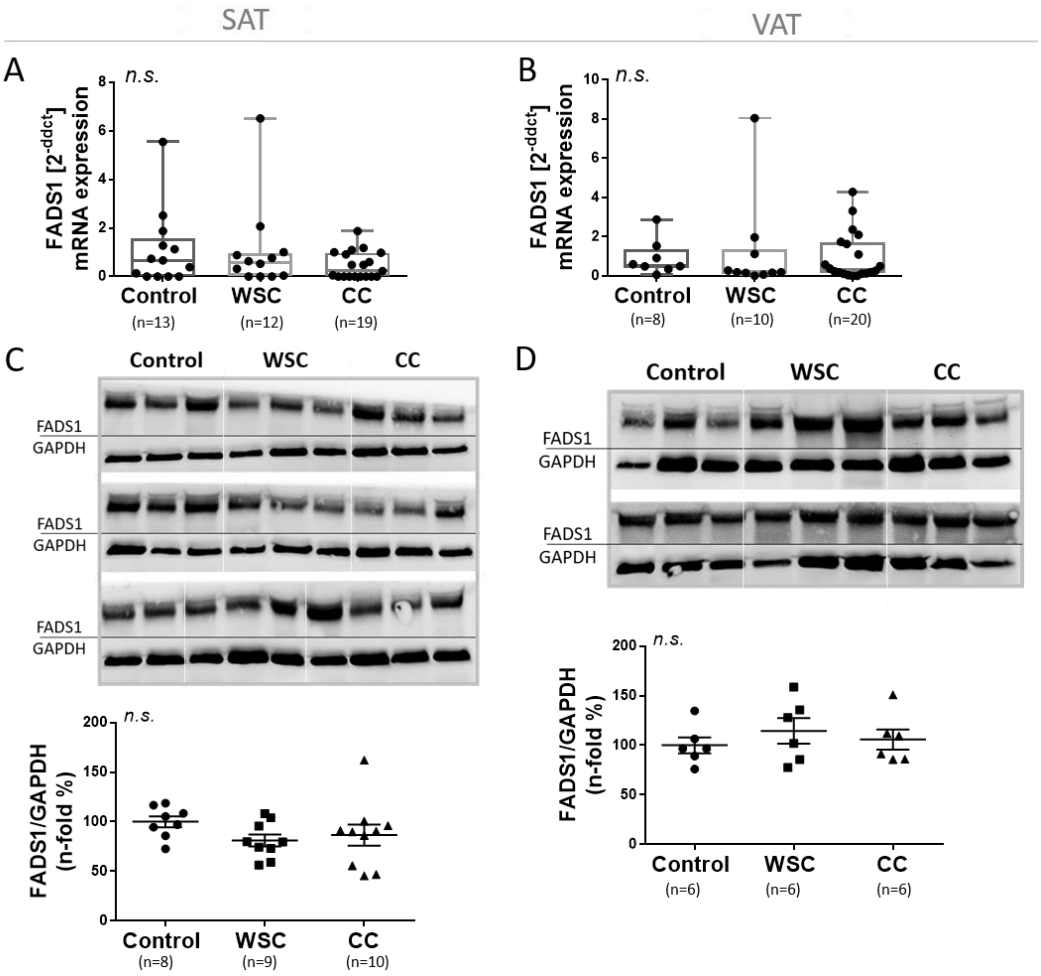


Figure 23: No differences in FADS1 mRNA and protein expression in SAT and VAT. (A, B) mRNA expression expressed as $2^{(-ddCT)}$, median [min-max]. (C, D) Protein content expressed as n-fold (%) of Control band intensity normalized to GAPDH, median [min-max]. Kruskal-Wallis test was applied for non-parametric data, *significant difference vs Control.

Although no differences in FADS1 mRNA and protein levels were observed, the visceral mRNA expression correlated negatively with TNF-α and CCL2 plasma concentrations in the total group (TNF-α r=-0.40, p=0.04; CCL2 r=-0.41, p=0.04, figure 24).

A

		all patients	Control	WSC	CC	WSC+CC
		n=25	n=6	n=8	n=13	n=20
Plasma TNF-α <> VAT FADS1 mRNA expression	Spearman r	**-0.4**	0.03	-0.19	-0.38	**-0.43**
	p-value	*0.04*	*>0.99*	*0.65*	*0.2*	*0.06*
Plasma CCL2 <> VAT FADS1 mRNA expression	Spearman r	**-0.41**	-0.37	-0.17	**-0.54**	**-0.39**
	p-value	*0.04*	*0.5*	*0.7*	*0.06*	*0.08*

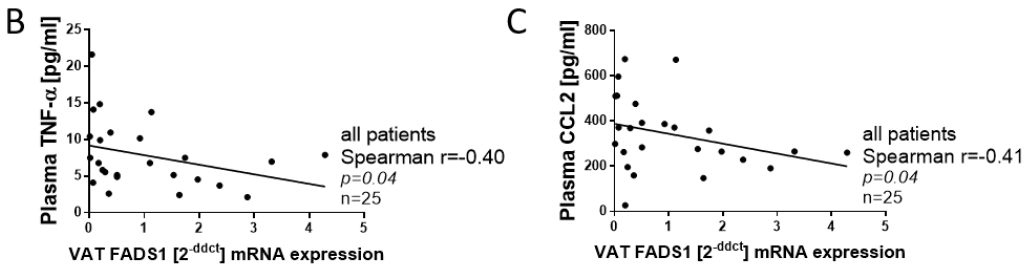


Figure 24: Negative correlation of FADS1 and plasma cytokines. (A) Spearman correlation of VAT FADS1 mRNA expression with TNF-α and CCL2 plasma concentrations [pg/ml] for all groups and exemplary graphs of the total group (B TNF-α) and (C CCL2).

The incubation of adipocytes and macrophages with the pro-inflammatory cytokines IL-6 and TNF-α revealed that IL-6 did not alter FADS1 expression in 3T3-L1 cells (figure 25 A) but significantly induced FADS1 in THP-1 cells (Ctrl 1.50±0.26, IL-6 2.10±0.36, p=0.01, figure 16 B). In contrast, TNF-α suppressed FADS1 in 3T3 cells (Ctrl 1.08±0.18, TNF-α 0.77±0.08, p=0.02, figure 25C) but not in THP cells (figure 25D). However, ex vivo in VAT explants of WSC and CC, TNF-α had no impact on FADS1 mRNA expression (figure 25E) or protein levels (figure 25F).

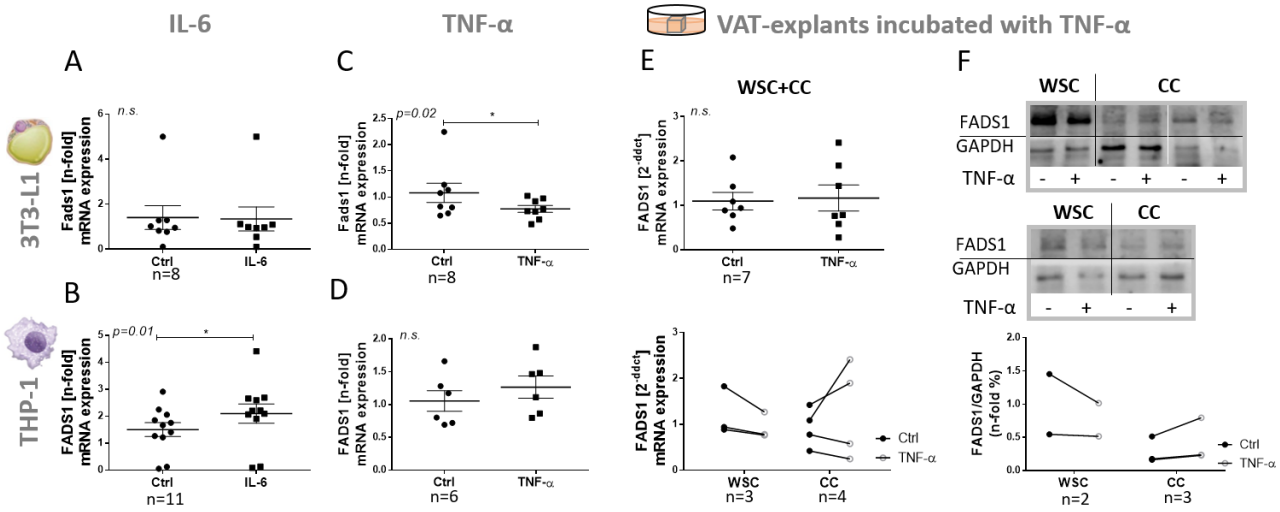


Figure 25: Pro-inflammatory cytokines suppress FADS1 expression in adipocytes and induce in Macrophages. FADS1 mRNA expression (n-fold of $2^{(-ddCt)}$) in 3T3 cells (upper panel A, C), THP-1 cells (lower panel B, D) and VAT explants (E, F) incubated with or without 100ng/ml IL-6 (A, B) or TNF-α (C-F). (E top) FADS1 mRNA expression in VAT explants incubated with or without 100 ng/ml TNF-α from WSC+CC and separated by groups (E bottom). (F) FADS1 immune blots of VAT explants incubated with TNF-α in 2 WSC and 3 CC patients normalized to GAPDH. *Significant difference by paired t-test (B, D, E) or Wilcoxon matched-pairs signed rank test (A, C).

No changes in FADS1 mRNA expression were observed when 3T3 and THP cells were incubated with the chemokines IL-8/Mip2 (figure 26A, B) or CXCL10 (figure 26C, D). Palmitate and LPS had no impact on FADS1 expression in 3T3 adipocytes (figure 26E) but palmitate induced FADS1 in THP macrophages (PA 2.04 [0.87-18.86] vs Ctrl 0.64 [0.26-3.31] and BSA 0.58 [0.37-3.54], p=0.0018, figure 26F) and LPS increased FADS1 expression up to 2-fold (Ctrl 1.05±0.17, LPS 1.96±0.39, p=0.0095, figure 26G). In a test VAT explants were incubated ex vivo with palmitate which also resulted in an induction of FADS1 (figure 26H). Since adipocytes (3T3L1) in contrast to macrophages (THP-1) were refractory to palmitate and LPS, the induction of FADS1 in VAT most likely reflects an induction in infiltrating inflammatory cells

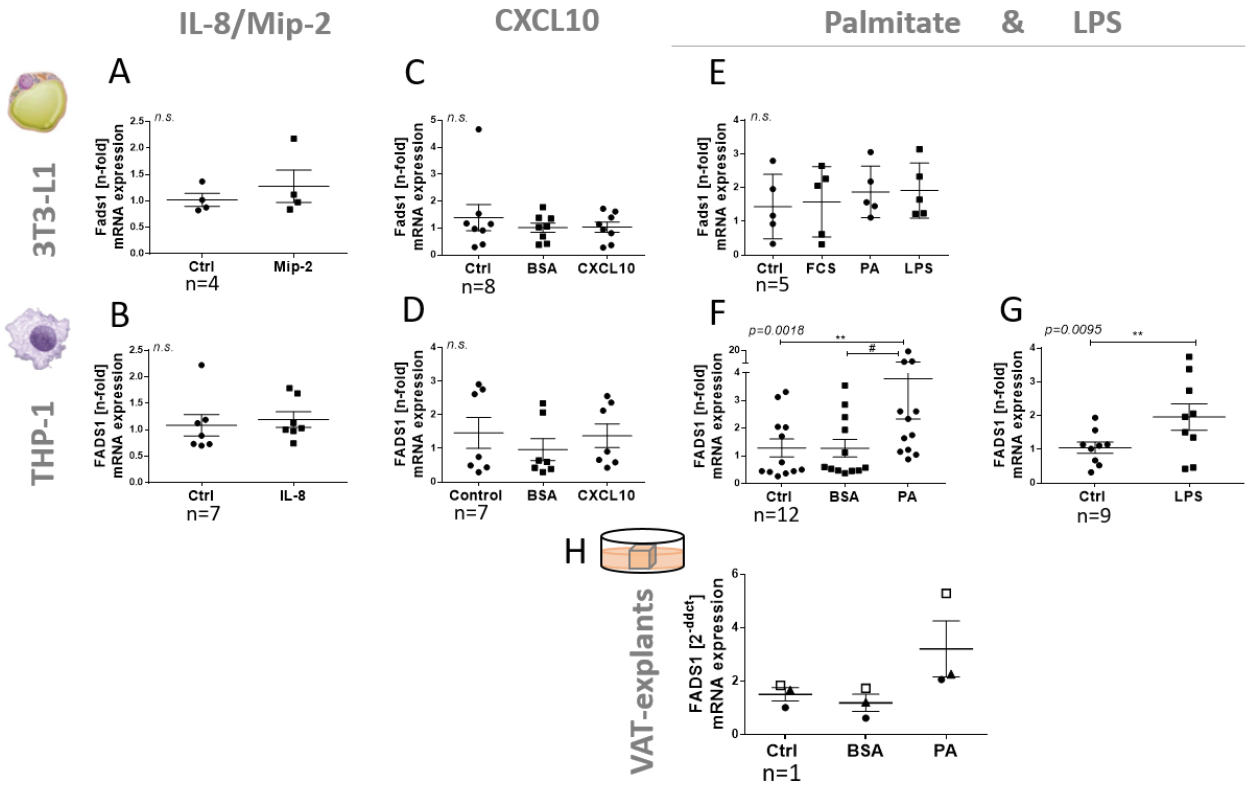


Figure 26: FADS1 is induced by palmitate but not regulated by IL-8 and CXCL10 in THP cells. FADS1 expression (n-fold of $2^{(-ddCt)}$) in 3T3 cells (upper row), THP-1 cells (middle) and VAT explants (bottom) incubated with or without 50ng/ml IL-8 (A, B), 50ng/ml CXCL10 (C, D), 100nM palmitate (E, F, H) and 100ng/ml LPS (E, G). (H) FADS1 expression in VAT explants from one patient (triplicate). Data expressed as mean±SEM. Significance was tested employing paired t-test (A, B, G), friedman-test (C, D, F) or repeated measures one-way ANOVA (E).

Systemic inflammation was associated with several changes in PUFA synthesis. A positive correlation was found between plasma concentrations of pro-inflammatory factors and metabolites of the AA synthesis pathway. From the enzymes involved in the synthesis of AA, EPA and DHA only FADS2 mRNA expression was lower in VAT of CC patients while ELOVL5 and FADS1 gene and protein levels were not altered in adipose tissue among groups. However, FADS2 and FADS1 VAT-mRNA levels correlated reversely with circulating pro-inflammatory factors and in vitro, TNF-α suppressed the expression of these enzymes in adipocytes. In contrast, in immune cells, FADS1 and ELOVL5 were induced by TNF-α and IL-6.

3.7 PGE_2 IN ADIPOSE TISSUE INFLAMMATION

PGE_2 was measured in VAT as this tissue has a greater inflammatory capacity than subcutaneous adipose tissue (Ibrahim, 2010). No differences in PGE_2 concentrations were observed among the study groups (figure 27A). When PGE_2 concentration was correlated with CRP serum levels, a positive correlation was found in the total group and in cancer patients (WSC+CC). The same positive pattern, though not statistically significant due to sample size, was found in WSC and CC though the Control group had a negative correlation (figure 27B).

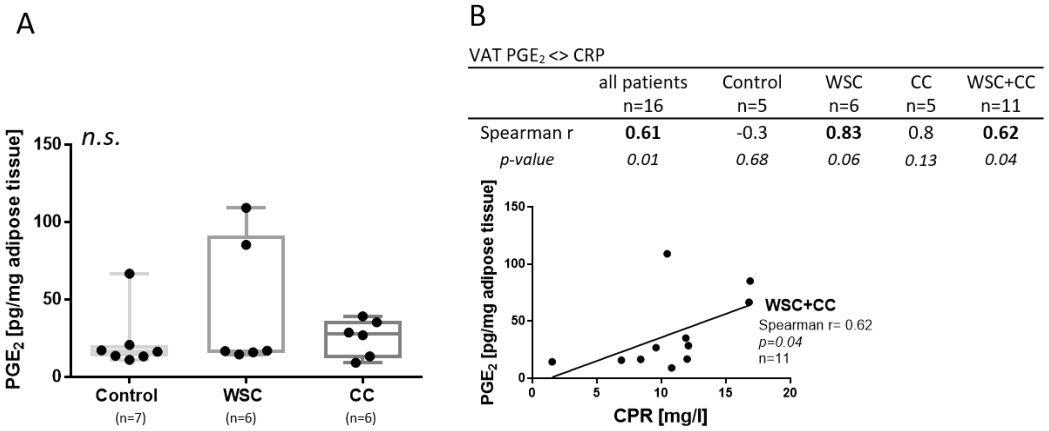


Figure 27: PGE_2 concentrations in VAT are not different among groups but correlate positively with CRP. (A) Prostaglandin E2 (PGE_2) [pg/mg adipose tissue wet weight]. (B) Spearman correlation of VAT PGE_2 with CRP serum concentrations for all groups and exemplary graphs of the cancer group WSC+CC.

The gene expression of the constitutive prostaglandin synthase COX-1 was measured in SAT and VAT with no differences among groups (figure 28A, B). However, it is possible that COX-1 is affected by the systemic inflammation typically observed in cancer cachexia since COX-1 in SAT correlated positively with CRP serum concentrations (figure 28C) and COX-1 mRNA levels in VAT with CXCL10 plasma concentrations in the total group and cancer group (all patients r=0.40, p=0.04; WSC+CC r=0.74, p=0.0001). This appears to be an associative effect apparent in cachectic patients but not in WSC (CC r=0.63, p=0.01; WSC r=-0.12, p=0.79; figure 28D).

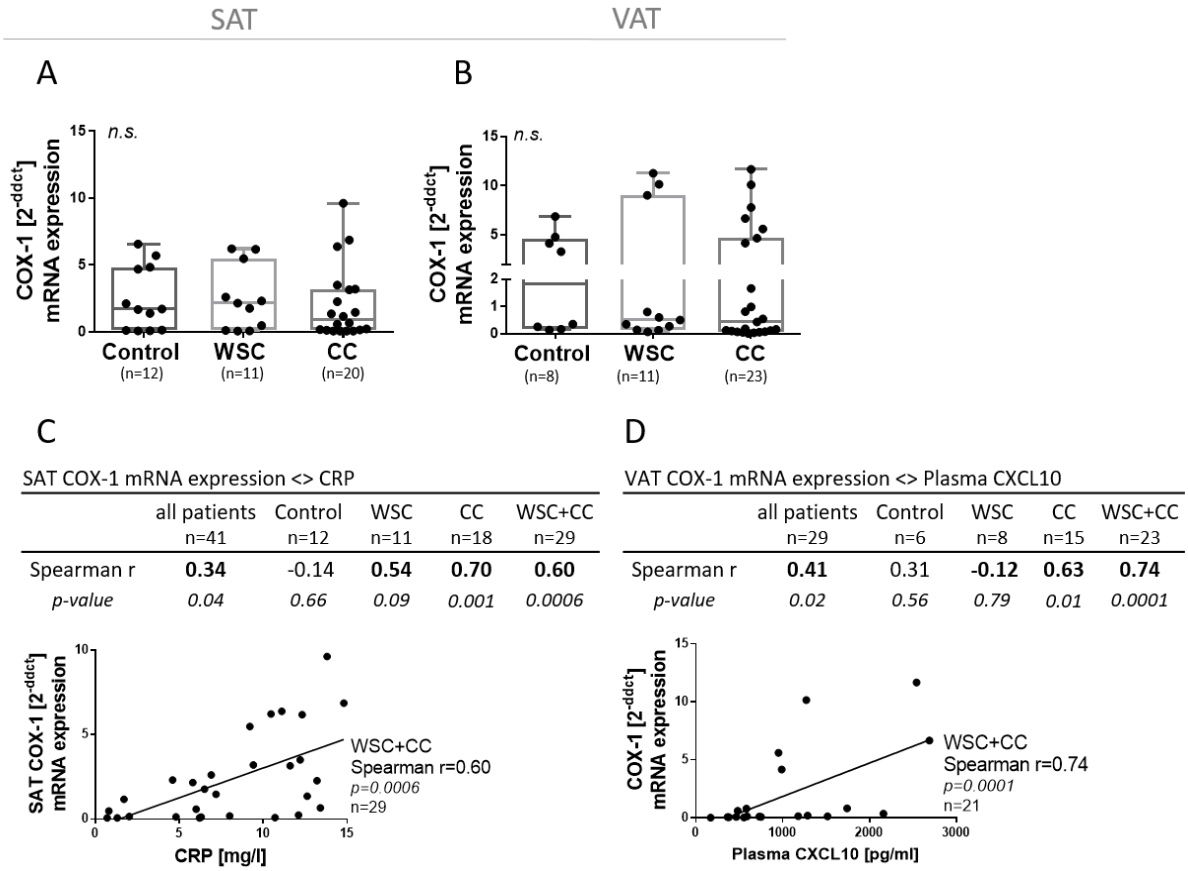


Figure 28: COX-1 in SAT and VAT correlate with systemic inflammatory factors. mRNA expression of COX-1 in SAT (A) and VAT (B), data expressed as $2^{(-ddCT)}$, median [min-max]. (C) Spearman correlation of VAT COX-1 expression with CRP serum concentrations [mg/l] and CXCL10 plasma levels (D) for all groups and exemplary graphs of the cancer group WSC+CC. Kruskal-Wallis test was applied for non-parametric data.

In VAT, mRNA levels of the inducible prostaglandin synthase COX-2 were assessed with higher expression in CC than compared to Control (Control 0.99 [0.01-15.88] vs CC 2.97 [0.75-29.46], p=0.04, figure 20A). The COX-2 expression correlated positively with the systemic inflammatory marker CRP (figure 29B) in all groups. COX-2 was correlated with the VAT IL-6 mRNA expression in the total group, cancer group and CC, where the strongest relationship was observed, but not independently in WSC (figure 29C). The

correlation of COX-2 with IL-6 was also observed in the Control group (figure 29C).

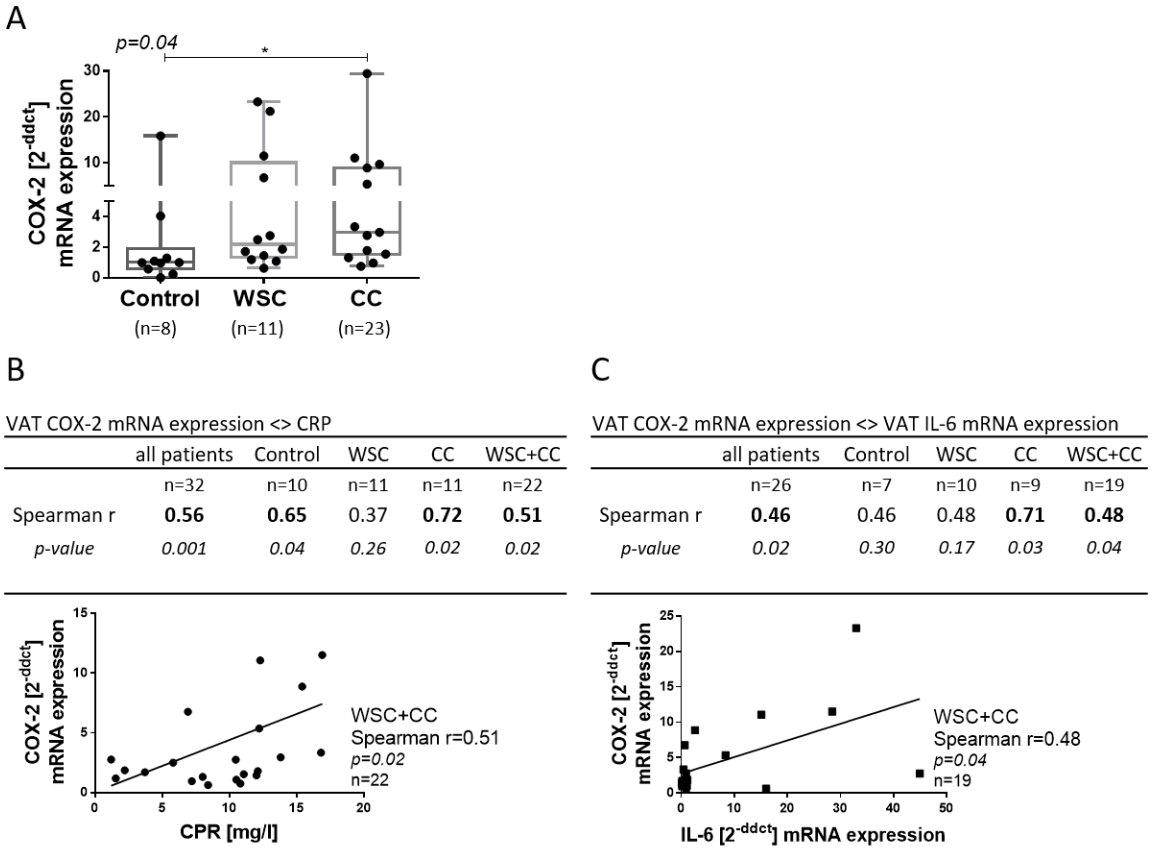


Figure 29: Higher COX-2 expression in VAT of cachectic cancer patients. mRNA expression of COX-2 in VAT (A) data expressed as $2^{(-ddCT)}$, median [min-max]. (B) Spearman correlation of VAT COX-2 expression with CRP serum concentrations [mg/l] and VAT IL-6 mRNA expression (C) for all groups and exemplary graphs of the cancer group WSC+CC. Kruskal-Wallis test was applied for non-parametric data, *significant difference vs Control.

As it was shown that TNF-α and IL-6 concentrations are higher in plasma of cachectic patients (Figure 11), the impact of these cytokines on COX-1 and COX-2 expression was tested in vitro in THP-1 macrophages. COX-1 was not regulated by TNF-α or IL-6 (figure 30A). COX-2 was induced 1.5-fold by IL-6 (figure 30B) this finding corresponded to the positive correlation of COX-2 with IL-6 mRNA levels in VAT (figure 29C). In contrast, TNF-α did slightly suppressed COX-2 about 20% (Ctrl 1.04±0.12, IL-6 2.56±0.24, TNF-α 0.76±0.12, $p<0.0001$; figure 30C).

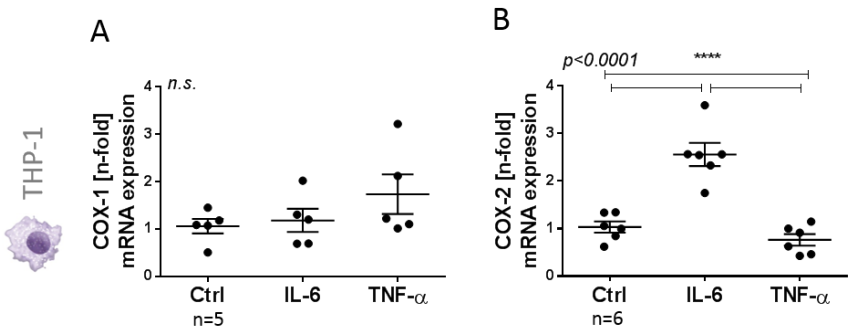


Figure 30: COX-2 but not COX-1 gene expression is modulated by IL-6 and TNF-α in THP macrophages. (A) COX-1 mRNA expression (n-fold of $2^{(-ddCt)}$) and COX-1 expression (B) in THP-1 cells incubated with or without 100ng/ml IL-6 and TNF-α. Significant difference by repeated measures one-way ANOVA.

Recently, the PGE_2-induced inflammation in macrophages in the context of the metabolic syndrome was studied and it was shown that insulin (INS) induced SCD in THP-1 macrophages and that PGE_2 and LPS inhibited this induction (Klauder et al. unpublished data). Additionally it was now evaluated how PGE_2 in combination with LPS and Insulin may regulate SCD since insulin resistance is a complication in cancer cachexia and adipocyte function is impaired during cancer cachexia.

PGE_2, as a single stimulus, statistically decreased SCD gene expression in THP-1 macrophages (Ctrl 0.84 [0.34-2.72] vs PGE_2 0.49 [0.31-1.20], p=0.0039) while in 3T3 adipocytes, PGE_2 reduction of scd-1 expression approached significance (Ctrl 0.64 [0.48-2.75] vs PGE_2 0.50 [0.32-1.52], p=0.054; figure 31B). However, these effects were not observed in the multi-stimulus setup using a smaller sample size. Independently, insulin and LPS alone did not influence scd-1 expression (figure 31C) but when cells were incubated with all three stimuli, scd-1 mRNA expression was reduced by up to 80% than compared to Ctrl or the single stimuli (INS LPS PGE_2 0.21±0.07 vs Ctrl 1.19±0.24, INS 0.84±0.22, LPS 1.01±0.23, PGE_2 0.88±0.19 figure 31C a). In 3T3 adipocytes that were incubated with a combination of PGE_2 and insulin (PGE_2 INS), scd-1 expression was suppressed by 50% compared to Ctrl (PGE_2 INS 0.57±0.21 (p=0.0004, figure 31C b). The same was found in samples incubated with LPS and insulin (LPS INS) or LPS and PGE_2 (PGE_2 LPS), though the post-test was not significant (LPS INS 0.62±0.16; PGE_2 LPS 0.63±0.18; figure 31C). TNF-α and TNF-α+PGE_2 reduced scd-1 mRNA levels significantly

when compared to Ctrl, LPS, INS, and PGE_2 ($p<0.0001$ c) and the same pattern was observed when compared to LPS INS, PGE_2 INS and PGE_2 LPS (figure 31C).

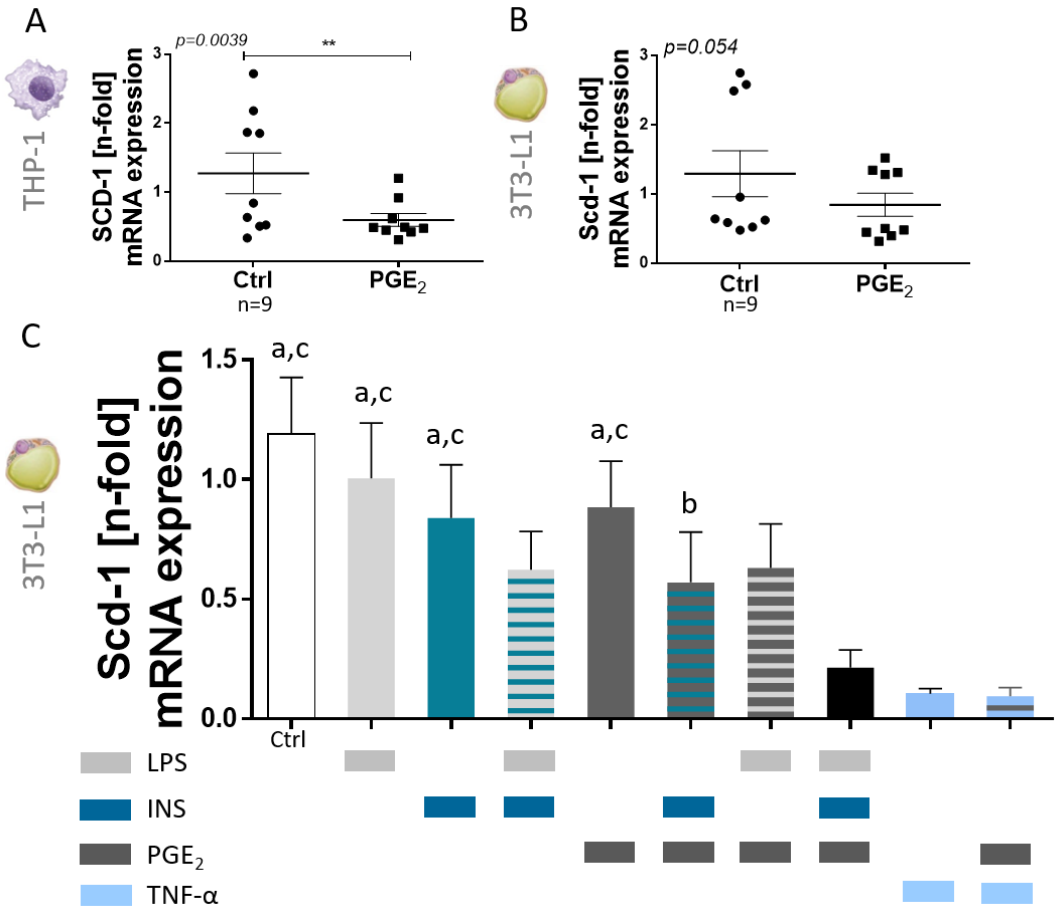


Figure 31: PGE_2 in combination with LPS and insulin down regulates SCD. SCD mRNA expression (n-fold of $2^{(-ddCt)}$) in THP-macrophages (A) and 3T3 cells (B) incubated with 1µM PGE_2. (A, B) data expressed as mean±SEM, significant difference tested by Wilcoxon matched-pairs signed rank test. (C) Scd-1 mRNA expression in 3T3 cells incubated with LPS, insulin, PGE_2, TNF-α. a=significant vs INS LPS PGE_2, Friedman test, Dunn´s posttest p=0.0004; b=significant vs Ctrl, Friedman test p=0.0006, Dunn´s posttest, n=6; c=significant vs TNF-α, one-way ANOVA, post-test: Tukey´s multiple comparisons test p<0.0001, n=5.

Insulin incubation increased SCD-1 protein expression significantly about 50% compared to Ctrl while PGE_2 and LPS had no impact on SCD-1 in 3T3 adipocytes (figure 32). LPS as well as PGE_2 did impair the insulin-stimulated SCD-1 increase, though that was only significant for PGE_2 INS (INS 147.9±13.2 vs LPS INS 126.5±14.4 and PGE_2 INS 106.6±18.2 figure 32). The combination of LPS INS and PGE_2 (68.2%) significantly

impaired the SCD-1 induction by insulin, and protein levels were also lower than compared to samples that were incubated with LPS INS (RM one-way ANOVA p<0.0001, $). When compared to PGE_2 INS the triple stimulus tended to reduce SCD-1 protein levels (Tukey´s post-test p=0.05). However, the incubation with TNF-α and TNF-α+PGE_2 resulted in the most effective downregulation of SCD-1 but PGE_2 did not further enhance the TNF-dependent repression. Protein contents were lower when compared to all other stimuli (§) though the post hoc tests were not significant for PGE_2 LPS (p=0.06) and LPS INS PGE_2 (p=0.07).

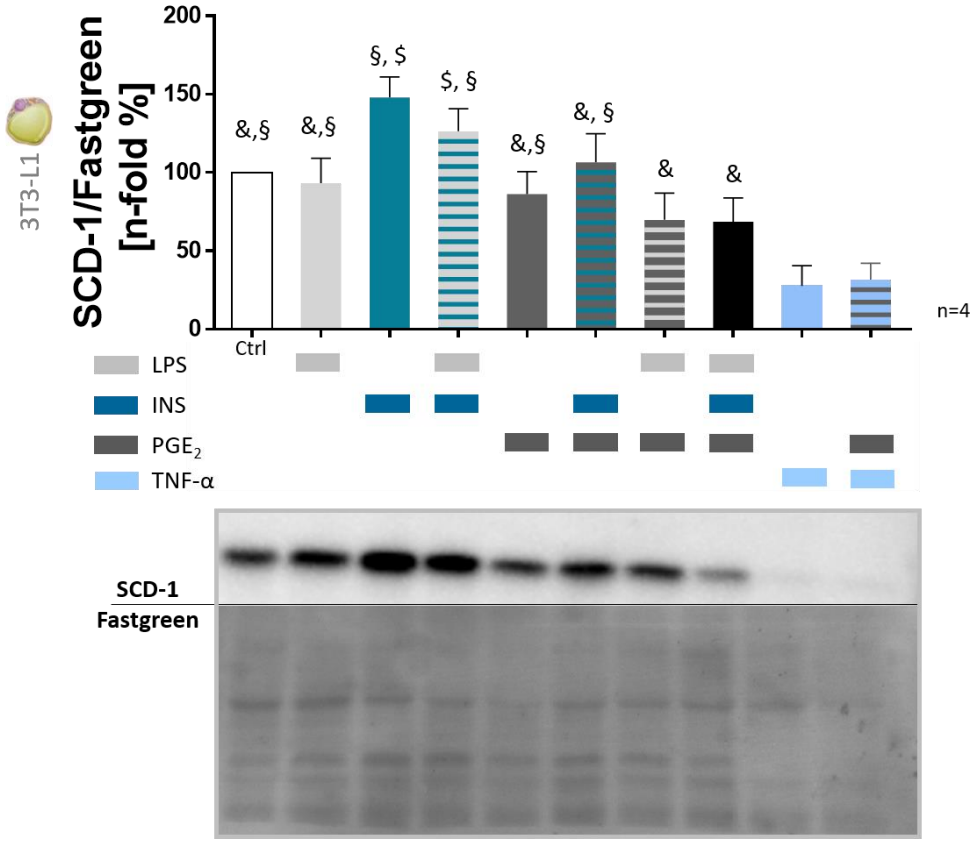


Figure 32: Up-regulation of SCD-1 by Insulin is suppressed by PGE_2 and LPS. Relative SCD-1 protein content in 3T3-L1 cell-lysates treated with LPS, insulin, PGE_2, TNF-α. Protein expression was normalized to Fastgreen staining (4 independent experiments). Data expressed as mean±SEM, &=significant vs INS; $=significant vs LPS INS PGE_2 §=significant vs TNF-α and TNF-α PGE_2 RM one-way ANOVA p<0.0001, Holm Sidak´s post-test.

VAT-contents of PGE_2 – a pro-inflammatory LM – were not altered between groups. In all groups PGE_2 tissue concentration as well as the mRNA expression of the synthesizing enzymes COX-1 and COX-2 was related to higher levels of inflammatory blood parameters. COX-2, not COX-1, was higher in VAT of CC. in vitro, COX-2 was induced by IL-6 and suppressed by TNF-α in immune cells. In 3T3-adipocytes, PGE_2 was shown to inhibit the insulin induced expression of SCD-1.

3.8 Systemic inflammation may drive chemokine increase in adipose tissue

Data in section 3.1 show that the distribution of inflammatory factors varies between tissues in cancer cachexia. Specifically, the pro-inflammatory cytokines TNF-α and IL-6 were higher in plasma (figure 11A) and the concentrations of chemokines such as IL-8 and CXCL10 were higher in adipose tissue of cachectic cancer patients (figure 12A). The results of section 3.5-3.7 demonstrate that TNF-α and IL-6 were able to regulate fatty acid modifying enzymes while chemokines had no effects. Therefore, this section investigates which factors may lead to the increase of chemokines in the adipose tissue in cancer cachexia.

In patients, plasma levels of TNF-α correlated positively with IL-8 concentrations in visceral adipose tissue in the total group ($r=0.51$, $p=0.003$), the cancer group ($r=0.49$, $p=0.01$) and in WSC ($r=0.78$, $p=0.01$) but not in CC or Control (figure 33A, B). The same relation was found for TNF-α in plasma and CXCL10 concentrations in VAT, TNF-α correlated significantly with CXCL10 in the total group ($r=0.46$, $p=0.01$), cancer group ($r=0.40$, $p=0.04$) and WSC ($r=0.79$, $p=0.01$, figure 33A, C).

A

		all patients	Control	WSC	CC	WSC+CC
		n=32	n=7	n=10	n=15	n=25
Plasma TNF-α <> VAT IL-8	Spearman r	**0.51**	0.64	**0.78**	0.41	**0.49**
	p-value	*0.003*	*0.14*	*0.01*	*0.13*	*0.01*
Plasma TNF-α <> VAT CXCL10	Spearman r	**0.46**	0.46	**0.79**	0.17	**0.40**
	p-value	*0.01*	*0.30*	*0.01*	*0.54*	*0.04*

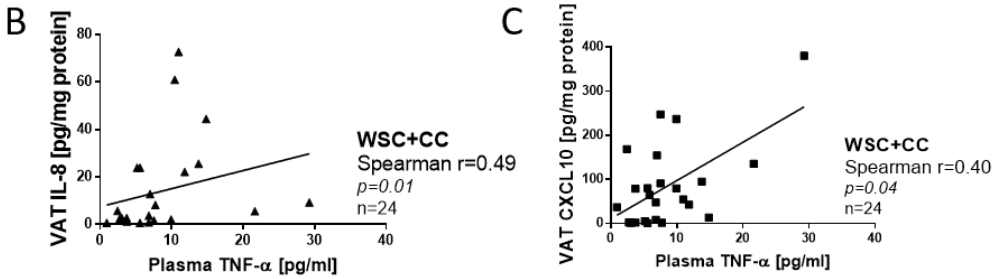


Figure 33: TNF-α in plasma correlates with higher chemokine levels in VAT of cancer patients. (A) Spearman correlation of plasma TNF-α [pg/ml] with VAT IL-8 [pg/mg protein] and VAT CXCL10 [pg/mg protein] for all groups and exemplary graphs of the cancer group WSC+CC (B IL-8, C CXCL10).

In vitro, TNF-α as well as IL-6 increased IL-8 expression (figure 34). In 3T3 adipocytes, TNF-α enhanced mip-2 expression by factor 15 (Ctrl 1.88±0.70 vs TNF-α 29.80±10.38, p=0.03) and incubation with IL-6 resulted in a 3-fold induction of Mip-2 (Ctrl 0.13±0.04 vs TNF-α 0.40±0.18, p=0.04; figure 34A, B). In THP macrophages TNF-α increased IL-8 by 2.5-fold (Ctrl 0.47 [0.26-23.34] vs TNF-α 1.16 [0.61-26.82], p=0.03; figure 34C) and IL-6 by 4.6 fold (Ctrl 0.32 [0.18-15.82] vs IL-6 1.48 [0.57-29.42], p=0.03; figure 34D). IL-8 protein concentrations were measured in lysates of VAT explants from WSC and CC after incubation with TNF-α (figure 34E). TNF-α tended to augment IL-8 concentrations in samples of WSC (p=0.07) and when WSC and CC were pooled (p=0.06) but not in CC as a single group (figure 34).

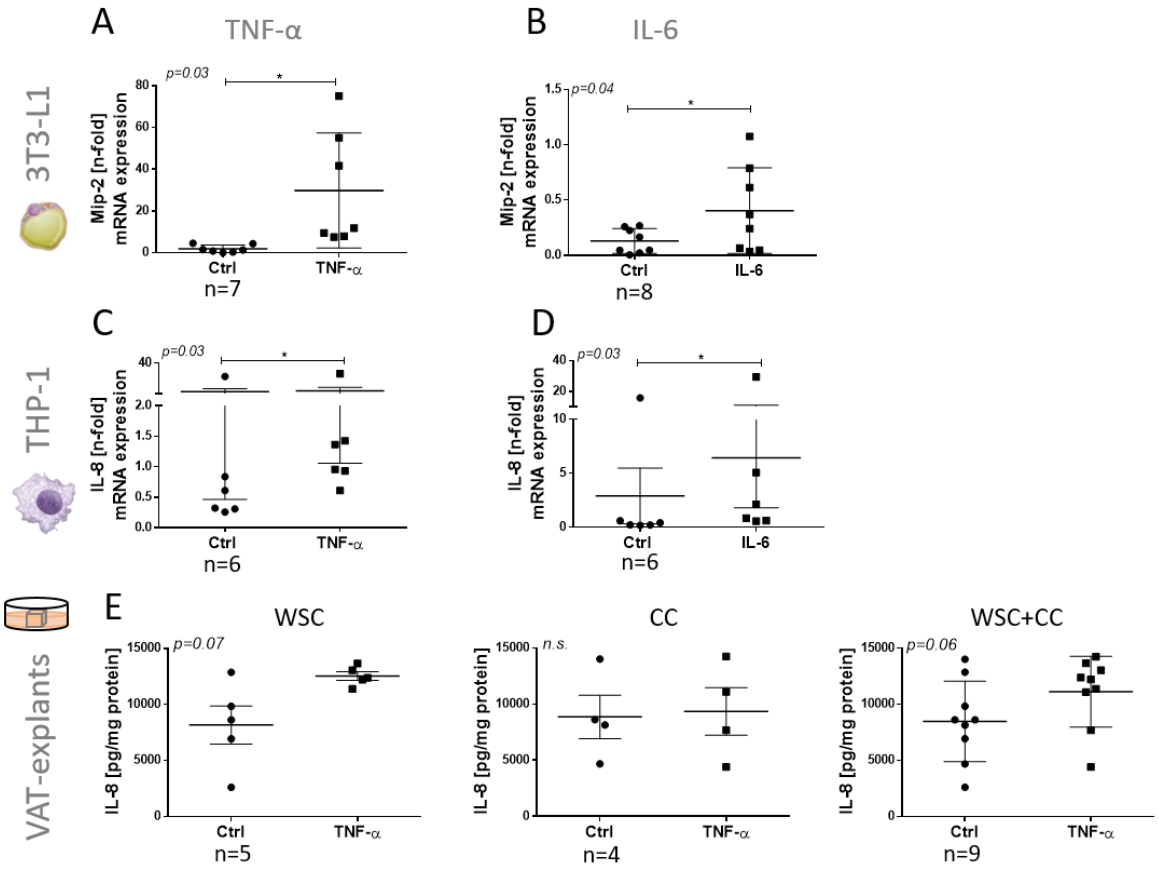


Figure 34: TNF-α and IL-6 increase IL-8/Mip-2 expression in adipocytes, macrophages and VAT explants. Mip-2 expression (n-fold of $2^{(-ddCt)}$) in 3T3 cells (upper panel), IL-8 expression in THP-1 cells (middle) incubated with or without 100ng/ml TNF-α (A, C, E) and IL-6 (B, D). (E) IL-8 protein levels [pg/mg protein] in VAT explants in WSC, CC and WSC+CC. *Significant difference by paired t-test (A, B, E) or Wilcoxon matched-pairs signed rank test (C, D).

CXCL10 mRNA expression was also increased by TNF-α and IL-6 in 3T3 adipocytes (figure 35 A, B) and THP-1 macrophages (figure 35C, D). The strongest induction could be observed in THP-1 cells as incubation with TNF-α resulted in a 12.4-fold increase in IL-8 mRNA levels (Ctrl 0.64±0.14 vs TNF-α 7.70±1.90, p=0.02; figure 35C). The qRT-PCR analysis of VAT explants showed that CXCL10 mRNA levels were consistently higher after TNF-α incubation in WSC and CC. This was not significant for the individual groups but when WSC and CC were pooled, a significant 5-fold increase in CXCL10 was found (Ctrl 1.09 [0.20-3.84] vs TNF-α 5.54 [0.72-33.96], p=0.01; figure 35E).

However, CXCL10 protein contents were not significantly higher in VAT explants after TNF-α incubation, with only the WSC group's TNF-α data tending to augment CXCL10 concentrations (p=0.06, figure 35F).

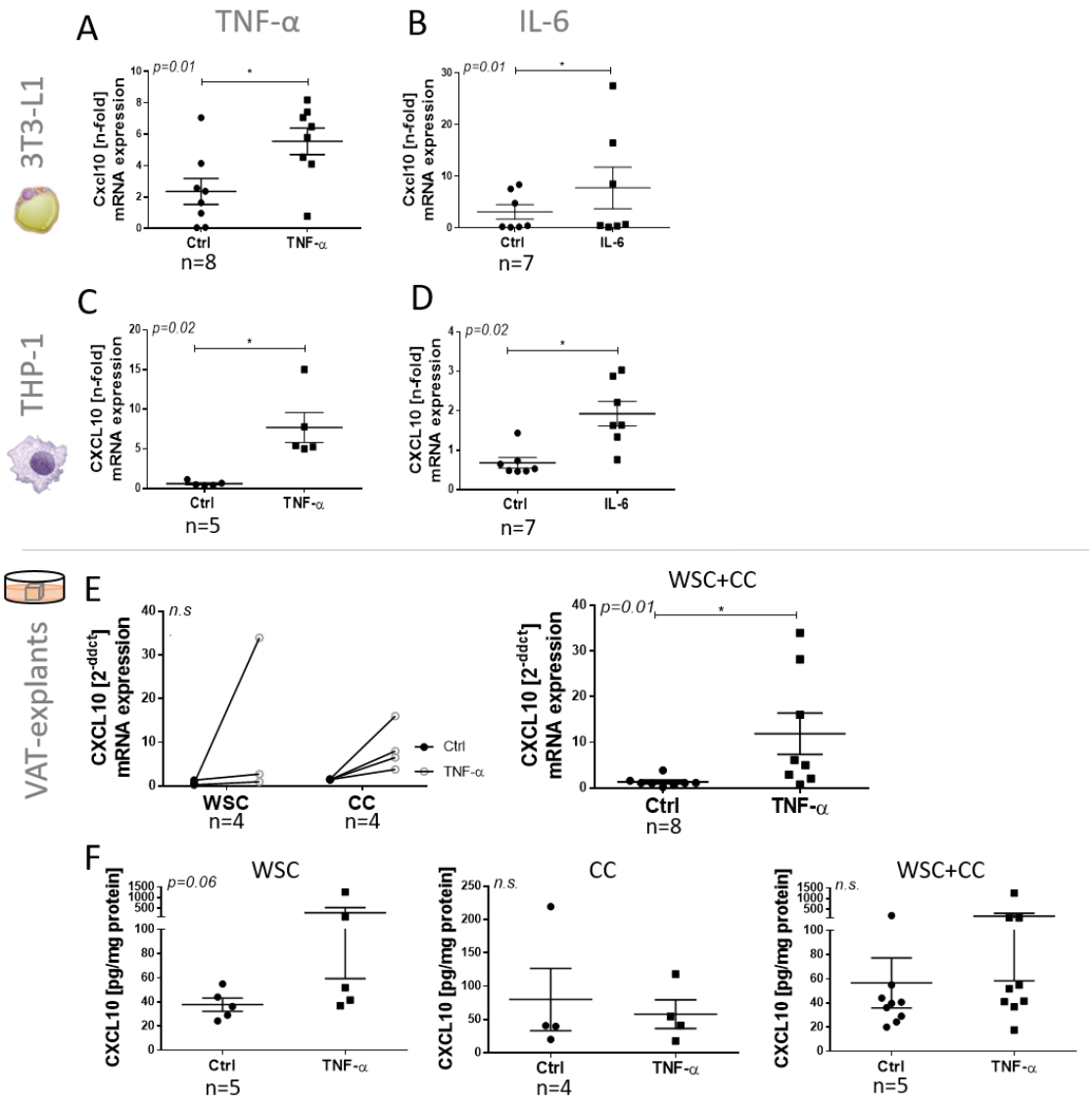


Figure 35: TNF-α and IL-6 increase CXCL10 expression in adipocytes, macrophages and VAT explants. CXCL10 gene expression (n-fold of $2^{(-ddCt)}$) in 3T3 cells (A, B), THP-1 cells (C, D) and VAT explants (E) incubated with or without 100ng/ml TNF-α (A, C, E, F) and IL-6 (B, D). (F) CXCL10 protein levels [pg/mg protein] in VAT explants in WSC, CC and WSC+CC. *Significant difference by paired t-test (A, C) or Wilcoxon matched-pairs signed rank test (B, D, E, F).

To test if higher palmitate levels in plasma of cachectic patients may contribute to the local adipose tissue inflammation, 3T3 adipocytes and THP macrophages were stimulated with palmitate and LPS as a positive control (figure 36). LPS, but not palmitate, induced IL-8 (figure 36A) and CXCL10 (figure 36B, C) in 3T3 adipocytes. In macrophages, palmitate enhanced IL-8 as well as CXCL10 expression up to 4-fold (figure 36D, F), while LPS incubation resulted in an even higher induction as a 10-fold increase in IL-8 and 13-fold increase in CXCL10 mRNA levels was observed (figure 36E, G). In a test experiment, palmitate did not induce IL-8 (figure 36H) but CXCL10 (figure 36I) expression in a triplicate of VAT explants from one patient.

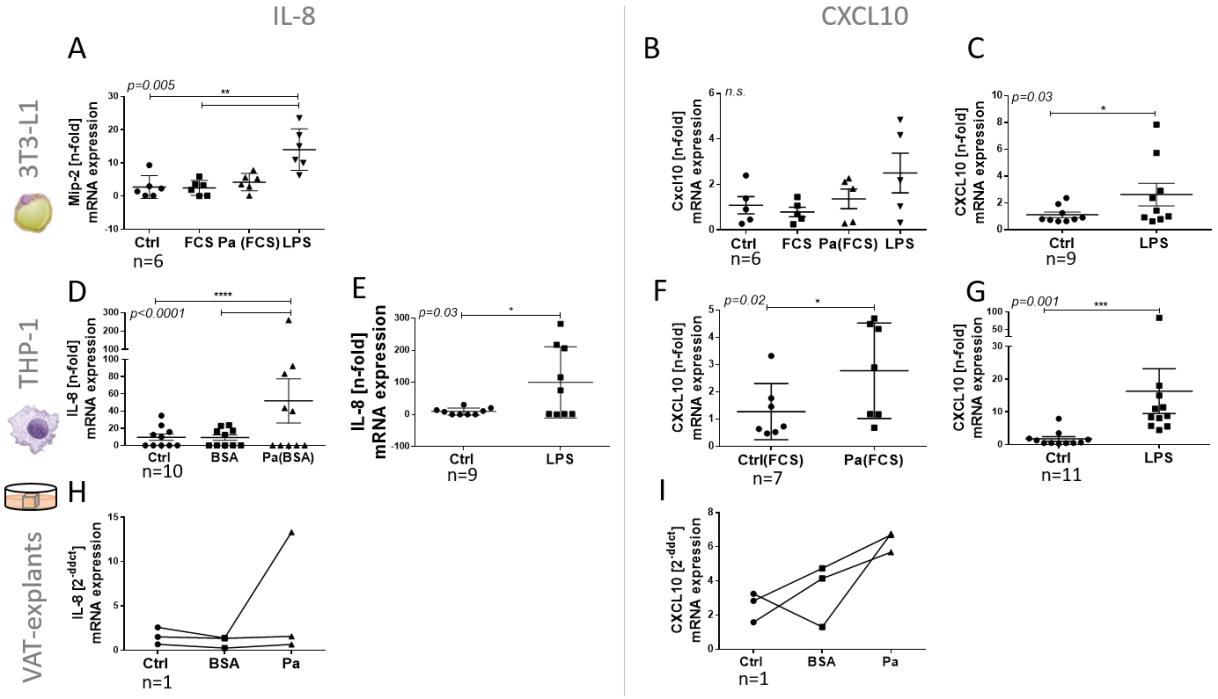


Figure 36: Palmitate increases IL-8 and CXCL10 in THP-1 macrophages. Gene expression of IL-8 (left panel) and CXCL10 (right panel) (n-fold of $2^{(-ddCt)}$) in 3T3 cells (A-C), THP-1 cells (D-G) incubated with or without 100nM palmitate (A, B, D, F) and 100ng/ml LPS (A-C, E, G). (H, I) IL-8 and CXCL10 expression in VAT explants from one patient (triplicate) incubated with palmitate. Data expressed as mean±SEM or mean±SD (E, F). Significance was tested employing repeated measures one-way ANOVA (A, B); Friedman-test (D); paired t-test (E) or Wilcoxon matched-pairs signed rank test (C, F, G.

Systemic inflammation is related to lower albumin levels as serum-CRP and plasma-IL-6 correlated negatively with albumin for all patients and within groups (figure 37A). Statistically significant relationships were observed for CRP and albumin in cachectic and non-cachectic cancer patients but not in Control. The correlations between CRP and IL-6 plasma with Control and CC achieving statistical significance but not with WSC.

Since myristic acid C14:0 was lower in blood samples of CC (appendix A) and it is one of the albumin ligands (Curry et al., 1998), SAT C14:0 levels were tested to see if they correlated with albumin concentration. As expected for physiologic conditions, only the Control group demonstrated a positive correlation of albumin with C14:0 (Control $r=0.61$, $p=0.03$) while no relationship was evident for WSC and CC or the combined cancer group (figure 37A, B). Thus, the results could be a hint for a lower fatty acid binding capacity in the cancer groups, which may contribute to more FFA e.g. palmitate leading to the observed increase in chemokines.

A

		all patients	Control	WSC	CC	WSC+CC
Albumin <> CRP		n=183	n=61	n=53	n=69	n=122
	Spearman r	**-0.4**	-0.17	**-0.32**	**-0.3**	**-0.4**
	p-value	*<0.0001*	*0.18*	*0.02*	*0.01*	*<0.0001*
Albumin <> Plasma IL-6		n=68	n=21	n=24	n=23	n=47
	Spearman r	**-0.36**	**-0.44**	-0.11	**-0.51**	**-0.36**
	p-value	*0.002*	*0.04*	*0.6*	*0.01*	*0.01*
Albumin <> C14:0		n=36	n=13	n=10	n=13	n=23
	Spearman r	0.27	**0.61**	0.08	0.02	0.05
	p-value	*0.1*	*0.03*	*0.84*	*0.96*	*0.34*

B

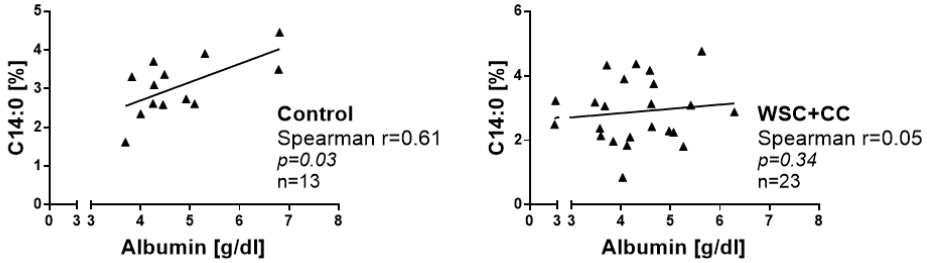


Figure 37: Negative correlation of albumin and systemic inflammation. (A) Spearman correlation of serum albumin concentrations [g/dl] with CRP [mg/l], plasma IL-6 [pg/ml] and C14:0 [%] for all groups. (B) Exemplary graphs of Control and cancer group WSC+CC.

Inflammatory factors are secreted by both adipocytes and immune cells and can act in an auto- or paracrine manner (Xie et al., 2010, Sárvári et al., 2015). Differentiated 3T3-L1 adipocytes were stimulated with supernatants of control or palmitate- stimulated THP cells (figure 38 (THP)) to evaluate the potential impact of inflammatory mediators released by the immune cells on adipocytes. Mip-2 expression, an IL-8 analog, was induced by conditioned medium independent from palmitate (figure 38 A). Palmitate medium taken from the THP cells increased CXCL10 mRNA expression in 3T3 adipocytes (figure 38B), while this effect was not visible when cells were incubated with unconditioned palmitate medium (figure 38B and 36B).

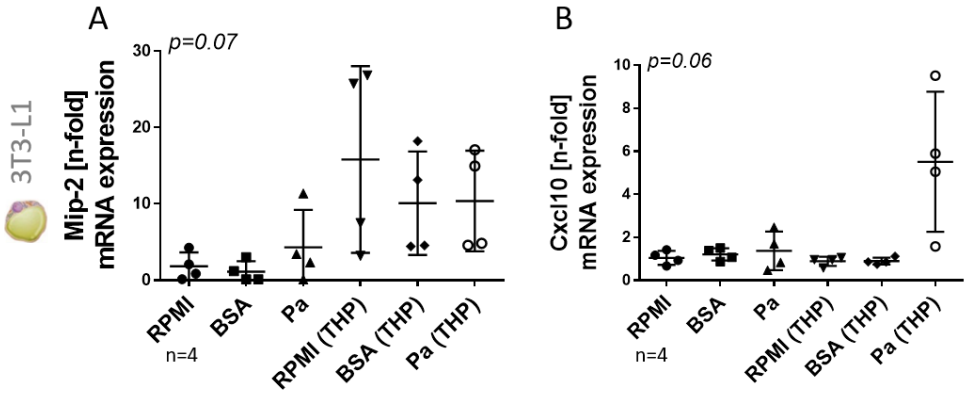


Figure 38: Chemokine expression in 3T3 adipocytes after treatment with THP-1 supernatants. Gene expression of Mip-2 (A) and CXCL10 (B) (n-fold of $2^{(-ddCt)}$) in 3T3 cells after cultivation for 24 h with RPMI culture medium from THP-1 macrophages that were prior stimulated with BSA or 100nM palmitate. As control media, served BSA and palmitate treated medium that was not exposed to THP cells. Data expressed as mean±SEM, repeated measures one-way ANOVA.

Prostaglandin E2 (PGE_2) was shown to be augmented in experimental cachexia models (Wang et al., 2005, Lira et al., 2010, Schrey and Patel, 1995), hence prostaglandin could further contribute to high chemokine concentrations in the adipose tissue of patients in cancer cachexia. For the treatment of THP-cells a PGE_2 concentration of 1µM that has been already established by Neuschäfer-Rube et al. (Neuschäfer-Rube et al., 2018). In the THP-macrophages of this sample, IL-8 mRNA levels were increased up to 17-fold by PGE_2 (Ctrl 1.18 [0.54-1.78] vs PGE_2 17.40 [4.91-100.6], p=0.02; figure 39A) but not CXCL10 (figure 39A). In the project of Julia Klauder it was already shown that PGE_2 and palmitate have a synergistic effect on IL-8.

In contrast, the induction of CXCL10 by palmitate was suppressed by PGE_2 (p=0.01, figure 39B). In 3T3 cells PGE_2 had no effect on Mip-2 or Cxcl10 expression (figure 39C, D). TNF-α increased Mip-2 (IL-8) as well as CXCL10 expression in 3T3 adipocytes (figure 34, figure 39E, F). While TNF-α enhanced Mip-2 expression up to 65-fold when compared to Ctrl, the combination with PGE_2 resulted in a 950-fold induction (Ctrl 1.63±0.71, TNF-α 104.2±35.2, TNF-α PGE_2 1519±441.3, p=0.04 &; figure 39E). Furthermore, PGE_2 could increase the effect of LPS on Mip-2 (figure 39E). The strongest induction of CXCL10 was achieved by TNF-α (RM ANOVA p=0.0005 §, figure 39F). PGE_2

reduced the TNF-α-effect by 90% (TNF-α 76.38±5.09, PGE_2TNF-α 8.2±1.03). When 3T3 cells were incubated with PGE_2 and LPS, the CXCL10 expression was lower than either the single stimuli or the Control (Ctrl 0.97±0.05, PGE_2 0.73±0.17, LPS 1.13±0.12 vs PGE_2 LPS 0.45±0.05; t-test p=0.04 # or RM ANOVA p=0.0005 &; figure 39F).

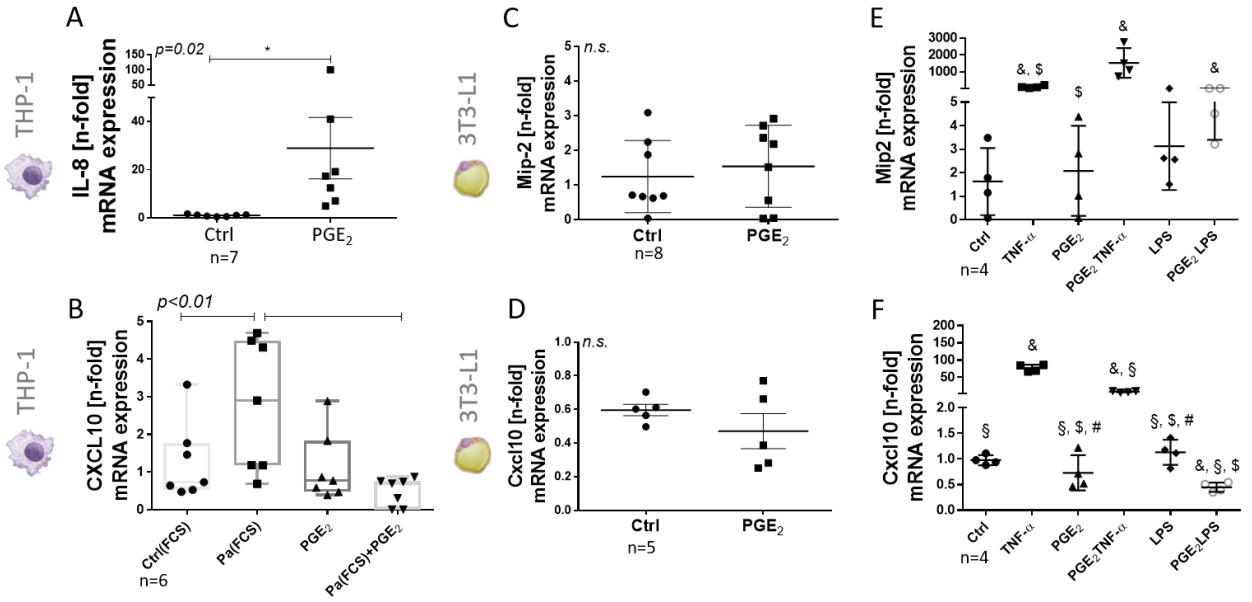


Figure 39: PGE_2 synergistically enhances IL-8 expression but suppresses CXCL10 in 3T3 and THP cells. IL-8 (top) and CXCL10 (bottom) mRNA expression (n-fold of $2^{(-ddCt)}$) in THP-macrophages (A, B) and 3T3 cells (C-F) incubated with 1µM PGE2, TNF-α and LPS. (B) THP cells incubated with PGE_2 and palmitate. Data expressed as mean±SD (A, C-F) and median [min-max] (B). Significant difference tested by Wilcoxon matched-pairs signed rank test (A, C); Friedman test (B); paired T-test (D, E, F) and reported measures one-way ANOVA (E, F). (E) RM ANOVA p=0.04, Tukey´s posttest not significant, &=significant vs Ctrl, $=significant vs TNF-α PGE_2 paired T-test p<0.05. (F) &=significant vs Ctrl, §=significant vs TNF-α, §=significant vs TNF-α PGE_2, RM ANOVA p=0.0005; #=significant vs PGE_2 LPS paired T-test p<0.05.

Chemokine protein levels in VAT showed a positive correlation with pro-inflammatory plasma cytokines (TNF-α, IL-6). Also in vitro and ex vivo, IL-8/mip-2 and CXCL10/cxcl10 were induced by TNF-α and IL-6. TLR-agonist LPS but not palmitate induced mip-2 and cxcl10 in 3T3-adipocytes while in immune cells both stimuli increased chemokine expression. The systemic inflammation might provoke an environment with impaired fatty acid binding which would lead to more FFA contributing to the raise in chemokines during cancer cachexia. Palmitate-conditioned medium derived from THP-1 cells enhanced chemokine expression in adipocytes. In immune cells PGE_2 and palmitate

synergistically augmented the induction of IL-8 while palmitate-induced CXCL10 expression was suppressed by PGE_2.

3.9 INFLAMMATION RESOLUTION IN CANCER CACHEXIA

Lipid mediators are important players in the resolution phase of inflammation (Serhan and Chiang, 2013). It was therefore investigated if lipid mediator formation and hence inflammation resolution is impaired in cancer cachexia. n3 PUFAs act as precursors for inflammation resolving compounds, namely resolvins, protectins and maresins, first described by Serhan and collogues (Serhan et al., 2009). Resolvins of the D-series derive from C22:6n3 docosahexaenoic acid (DHA). Contrary to the expectations, the plasma concentration of resolving D1 was even higher in CC than in Control (Control 98.3 [43.34-295.4], WSC 275.6 [18.9-547.9], CC 561.2 [64.0-2687], p=0.04, figure 40).

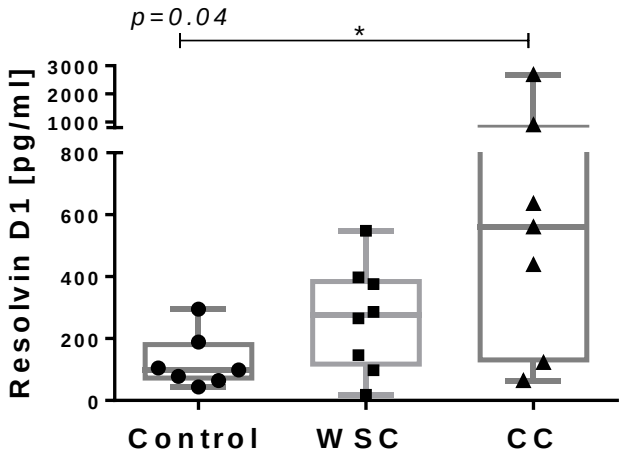


Figure 40: Higher serum concentrations of resolving D1 in cancer cachexia. Resolvin D1 [pg/ml] data expressed as median [min-max], Kruskal-Wallis test.

The lipoxygenase ALOX15 is one of the catalyzing enzymes in the synthesis of D-resolvins (1.5.1). In SAT the gene expression of ALOX15 did not differ among the study groups (figure 41A), while in VAT of CC, ALOX15 mRNA expression was about 10-times higher than in the Control group (Control 0.79 [0.09-10.05], WSC 3.54 [0.03-35.57], CC 8.61 [1.23-285.5], p=0.0062, figure 41B). Interestingly in contrast to gene expression,

immune blotting revealed that ALOX15 protein levels were lower in CC when compared to Control in SAT (figure 41C) and also lower than in WSC in VAT (figure 41D).

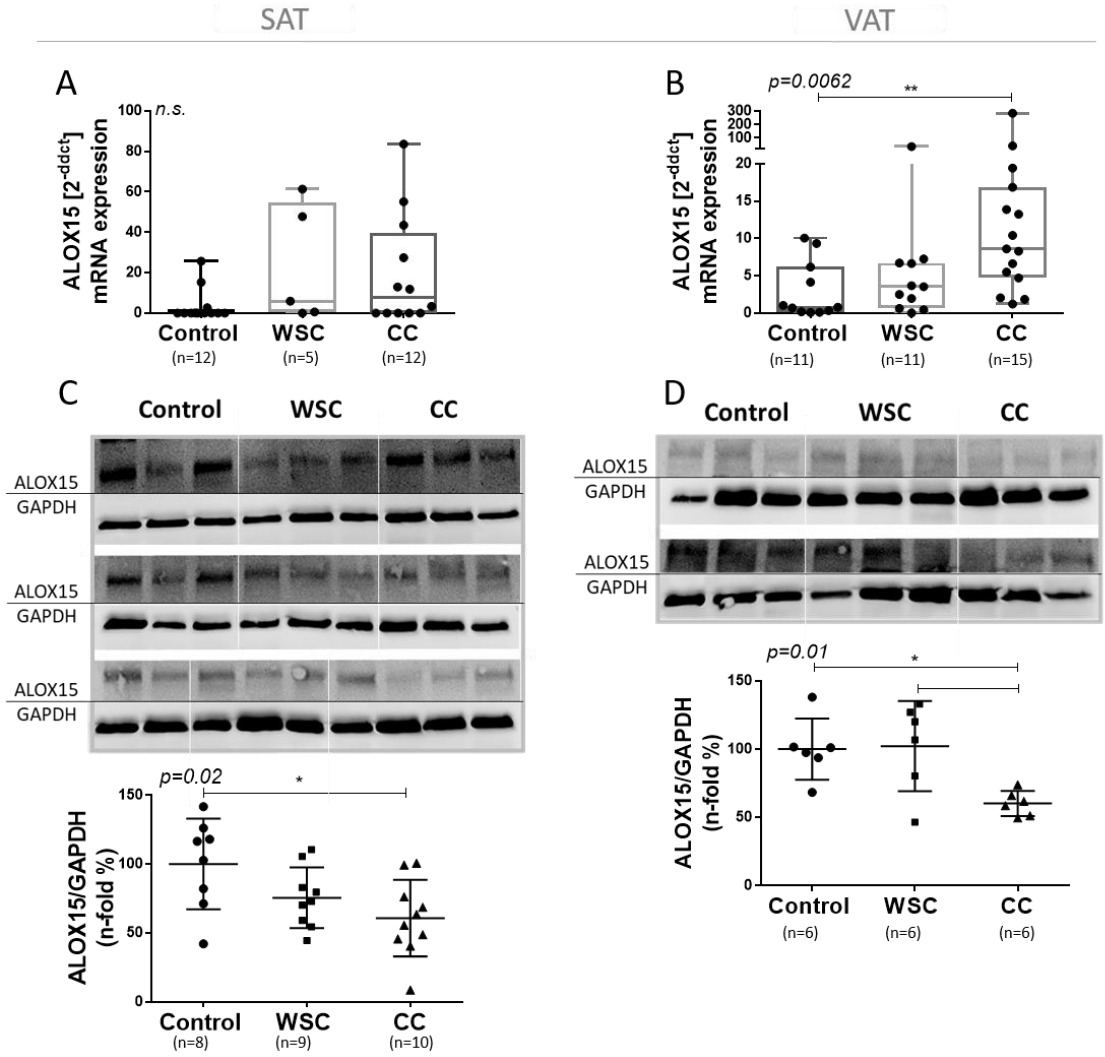
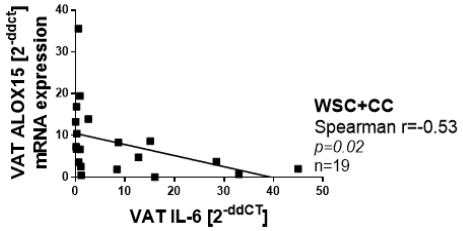


Figure 41: Lower ALOX15 protein levels in SAT and VAT of cachectic patients. ALOX15 mRNA and protein expression in SAT and VAT. (A, B) mRNA expression expressed as $2^{(-ddCT)}$, median [min-max]. Protein content expressed as n-fold (%) of Control band intensity normalized to GAPDH, (C, D) mean±SD. One-way ANOVA significance test was applied for parametric data (C,D) and Kruskal-Wallis test for non-parametric data (A, B).

Although ALOX15 mRNA levels were higher in VAT of cachectic patients, in all groups except Control, a negative correlation of ALOX15 mRNA levels with IL-6 mRNA levels was observed in VAT (figure 42A). This finding was supported by *in-vitro* data showing that IL-6 incubation of differentiated U937 macrophages reduced ALOX15 gene expression (Ctrl 1.00 [0.46-4.76] vs IL-6 0.60 [0.17-4.28], $p<0.05$, figure 42B). The incubation of U937 cells with IL-8 and PGE_2 had no effects on ALOX15 expression (figure 42C, D).

A

VAT ALOX15 mRNA expression <> VAT IL-6 mRNA expression					
	all patients n=27	Control n=8	WSC n=10	CC n=9	WSC+CC n=19
Spearman r	**-0.39**	-0.45	**-0.71**	**-0.63**	**-0.53**
p-value	*0.04*	*0.27*	*0.03*	*0.08*	*0.02*



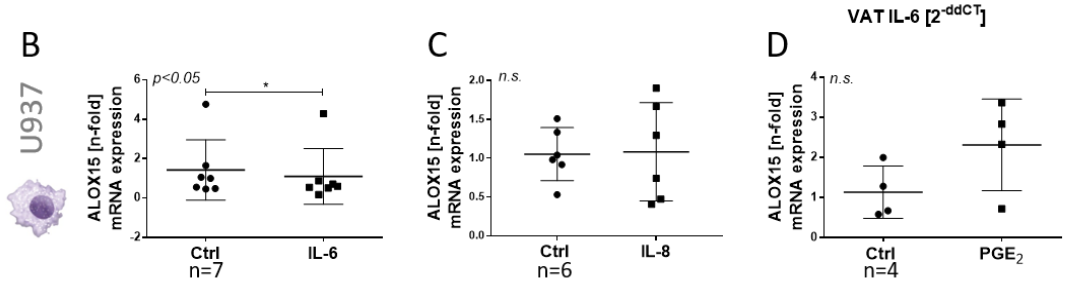


Figure 42: Negative correlation of ALOX15 and IL-6 gene expression in visceral adipose tissue. (A) Spearman correlation of VAT ALOX15 and VAT IL-6 mRNA expression for all groups and exemplary graph of the cancer group WSC+CC. (B-D) ALOX15 gene expression (n-fold of $2^{(-ddCt)}$) in U937 cells incubated with or without IL-6 (B), IL-8 (C) and PGE_2 *Significant difference was tested by Wilcoxon matched-pairs signed rank test (B) and paired T-test (C, D).

In the *in-vitro* system, TNF-α did not regulate ALOX15 gene expression (figure 43A) and there was no impact of TNF-α on ALOX15 in visceral adipose tissue explants of the cancer group or WSC and CC independently (figure 43B). Alox15 protein levels were also not affected by TNF-α in explant culture (figure 43C).

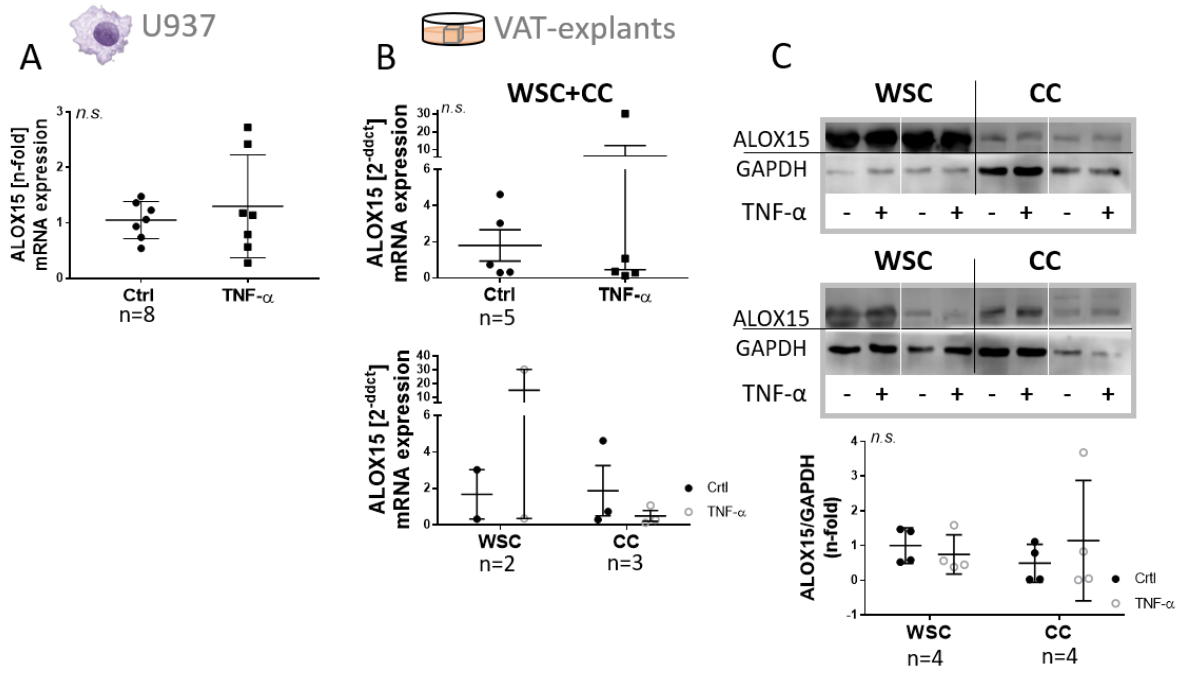


Figure 43: ALOX15 is not regulated by TNF-α in U937 macrophages and VAT explants. ALOX15 mRNA expression (n-fold of $2^{(-ddCt)}$) in U937 cells mean±SD (A) and in VAT $2^{(-ddCT)}$, mean±SEM incubated with or without 100ng/ml TNF-α. (B top) VAT explants from WSC+CC and separated by groups (B bottom). (C) ALOX15 immune blots of VAT explants incubated with TNF-α in 4 WSC and 4 CC patients normalized to GAPDH.

Another important enzyme involved in lipid mediator formation is the lipoxygenase ALOX5 along with its activating protein ALOX5AP (1.5.1). ALOX5 and ALOX5AP gene expression were measured in SAT and VAT and are reported in figure 44. No differences in ALOX5 expression could be observed in SAT among groups (figure 34A), though in VAT, ALOX5 mRNA levels were significantly lower in CC when compared to WSC (Control 1.23 [0.04-8.44], WSC 2.75 [1.14-5.58], CC 0.89 [0.0-3.44], p=0.02, figure 44B). It was expected that ALOX5AP expression in VAT would follow this pattern. However, the data show that ALOX5AP expression was significantly higher in CC compared to WSC (Control 0.45 [0.0-5.83], WSC 0.71 [0.0-2.12], CC 5.35 [0.17-14.24], p=0.04, figure 44D). SAT ALOX5AP mRNA levels did not differ among groups (figure 44C).

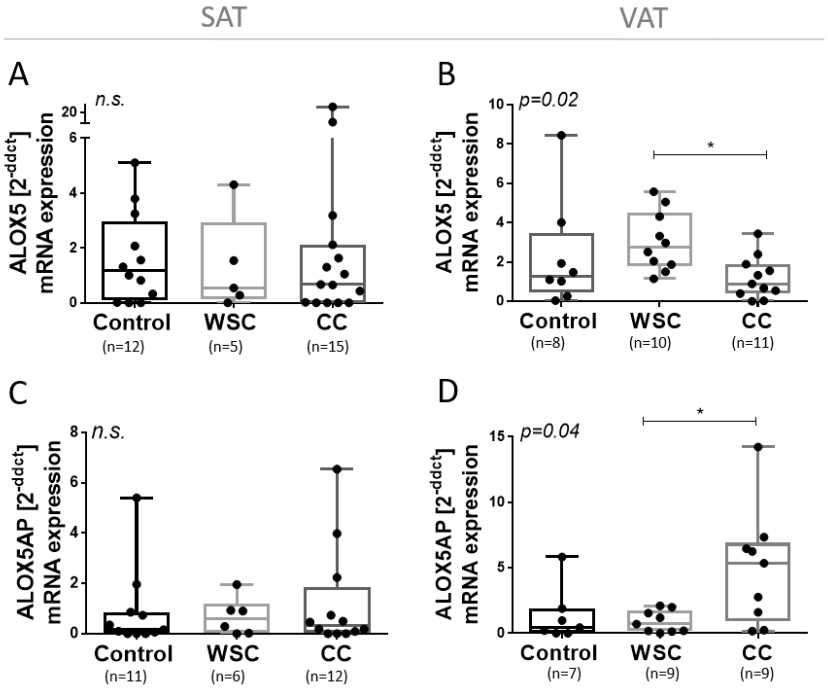


Figure 44: Lower ALOX5 and higher ALOX5AP expression in VAT of cachectic patients. mRNA expression of ALOX5 (A, B) and ALOX5AP (C, D) in SAT (A, C) and VAT (B, D). Data expressed as $2^{(-ddCT)}$, median [min-max], Kruskal-Wallis test.

When ALOX5 mRNA expression in VAT was correlated with IL-6 mRNA expression, a positive correlation was found in the total group (r=0.54, p=0.003) which is explained by a strong correlation coefficient in the Control group (r=0.88, p=0.007, figure 45A). In SAT, ALOX5 correlated positively with palmitate C16:0 in the total group, though the sample size was relatively small and one case may have a large influence on this observation (r=0.69, p=0.01, n=13, figure 45B). Then the impact of the pro-inflammatory cytokines IL-6 and TNF-α on ALOX5 and ALOX5AP was tested in vitro in U937 macrophages (figure 45C, D, E, F). Although a positive correlation of ALOX5 and IL-6 was observed in the Control group, in vitro IL-6 suppressed ALOX5 expression (Ctrl 1.64±0.58 vs IL-6 1.10±0.40, p=0.04, figure 45C) while ALOX5AP was induced by IL-6 (Ctrl 1.70±0.46 vs IL-6 2.47±0.72, p=0.02, figure 45D). As IL-6, TNF-α incubation resulted in a reduction of ALOX5 (Ctrl 1.12±0.29 vs TNF-α 0.57±0.26, p=0.0015, figure 45E) but induction of ALOX5AP (Ctrl 0.68 [0.42-3.67] vs TNF-α 2.57 [0.89-6.03], p=0.03, figure 45 F).

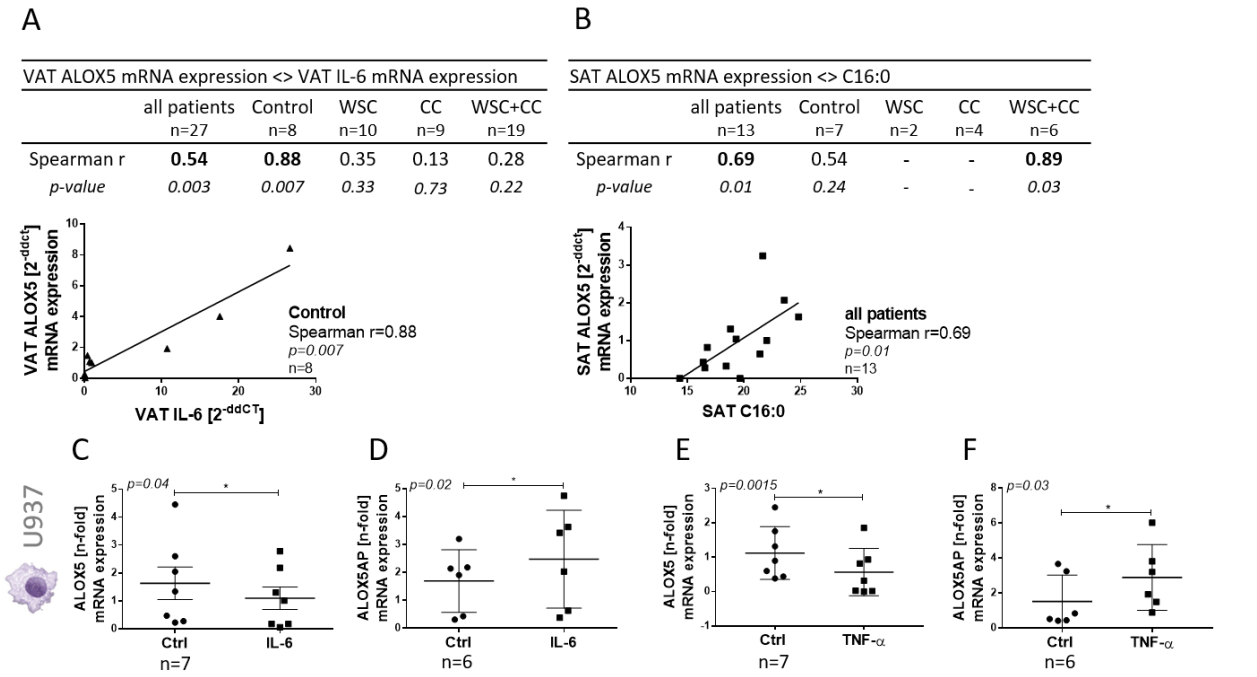


Figure 45: Impact of inflammatory factors on ALOX5 and ALOX5AP. (A) Spearman correlation of VAT ALOX5 and VAT IL-6 mRNA expression for all groups and exemplary graph of the Control group, (B) Spearman correlation of SAT ALOX5 and SAT C16:0 for all groups and exemplary graph. ALOX5 (C, E) and ALOX5AP (D, F) gene expression (n-fold of $2^{(-ddCt)}$) in U937 cells incubated with or without IL-6 (C, D) and TNF-α (E, F). Data expressed as mean±SEM *Significant difference was tested by paired T-test (C-E) and Wilcoxon matched-pairs signed rank test (F).

ALOX5AP VAT mRNA levels correlated positively with VAT IL-8 protein concentrations in the total group (r=0.45, p=0.03) and the cancer group (WSC+CC r=0.68, p=0.008). In WSC and CC the correlation was also found though it was not statistically significant (WSC r=0.71, p=0.09; CC r=0.58, p=0.10, figure 46A). When U937 cells were incubated with IL-8, both ALOX5 as well as ALOX5AP were induced (figure 46B, C) while PGE_2 did not influence ALOX5 or ALOX5AP gene expression (figure 46 D, E).

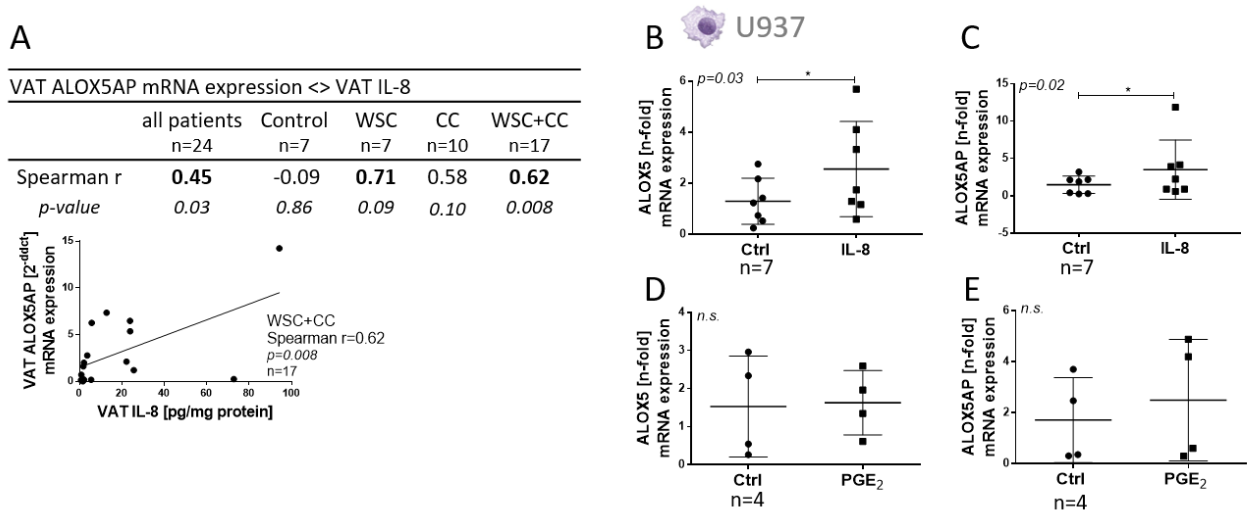


Figure 46: IL-8 correlates with ALOX5AP in VAT and increases ALOX5 and ALOX5AP in U937 macrophages. (A) Spearman correlation of VAT ALOX5AP mRNA expression and VAT IL-8 [pg/mg protein] for all groups and exemplary graph of the cancer group WSC+CC. ALOX5 (B, D) and ALOX5AP (C, E) gene expression (n-fold of $2^{(-ddCt)}$) in U937 cells incubated with or without IL-8 (B, C) and PGE_2 (D, E). Data expressed as mean±SD *Significant difference was tested by paired T-test (B, D, E) and Wilcoxon matched-pairs signed rank test (C).

Lipid mediators act via distinct receptors such as the resolving receptor chemokine like receptor 1 (CMKLR1) (Yoshimura and Oppenheim, 2011). The data shows that CMKLR1 mRNA levels were not different between groups (figure 47A, B). However, in VAT, gene expression correlated negatively with high plasma concentrations of TNF-α and CXCL10 in the total group, the cancer group and CC but not WSC (figure 47C, D). No pairing was possible in Control due to the small sample size. In THP macrophages, TNF-α, IL-6 and PGE_2 did not regulate CMKLR1 (figure 47E, F, H) while IL-8 incubation resulted in a 1.5-fold increase in CMKLR1 expression (Ctrl 1.10±0.27 vs IL-8 1.60±0.32, p=0.02, figure 37G).

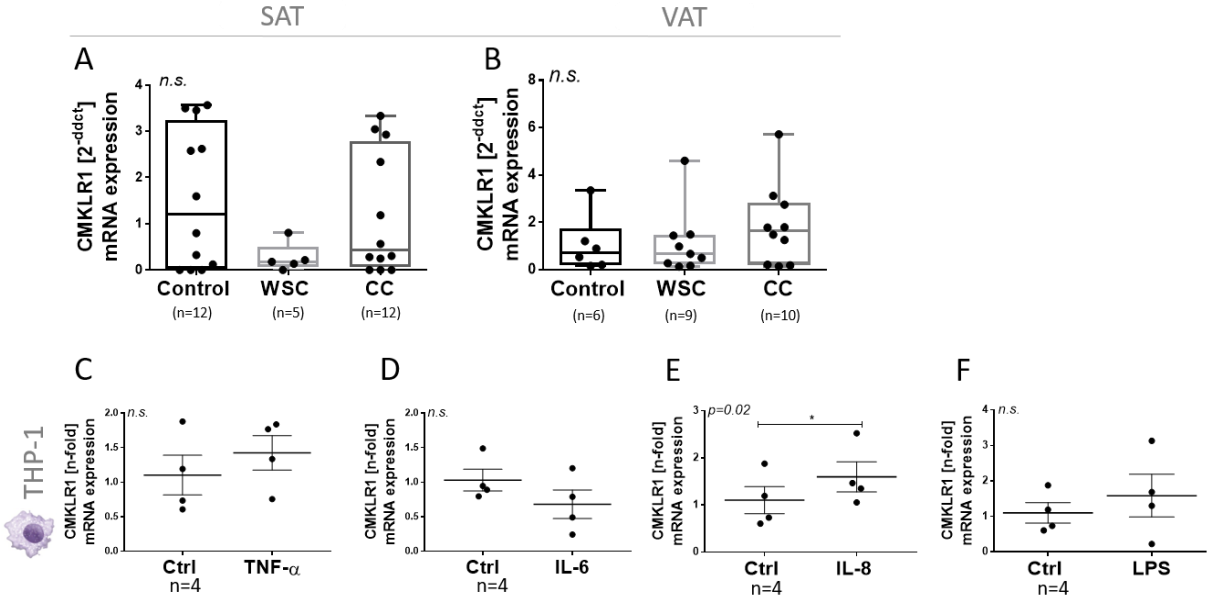


Figure 47: Negative correlation of VAT CMKLR1 gene expression with plasma concentration of TNF-α and CXCL10 and induction by IL-8. mRNA expression of CMKLR1 in SAT (A) and VAT (B). Data expressed as $2^{(-ddCT)}$, median [min-max], Kruskal-Wallis test. Spearman correlation of VAT CMKLR1 mRNA expression with plasma TNF-α [pg/ml] and CXCL10 [pg/ml] for all groups and exemplary graph of the cancer group WSC+CC. CMKLR1 gene expression (n-fold of $2^{(-ddCt)}$) in U937 cells incubated with or without (E) TNF-α, (F) IL-6, (G) IL-8 and (H) PGE_2. Data expressed as mean±SEM *Significant difference was tested using paired T-test.

The results regarding inflammation resolution were controversial. Although protein levels of ALOX15 which is involved in resolving synthesis were lower in adipose tissue of CC, the plasma resolving D1 concentration was higher in the cachectic group. The gene expression of ALOX5 was lower in VAT of CC whereas the expression of its activating protein appeared to be higher. Correlation analysis and in vitro experiments mostly showed a positive association of ALOX5 and ALOX5AP with pro-inflammatory cytokines and chemokines. VAT mRNA levels of the resolving receptor CMKLR1 negatively correlated with plasma concentrations of TNF-α and CXCL10 while IL-8 induced CMKLR1 in vitro.

4 DISCUSSION

4.1 HYPOTHESIS AND MAJOR FINDINGS

Systemic and adipose tissue inflammation in adipose tissue are pivotal factors in cancer cachexia. Pro-inflammatory cytokines and chemokines as well as n6 and n3 fatty acids and their derivatives –bioactive lipid mediators- transduce pro- and anti-inflammatory signaling. Cancer patients, especially cachectic patients, exhibit alterations in plasma FA profile that promotes inflammatory pathways. Besides an altered dietary ingestion of exogenous FA, changes in the endogenous synthesis in subcutaneous and visceral adipose tissue (and liver) could contribute to the shift in FA composition. This shift of the fatty acid profile to a more pro-inflammatory pattern may induce pro-inflammatory cytokines and chemokines that modulate the expression and function of enzymes, which synthesize fatty acids and their derivatives. This would enhance and perpetuate the chronic systemic and local inflammation.

The cachectic cancer patients showed a systemic inflammation of that was characterized by higher levels of circulating TNF-α, IL-6, IL-8 and CRP than weight stable or control patients. Adipose tissue inflammation was more profound in the visceral depot with increased levels of the chemokines CXCL10 and IL-8 respectively. The profile of circulating fatty acids was shifted from n6 and n3 PUFAs to a more saturated pattern while only slight changes were observed regarding FA profile in SAT of CC. The expression of enzymes involved in FA elongation and desaturation such as FASN or FADS2 was lower in adipose tissue of CC and related to systemic inflammatory factors. In vitro, the expression of desaturases and elongases in adipocytes and macrophages was regulated differently by cytokines, LPS or palmitate. The synthesis pathway of pro-inflammatory LM PGE_2 seems to be activated in VAT of cachectic patients. The higher plasma concentration of the pro-resolving LM resolvin D2 in CC cannot compensate the overall inflammatory status and the results indicate that inflammatory cytokines interfere with synthesis pathways of pro-resolving LM.

4.2 THE BRAZILIAN COHORT IN THE INTERNATIONAL CONTEXT

The study of cancer associated cachexia in a Brazilian cohort is important because of two factors; firstly the incidence of colorectal cancer and stomach carcinoma are in the most common cancer types presenting in that country (globocan2012 1) and secondly; that these cancer sites are highly associated with patients with cachexic status. While research efforts and medical treatment are increasing our understanding, progressing and response to these cancers, the mortality for these sites are high relative to other sites (globocan2012, (Ferlay et al., 2015)).

Internationally, stomach cancer incidence declined worldwide during the last decade (Karimi et al., 2014). However Brazil had an increase of CRC incidence in men from 2002 to 2012 (Arnold et al., 2017). Although the worldwide CRC incidence is higher in men than in women (men 23.6%, women 16.3%) in Brazil, the incidence rates between sexes are similar (men 21.1%, women 18.6 %) (WHO 2018, (GLOBOCAN, 2018)). The increase of the Brazilian incidence rates was associated with a higher human development index linking CRC to the rise of prosperity in the country (Arnold et al., 2017). It was shown that a higher BMI (>22) accounts for 3.8% of all newly diagnosed cancers in Brazil in 2012 and this number is estimated to rise to 4.6% in the year 2025 (Rezende et al., 2018). Furthermore, the intake of red meat which is a risk factor for CRC and other cancers (Mehra et al., 2017, Cross et al., 2010, Norat et al., 2005) is traditionally high in Brazil relative to many other nations.

The mean age at diagnostic of stomach and CRC is about 70 years (Kasi et al., 2015, De Pergola and Silvestris, 2013, Howlader and N, 2011), the sample in the present study showed that non-cachectic as well as cachectic patients are younger with a mean age of 61.5 years for stomach cancer and 63 for CRC. The recommendation for a medical check-up is from the age of 50 years in Brazil which is comparable to North America and Europe, but giving that the risk for some cancers is higher in the developed areas of Brazil (Rezende et al., 2018), it could be argued that preventive check-ups should be carried out earlier.

Among the cancer patients recruited 60% were cachectic which may reflect the types of cancer site focused on, the stage of cancer progression at which the patient was eventually diagnosed, public awareness, or lack of, the symptoms of cancer or an unintentional recruitment bias of the present study. In discussions with the physicians involved in this study, it was commented that the weight loss is a symptom that evokes attention and is in some cases reason for medical consultation which eventually results in the detection of the tumor. In this stage cachexia has often progressed, thus more should be done for prevention and earlier detection to low the mortality rates which are about 50% for CRC and 70% for stomach cancer (GLOBOCAN, 2018).

4.3 ALTERATIONS IN SYSTEMIC PARAMETERS IN CANCER CACHEXIA

Due to its feasibility the classification of Fearon et al. was employed as less parameters are required in comparison to other classifications (Fearon et al., 2011). Cancer cachexia of the CC group was confirmed by lower BMI and striking weight loss over the previous 6 months accompanied by lower life quality and anorexia (table 4). In addition, cachectic patients presented a systemic inflammation with high CRP plasma levels (>5mg/l) (table 2). CRP is an acute phase protein contributing to systemic inflammation and the loss of protein stores, as its synthesis requires large amounts of essential amino acids (Reeds et al., 1994). Consequently albumin appeared to be diminished in cachectic patients and acts as a negative first phase protein (table 5). In the plasma of CC the pro-inflammatory cytokines TNF-α and IL-6 were elevated (figure 11). High plasma concentrations of these classic pro-inflammatory cytokines contribute to systemic inflammation by inducing CRP (Rhodes et al., 2011), fibrinogen, hepcidin and a number of other positive acute phase proteins (Gruys et al., 2005). The lower anorexia score (more anorexia) as well as the reduction of haemoglobin and albumin in the CC group is indicative for the malnutrition during cachexia (Evans et al., 2008, Levitt and Levitt, 2016, Zhang et al., 2017). The reduction of hemoglobin in CC compared to the other groups might be explained by a disturbed iron resorption caused by an up-regulation of hepcidin due to inflammation (D'Angelo, 2013).

Systemic inflammation is a feature of metabolic extremes, in obesity as well as in cachexia (Johnson et al., 2012, Onesti and Guttridge, 2014, Vegiopoulos et al., 2017). In all groups the median BMI pre-surgery was higher than 25kg/m^2 (table 1) and one could assume that a preexisting inflammation in obese patients might favor the progression of cachexia despite an elevated starting weight. In accordance with such a hypothesis, it was described that cancer patients with overweight or obesity have a higher propensity to become cachectic during cancer progression (Gonzalez et al., 2014).

Although lipid mobilization is increased during cancer cachexia (Ebadi and Mazurak, 2014), total triglycerides, cholesterol, LDL and HDL fractions were lower in serum of CC when compared to the other groups (table 2). Decreased lipid levels in cancer cachexia have been described previously (Fujiwara et al., 2014, Malaguarnera et al., 2006) and may result from reduced food intake (Tisdale, 2009) or altered LPL activity (Nara-Ashizawa et al., 2001, Kusunoki, 2013). Furthermore, it was shown that inflammation possibly leads to intestinal malabsorption (Mulligan et al., 1992, Argilés et al., 1989, Elmehdawi, 2008) and inflammation was also associated with changes in the cholesterol and lipid transporting apolipoproteins (Sirniö et al., 2017). Apolipoprotein A1 which is the molecular backbone of HDL and was shown to be inversely correlated with CRP and cytokines in CRC (Sirniö et al., 2017) and also in the present study a negative correlation of apolipoprotein A1 and CRP was observed in the CC group (appendix D).

4.4 PRO-INFLAMMATORY CYTOKINES – CAUGHT IN A POSITIVE FEEDBACK LOOP

The cross talk between tumor, blood and adipose tissue is a crucial factor for the manifestation of systemic and local adipose tissue inflammation and the progression of the syndrome. The higher plasma concentrations of the pro-inflammatory cytokines TNF-α and IL-6 in CC (figure 11) can be explained by several mechanisms. IL-6 can be released from epithelial tumors and act in a paracrine manner on other tumor cells or host cells (Schafer and Brugge, 2007), by the activation of JAK family tyrosine kinases, IL-6 signaling is mediated via STAT3, PI3K and MAPK (Heinrich et al., 1998, Costa-Pereira, 2014).

It is also possible that tumor infiltrating macrophages releases IL-6 which in turn stimulates colon tumor cells to produce more IL-6 (Li et al., 2009). Ex vivo, TNF-α did increase IL-6 expression in VAT explants from cancer patients (appendix C). Furthermore it was previously demonstrated in vitro that TNF-α increases IL-6 secretion in myeloma cells (Lee et al., 2013). TNF-α, IL-6 as well as IL-1 belong to the inflammatory triad which act tumorigenic by increasing ROS, DNA damage or promoting proliferation and survival of tumor cells (Tarasiuk et al., 2018). Adipocyte-derived IL-6 augmented malignancy in breast cancer (Gyamfi et al., 2018) and IL-6 drives the progression of cancer cachexia by enhancing the expression of lipolytic and browning genes in men and mice (Han et al., 2018).

High TNF-α serum levels are characteristic for several cancer types and are associated with severity of the disease and lower survival (Michalaki et al., 2004, Pavese et al., 2010). It leads to muscle wasting by inducing protein degradation via the ubiquitin-proteasome-system and contributes to adipose tissue depletion as it enables lipolysis by the phosphorylation of perilipin1 (Patel and Patel, 2017, Das and Hoefler, 2013). There are multiple origins of TNF-α in the scenario of cachectic cancer patients. It is described that colon cancer cells can release TNF-α, IL-6 and other cytokines then activate and recruit circulating monocytes and tissue resident macrophages that further release TNF-α (Zins et al., 2007, Chanmee et al., 2014).

The adipose tissue also faces increased immune cell infiltration during cancer cachexia (Batista et al., 2016, Machado et al., 2004). The majority (59 patients) of the cachectic group was overweight or obese before manifestation of cachexia and it was shown that each excess kilogram of fat accounts for an additional burden of 20-30 million macrophages (Lumeng and Saltiel, 2011). In obese, these macrophages exhibit mostly M1 polarization which is associated with increased secretion TNF-α, IL-6 or CCL2 (Ouchi et al., 2011) and in obese CRC patients peritumoral adipose tissue was the source of circulating cytokines (Amor et al., 2016). Our group has demonstrated that in cancer cachexia M1 polarization is more prominent than M2 polarization in the tumor infiltrate (de Matos-Neto et al., 2015).

The numerous inflammatory pathways in cancer cachexia that are activated by IL-6 and TNF-α or lead to the increase of these cytokines create a feed-forward dynamic which is crucial for the manifestation and progression of cancer cachexia.

4.5 LOCAL ADIPOSE TISSUE INFLAMMATION IS CHARACTERIZED BY INCREASED CHEMOKINE PRODUCTION

In addition to the pro-inflammatory cytokines the plasma concentration of IL-8 - a chemokine was also higher in CC and increased IL-8 blood levels have already been described in cachexia (Pfitzenmaier et al., 2003). The tumor seems to play an important role for the observed high IL-8 levels snice it was shown that IL-8 is expressed in many solid tumors and hematological malignancies (Xie, 2001, Song et al., 2009). Tumor derived IL-8 is a potent chemoattractant that recruits neutrophils and myeloid-derived suppressor cells to the tumor microenvironment by paracrine signaling (Long et al., 2016).

IL-8 synthesis is induced via the NFκB pathway (Kim et al., 2010) and it acts via the CXCR1 or CXCR2 receptor activating a variety of downstream pathways such as JAK/STAT3, MAPK or PI3K (Long et al., 2016, Waugh and Wilson, 2008, Balkwill, 2004). In the pathological state of neoplasia, IL-8 recruits TAN-N2 neutrophils that activate tumor angiogenesis, promote metastasis by tissue remodeling and suppress T-cells (David et al., 2016). Transgenic mice carrying human CXCL8 gene developed colonic and gastric carcinomas associated with MDSC (myeloid derived suppressor cells) recruitment (Asfaha et al., 2013) and in prostate cancer patients increasing IL-8 and IL-6 serum levels were related to higher levels of MDSCs and impaired T-cell function (Chi et al., 2014).

Cytokines in the tumor microenvironment like TNF-α, -β, IL-6 or IL-1β contribute to tumor malignancy by promoting the transition from epithelial cells to invasive mesenchymal like cells (Tan et al., 2014). This process increases IL-8 production (Bates et al., 2004) and it was also shown that IL-8 itself promotes the transition process in colon cancer cells in a positive feedback loop (Cheng et al., 2014). Thus, the high IL-8 plasma levels in the cachectic group may derive from and increase the progression of the tumor.

Besides systemic inflammation, the cachectic group presented a profound local inflammation in the visceral adipose tissue driven by IL-8 and CXCL10, respectively. IL-8 concentration in VAT was higher in CC than in Control while no differences were observed in SAT (figure 12A). Furthermore, VAT concentrations were higher than SAT IL-8 levels (figure 12A). These findings corroborate data of Bruun et al. who characterized the distribution of IL-8 between adipose tissue depots (Bruun et al., 2004). Although adipocytes are capable to synthesize and secrete IL-8 (figure 12B), in metabolic extremes the increase of IL-8 in the adipose tissue is mostly due to non-fat cells (Steppan et al., 2001, Nieman et al., 2011, Straczkowski et al., 2002).

CXCL10 which is a cytokine released to recruit Th1-cells and other immune cells (Clark-Lewis et al., 2003). In both SAT and VAT CXCL10 levels were higher in CC than in Control patients (figure 12A) and similar to IL-8, CXCL10-VAT-concentration appeared to be higher than in SAT (about 8.4-fold). Under pro-inflammatory conditions CXCL10 is induced by adipocytes (Krinninger et al., 2011, Herder et al., 2007) as well as immune cells (Dyer et al., 2009, Luster and Ravetch, 1987, Lo et al., 2010) via the NFκB or the JAK/STAT pathway. CXCL10 binds to the CXCR3 receptor which is expressed on Th1-cellls, natural killer cells, macrophages and B-cells (Loetscher et al., 1998, Qin et al., 1998).

The increase of IL-8 and CXCL10 in the adipose tissue results probably from both, increased synthesis in adipocytes and the release from the infiltrating immune cells. Also the cross-talk between the cell types is important as in vitro results of the present showed that palmitate induced increase of cxcl10 and mip-2 in adipocytes was only observed using conditioned medium from macrophages (figure 28). However, since the chemokine concentration of isolated adipocytes was not altered between the groups (figure 2B), it can be concluded that the infiltrate plays a more important role in chemokine secretion in the adipose tissue.

CXCL10 and IL-8 VAT-concentrations correlated with plasma levels (figure 13) indicating that interactions between adipocytes and the infiltrate can additionally enhance systemic as well as local tissue inflammation (Catalán et al., 2013). The increase of the cytokines TNF-α and IL-6 further seems to increase the chemokine levels as in vitro and

ex vivo, IL-8/mip-2 and CXCL10/cxcl10 were induced by TNF-α and IL-6 (Figure 34, Figure 35) and chemokine protein levels in VAT showed a positive correlation with pro-inflammatory plasma cytokines TNF-α and IL-6 (Figure 33). It was further shown that the cytokine induced increase of IL-8/mip-2 can be enhanced synergistically by palmitate or PGE_2 in macrophages and adipocytes (Figure 39A, E). That was not the case for CXCL10 which was suppressed by PGE_2, though the TNF-α effect on CXCL10 was not fully blocked and still much higher than baseline levels (Figure 39B, F). Nevertheless the high CXCL10 concentrations in the adipose tissue indicate that CXCL10-suppressing mechanisms are less important than inducing processes. However, CXCL10 suppression by PGE_2 might be relevant in the circulation and tumor microenvironment where PGE_2 was shown to impair the recruitment of the natural killer cell surveillance (Van Elssen et al., 2011).

IL-8 and CXCL10 seem to play an important role in the local adipose inflammation in cancer cachexia. Paracrine interactions between adipocytes and the infiltrate as well as pro-inflammatory cytokines and LM e.g. PGE_2 lead to the high chemokine concentration in adipose tissue. This generates an inflammatory environment that can activate and recruit immune cells in a self-enhancing and self-perpetuating way, which in turn secrete other inflammatory factors making the adipose tissue an important contributor to inflammation.

4.6 PLASMA FA COMPOSITION RATHER THAN ADIPOSE TISSUE FA COMPOSITION FAVORS INFLAMMATION IN CANCER CACHEXIA

Although serum triglycerides were lower in the CC group reported here (table 2), it has been demonstrated in a subset this cohort that the relative amount of FFA (FFA/TAG) in the circulation is higher in CC than in the other groups (unpublished data, not shown). The analysis of the fatty acid profile revealed that the percentage of the saturated FA palmitic (C16:0) and stearic acid (C18:0) was higher in cachectic patients compared to controls. A similar pattern was observed regarding the MUFAs palmitoleic (C16:1) and oleic acid (C18:1n9).

Dietary PUFAs C18:3n3 and C18:2n6 were lower in both cancer groups probably due to the consumption of less overall food or a shift in food choices as indicated by the altered anorexia score in those groups (table 4). In both SAT and plasma, the same pattern of the n6/n3 ratio was found. In cachectic patients the ratio was higher than in the Control though in plasma that was not significant (Figure 14B, appendix A). In the literature, dietary n6/n3 ratios of 5/1 – 2.5/1 have shown to have beneficial effects whereas higher ratios are associated with adverse consequences such as inflammation (Simopoulos, 2002). The n6/n3 ratio of the PUFA intake of a subset of this cohort was with 7/1 in WSC and CC (WSC 7.7±1.1, CC 7.7±0.8, unpublished data) higher than the recommendation and may be an additional factor for an unfavorable FA profile in cancer and cancer cachexia. In fact, the beneficial n3 PUFAs DPA C22:5n3 and DGLA C20:3n-6 were lower in CC than compared to Controls [appendix A].

Studies that specifically investigate the circulating fatty acid profile of cancer cachexic patients are rare with most previous work concentrating on comparisons between cancer cases and controls thus comparison requires caution. In contrast to the present study, results from the Singapore Chinese Health study showed no differences in C16:0 and C18:0 between cancer cases and controls and lower levels of MUFAs (Butler et al., 2017). However, in line with our results, the Singapore study also detected lower levels of the dietary PUFAs and C20:3n-6 in colon cancer patients (Butler et al., 2017). In addition, Murphy et al. found a positive correlation between the time to death of advanced

cancer patients and lower amounts of both n3 and n6 FAs (Murphy et al., 2010, Huang et al., 1998).

Dihomo-γ-linolenic acid C20:3n6 has anti-inflammatory properties because it can be converted to anti-inflammatory LM such as 1 series prostaglandins (PGD_1, PGE_1) and 15-HETE (Wang et al., 2012). These anti-inflammatory compounds were shown to suppress chronic inflammation, development of atherosclerotic plaques or growth and differentiation of tumor cells (Wang et al., 2012). In another CRC case and control study, Hodge et al. demonstrated that DPA C22:5n3 is inversely associated with CRC risk though absolute DPA C22:5n3 levels did not differ between cancer cases and controls thus implying a protective role of DPA (Hodge et al., 2015). However, again cachexia was not considered in the research (Hodge et al., 2015). In vitro studies, DPA down regulated IL-6 in macrophages, inhibited TNF-α, improved cell membrane integrity and DPA–supplementation in vivo increased the concentration of EPA and DHA (Tian et al., 2017, Miller et al., 2013). Thus, the lower plasma levels of C20:3n6 and C22:5n3 in cachectic patients of this study might further contribute to the inflammatory status.

Unlike in the present study, the findings of Hodge et al. did not show an increase in mean SFAs or MUFAs levels in the case group, however, higher levels of C16:0 were associated with a higher CRC risk (Hodge et al., 2015). SFAs transmit pro-inflammatory processes via the toll-like receptor signal cascades, long PUFAs can interfere with these pathways (A, 2014). But since our results showed that PUFA concentration is reduced and SFA levels are even higher in cachectic patients the balance of anti- and pro-inflammatory actions is disturbed in cancer cachexia. The analysis of the relative FA percentages in SAT showed lower levels of GLA C18:3n6 and ALA C18:3n3 in CC while no differences were observed regarding the levels of abundant FAs (table 6).

Two other studies have measured fatty acids in adipose tissue under neoplastic conditions – one using a preclinical rat model of colon cancer and the other evaluated FA profile in SAT of CRC patients (Cottet et al., 2015, Ebadi et al., 2017). Despite the variation in the populations used, these studies concurred with this data as both failed to detect major differences in mean FA levels between groups (Cottet et al., 2015, Ebadi et al., 2017). However, in the patient model, CRC risk was associated with higher levels of C16:1

or higher ratios of n6/n3 (Ebadi et al., 2017) which is important as the latter index was higher in the cachectic group compared to Controls of the present study (figure 14B). A study that addressed gastric ulceration and gastric cancer risk investigated differences between H.pylori positive and negative individuals. H.pylori infection is a risk factor for gastric cancer (Huang et al., 1998) and Pagkalos et al. demonstrated that levels of beneficial PUFAs such as C20:3n6, C22:5n3 and C22:6n3 were higher in subcutaneous adipose of H.pylori negative patients than in H. pylori carriers (Pagkalos et al., 2009).

The fact that only minor changes in the FA profile could be observed between weight stable and cachectic patients might be due to the low turnover rate of adipose tissue (van Staveren et al., 1986). FA composition in adipose tissue can reflect the dietary intake over a period of 2-3 years (Beynen et al., 1980) while in blood FA profile changes within days or weeks (Skeaff et al., 2006). Although fat depletion in cachectic patients is characterized by a rapid breakdown of triglycerides and release of FA (Ebadi and Mazurak, 2014), the data show that the composition of FA within the tissue seems to be relatively stable even under cachectic conditions.

The decrease in plasma PUFA levels, in particular C22:5n3 and C20:3n6 and the concomitant increase in saturated fatty acids may contribute to shift the immune response to a more pro-inflammatory state in CC patients.

4.7 SYSTEMIC RATHER THAN LOCAL PRODUCTION OF SFA DRIVES PRO-INFLAMMATORY RESPONSE IN ADIPOSE TISSUE

In order to discover the underlying mechanisms of the altered FA profile in cancer cachexia the expression patterns of enzymes involved in fatty acid synthesis, elongation and saturation have been investigated. Saturated fatty acids are generated by the action of FASN in the de novo lipid synthesis (Hillgartner et al., 1995, Semenkovich, 1997). FASN protein levels in SAT were not different between the groups while in VAT FASN levels were lower in WSC and CC when compared to Control (Figure 15). However, the serum levels of C16:0 which is a product of the FASN reaction, are higher in the cancer groups.

This might be due to the fact that FASN in the liver is much more important for the whole-body lipogenesis than FASN of the adipose tissue (Swierczynski et al., 2000).

In the cancer groups VAT-FASN-protein-levels correlated inversely with CRP serum concentrations (figure 6) and SAT percentages of C12:0 and C14:0 were also inversely correlated with CRP and circulating inflammatory factors such as IL-6 and CCL2. Furthermore, the total of SFAs in SAT correlated negatively with SAT-IL-6 protein levels (table 7). These results are in contrast to a study of Berndt et al. who showed a positive correlation of FASN mRNA levels in adipose tissue with cytokine concentration within the tissue (Berndt et al., 2007). However, it was also shown that FASN correlated with fat area (Berndt et al., 2007). Thus, it is more likely that the observed lower FASN mRNA levels in CC are associated with the loss of fat mass in this group.

The tumor burden itself could also be an additional factor for the reduced FASN expression. Notarnicola et al. demonstrated 2-fold reduction of FASN gene expression and activity in adipose tissue adjacent to tumor region compared to distant adipose tissue in patients with CRC (Notarnicola et al., 2012). It is possible that VAT is more affected by the tumor induced impairment of lipid metabolism than SAT and hence this might explain why alterations in FASN expression were only observed in VAT but not SAT.

The conversion of the SFAs C16:0 and C18:0 to the MUFAs palmitoleic acid C16:1 and oleic acid C18:1 is catalyzed by the stearoyl-CoA desaturase (Miyazaki et al., 2001). It has been described that these MUFAs have an anti-inflammatory potential while the saturated precursors activate toll-like receptors and thereby activate pro-inflammatory pathways (Rocha et al., 2016, Souza et al., 2014, Carrillo et al., 2012). The C16:1/C16:0 ratio – an index for SCD activity – tended to be lower in CC compared to Control (figure 14C) pointing to a lower activity, in particular a lower desaturation rate from C16:0 to C16:1. This is also supported by the high levels of palmitate in the circulation but was not reflected by the SCD mRNA and protein expression in SAT with higher SCD mRNA levels in CC when compared to Control and unchanged protein expression (Figure 17A, B).

SCD is regulated by several factors, it is induced by its substrate palmitate, insulin and glucose while PUFAs and leptin are SCD suppressors (Ralston et al., 2015, Sampath and Ntambi, 2005). Thus, the high plasma palmitate levels could contribute to the increase

in SAT mRNA levels, since test wise an ex vivo culture experiment showed the induction of SCD in human VAT explants by exogenous palmitate stimulation (Figure 20G). In accordance with such a scenario the up-regulation of SCD by palmitate in isolated human adipocytes was already described (Collins et al., 2010).

However, inflammation seem to have a contrary effect on SCD. The In vitro results of the present study showed an inhibition of SCD mRNA and protein by inflammatory factor TNF-α in adipocytes (Figure 18A). That might be a possible scenario explaining the accumulation of palmitate and stearate in CC. But VAT adipose tissue explants did not respond to TNF-α stimulation (Figure 18C, D), though chronically elevated TNF-α levels might have decreased the responsiveness.

Cancer cachexia is often associated with insulin resistance (Vegiopoulos et al., 2017) and the in vitro data showed an induction of SCD-1 in 3t3 adipocytes (figure 31, 32). However, it was also demonstrated that several inflammatory factors that are described to be increased in cancer cachexia such as TNF-α, PGE_2 and LPS suppress the insulin effect on SCD-1 (figure 31, 32) hence the role of insulin in SCD regulation in adipose tissue of cachectic cancer patients is unclear.

Contrary to what would be expected, plasma levels of oleate were also increased in these patients. Since oleate is a prime substrate for mitochondrial β-oxidation, the latter observation may, however, reflect the shift from aerobic ATP production to glycolytic ATP production in cancer (Ren and Schulz, 2003, Seyfried, 2015). Besides the described anti-inflammatory properties of oleate, there is also evidence that oleate can promote inflammation in macrophages (Nguyen et al., 2007) and could be involved in immune cell activation and infiltration in cancer cachexia. A conclusion of the consequences of SCD regulation in CC is difficult due to the controversial results. In both SAT and VAT show large variance in SCD protein content (Figure 17B,D) indicating that the individual regulation differs immensely between patients which might be due to the short half-life of the protein (Toyama et al., 2007). It is also questionable if the SCD regulation in the adipose tissue really plays a major role in cancer cachexia as its metabolic importance is mostly described in the liver (Flowers and Ntambi, 2008) and a distinct SCD up-regulation was shown in many cancers (Igal, 2010).

However, it seems plausible that an increase in circulating levels of SFAs might further drive inflammation due to the activation of toll like receptors and pro-inflammatory signal cascades. Indeed, palmitate induced the production of CXCL10 and IL-8 in macrophages (Figure 36D, F) and CXCL10 expression in visceral adipose tissue explants (Figure 36I). In fact, in all groups with the highest correlation coefficient in the CC group SAT C18:1 or total MUFAs were positively correlated with SAT concentrations of the chemoattractants IL-8 and CXCL10 or plasma IL-8 (Figure 19A).

Although SCD was suppressed by TNF-α in 3T3-adipocytes which is a model for white fat cells, the rise in palmitate in the cancer groups does not seem to be due to changes in SCD regulation in adipose tissue. SFAs appear to have a different origin but, reaching the adipose tissue via the circulation, could contribute to an increase in pro-inflammatory chemokine production in SAT and VAT.

4.8 DIFFERENTIAL EFFECTS OF INFLAMMATION ON PUFA SYNTHESIZING ENZYMES IN IMMUNE AND FAT CELLS

The shift from n3 to n6-FAs in plasma and adipose tissue of cachectic patients (figure 14B) as well as the positive correlation of several n6-metabolites of the AA pathway in adipose tissue with circulating TNF-α suggest (table 8) that pathways involved in n6-PUFA synthesis are modulated by systemic inflammation. The main enzymes involved in AA synthesis are FADS2, ELOVL5 and FADS1 (1.5.1 figure 6). In VAT but not SAT, FADS2 mRNA expression was lower in CC when compared to Control (Figure 21A,B) while tissue expression of ELOVL5 and FADS1 was not altered between groups (figure 21 B,C and 23).

In the literature it was demonstrated that diet induced acute weight loss in obese women resulted in reduction of FADS1 and FADS2 gene expression in adipose tissue (Dahlman et al., 2005, Mangravite et al., 2007). This could also be a factor in the present study as rapid weight loss is also a key feature of cancer cachexia with the limitation that the present study only evaluate one time point while Dahlmann and Mangravite et al. could

compare the expression before and after weight loss (Dahlman et al., 2005, Mangravite et al., 2007).

FADS2 is important in the AA synthesis as it catalyzes the rate-limiting desaturation of LA C18:2n6 to GLA C18:3n6 (Stoffel et al., 2014). Although GLA levels were lower in SAT of CC, FADS2 gene expression was not impaired in this fat depot and further studies are needed to assess GLA contents in VAT to match the data of FADS2 and the reaction product. Nevertheless, there are results pointing to an altered FADS2 activity in SAT. FADS2 desaturases EDA C20:2n6 to DGLA C20:3n6 and it was shown that the ratio of C20:3n6/C20:2n6 – an index for FADS2 activity was lower in CC compared to WSC (figure 14E). In accordance to that, mice lacking FADS2 in the adipose tissue showed similar changes in WAT and serum FA profile to what was found in the present study (Suitor et al., 2017). However, also in the murine FADS2 knockout, the alterations in FA composition were only marginal. Hence, the authors concluded that alternative elongation/desaturation pathways exist in the adipose tissue and the liver has a compensatory effect in regulation of serum FAs (Suitor et al., 2017).

The effect of systemic inflammation in cancer cachexia on enzyme expression might be masked by high deviations between the individuals. In addition, adipose tissue is composed of different cell types. While pro-inflammatory cytokines repressed FADS1 and FADS2 in adipocytes (Figure 22A, 25C) they induced these enzymes and ELOVL5 in macrophages (figure 22D, I and 25B). Thus, the resulting whole tissue expression levels might appear to be unaltered. In accordance with the repression of FADS1 by TNFα in adipocytes, in the whole study population FADS1 and FADS2 expression of the visceral depot was negatively correlated with TNF-α plasma concentrations and other systemic inflammation markers such as CCL2 and CRP (figure 21E and 24A-C). Over all, Du et al. who found a relation between lower FADS1 contents and poor survival prognosis in esophageal squamous cell carcinoma (Du et al., 2015).

SAT and VAT Chemokine concentrations of IL-8 and CXCL10, though elevated in SAT and VAT of CC, did not affected the expression of FADS2, ELOVL5 or FADS1 in vitro (table 9, Figure 26A-D). Hence, the pro-inflammatory cytokines TNF-α and IL-6 seem to be more relevant for the regulation of FA modifying enzymes.

Palmitate induced FADS1 expression in macrophages and in adipose tissue explants (Figure 26F, H) as well as LPS (Figure 26G), which is an even stronger Toll-like receptor activator (Lu et al., 2008b, Zuany-Amorim et al., 2002). Gastrointestinal cancer is often accompanied by a disturbance of the physiological endothelial barrier function, this could increase blood endotoxin concentration (Kang et al., 2013, Puppa et al., 2011, Klein et al., 2013). Therefore, endotoxins such as LPS might be an additional factor that alters the expression of fatty acid modifying enzymes.

It was tested if the alterations in n6-FA synthesis and systemic inflammation influenced PGE_2 metabolism in cancer cachexia. During cancer cachexia PGE_2 can be released from many tissues such as adipose tissue, liver and from the tumor itself (Howe et al., 2013, Martín-Sanz et al., 2017, Eberhart et al., 1994) where inducible synthesizing enzyme COX-2 is increased via the NFκB-pathway (Sano et al., 1995). In fact, PGE_2 tissue concentration and the mRNA expression of the constitutive synthesizing enzyme COX-1 were positively correlated with CRP and plasma CXCL10 in cancer patients independent from cachexia (figure 27B and 28C,D) though VAT-PGE_2-concentrations and COX-1 mRNA levels did not differ between groups (figure 27A and 28A,B). COX-2 seems to play a bigger role in PGE_2 production in cancer cachexia as mRNA levels were higher in VAT samples of CC (figure 29A,B).

The expression levels of COX-2 were related to higher levels of CRP and VAT-IL-6-concentration with the highest correlation coefficient in CC (figure 29B, C). These results indicate that COX-2 in adipose tissue of cachectic patients is induced by IL-6. In line with that, in vitro studies showed that IL-6 stimulation increased COX-2 expression by 2.5-fold in macrophages (figure 30B). Therefore, it is possible that the infiltrated immune cells are the main contributors in PGE_2 production since it was shown before that PGE_2 can enhance inflammatory response of macrophages in an autocrine positive feed forward or negative feedback loop (Aoki and Narumiya, 2012). Additionally the higher COX-2 expression could results from toll-like receptor activation by complexes of fetuin A and SFAs (Howe et al., 2013, Pal et al., 2012) which were found to be increased in CC. The increase of COX-2 in VAT of CC can contribute to adipose tissue wasting as it was shown previously that COX-2 action promotes WAT-browning (Vegiopoulos et al., 2010).

Several animal and clinical studies showed a relation between prostaglandins and the severity of cancer or cancer cachexia (Wang et al., 2005, Lira et al., 2010, Schrey and Patel, 1995) and COX-2 inhibition reversed weight loss in cachectic patients and animal models (Mantovani et al., 2010, Lai et al., 2008, Davis et al., 2004). Endogenous and ingested n3-PUFAs reduce the amount of PGE_2 (Gravaghi et al., 2011, Gärtner et al., 2016) but since the percentages of these FA were lower in CC it may be concluded that physiological regulation of pro-inflammatory lipid mediators is impaired in cancer cachexia.

Since FADS2 was repressed in VAT of CC patients as well as by TNFα treatment in 3T3L1 cells, the FADS2-mediated desaturation steps in PUFA synthesis could be impaired in adipose tissue of CC. By contrast the elongation appeared not be affected or rather induced in immune cells. However, it remains elusive whether changes in the local capacity to produce PUFA would impact the production of PUFA-derived lipid mediators at all, since circulation FA might have a larger impact on the lipid mediator production than adipose tissue-derived fatty acids.

4.8 INFLAMMATION RESOLUTION – IMPAIRED BUT NOT SWITCHED OFF

In contrast to pro-inflammatory n6-derived LM are LM deriving from n3-PUFAs known for their ability to resolve inflammation (Titos and Clària, 2013). A higher availability of n3-FA, which are precursors for anti-inflammatory LM, increases their endogenous production (Laviano et al., 2013). Consequently, it was assumed that levels of circulating resolving compounds are reduced in plasma of CC patients since contents of n3-PUFAs were found to be lower. Against that expectation the plasma concentration of resolvin D1 was higher in CC when compared to Control (figure 40).

Resolvin D1 is synthesized out of DHA C22:6n3 (Serhan et al., 2009) but neither in plasma nor in adipose tissue C22:6n3 was detected. Most studies are measuring resolving LM after a dietary intervention with n3-PUFAs but less data investigated physiologic or pathophysiologic endogenous LM levels (Schwab et al., 2007, Clària et al., 2011). It is possible that the higher resolvin D1 concentrations are an approach to compensate the inflammatory status. This was also described by Eickmeier et al. who

found positive correlations of RvD1 with pro-inflammatory factors in lung disease of cystic fibrosis (127). ALOX15 and ALOX5 are involved in RvD1 synthesis as well as COX-2 via an alternative pathway triggered by aspirin (1.5.1 figure 7). Within the adipose tissue both infiltrating immune cells as well as adipocytes possess enzymatic equipment for LM synthesis (Eickmeier et al., 2017, López-Vicario et al., 2015).

Although mRNA levels of ALOX15 were higher in VAT of CC (figure 31B), in all cancer patients ALOX15 mRNA levels were negatively correlated with VAT-IL-6 gene expression and also IL-6 stimulation in vitro resulted in a suppression of ALOX15 in U937 macrophages (figure 32B). These results in combination with the lower ALOX15 protein levels in SAT and VAT of CC (figure 41C, D) suggest a reduced production of ALOX15 dependent LM such as D-series resolvins or lipoxins in die adipose tissue of cachectic cancer patients.

ALOX5 is involved in synthesis pathways of resolving LM such as RvD1 but in reaction with its activating protein ALOX5AP it is also involved in the synthesis of pro-inflammatory LM such as leukotrienes (A, 2014, O'Connor et al., 2014). Other findings further support the idea that the RvD1 synthesis pathway could be suppressed in CC since ALOX5 mRNA levels in VAT of CC were lower compared to Control and in vitro down-regulated by pro-inflammatory cytokines (figure 44B, 45C, E). On the other hand the higher VAT ALOX5AP expression in CC (figure 44D) and its induction by cytokines (figure 45D, F and 46C) may be indicative for increased leukotriene production.

LM function is transduced via specific receptors on immune cells (Freire and Van Dyke, 2013, Serhan and Chiang, 2013). CMKLR1 mediated resolvinE1 signaling attenuate inflammation by increasing phagocytosis of macrophages and inhibiting migration of PMNs and dendritic cells (Fredman and Serhan, 2011). IL-8 stimulation of U937 macrophages increased CMKLR1 expression (figure 47G) but although IL-8 concentration is high in VAT of cachectic patients and other cytokines are described to enhance CMKLR1 expression in adipocytes (Fredman and Serhan, 2011) , CMKLR1 mRNA levels in the adipose tissue of patients did not differ between groups (figure 47 A, B).

There is evidence for an impaired anti-inflammatory capacity in cancer cachexia since the levels of n3-FA precursors are lower as are protein levels of ALOX15, an important key enzyme of anti-inflammatory LM formation. The origin of the higher resolvin D1 levels is unclear. The increase seems to be a systemic attempt of the organism to cope with the chronic inflammation which, however, it fails to resolve.

5 CONCLUSION

Cancer cachexia is an inflammatory condition. Pro-inflammatory cytokines drive systemic inflammation and promote the increase of chemokines in white adipose tissue leading to infiltration of immune cells and local adipose tissue inflammation. Pro-inflammatory saturated fatty acids and lipid mediators can further contribute to the increase in chemokines in the adipose tissue. The synthesizing pathways of anti-inflammatory lipid compounds which could counteract seem to be altered in cancer cachexia contributing to persistence of inflammation and progression of syndrome.

APPENDIX A FATTY ACID COMPOSITION PLASMA

Appendix 1 Fatty acids in plasma, Data by Daniela Riccardi

	Control (n=14)	WSC (n=14)	CC (n=14)	P
14:0	1.74 [0.48; 2.8]	1.00 [0; 1.52]	0 [0; 1.21]*	0.019
16:0	24.10 [21.43; 26.41]	27.83 [26.98; 28.63	27.98 [26.02; 30.89]*	0.006
16:1	0.70 [0.35; 1.09]	1.08 [0.59; 1.43]	1.20 [0.79; 1.77]	0.065
17:0	0.08 [0; 0.22]	0.16 [0; 0.28]	0 [0; 0.12]	0.533
18:0	13.25 [11.9; 15.29]	13.93 [12.97; 17.0	15.37 [14.01; 18.8]*	0.025
18:1c9	20.56 [19.4; 21.4]	27.39 [26.71; 29.90	28.55 [25.66; 29.83]*	<0.001
18:2n6	21.59 [21.24; 25.61]	15.63 [13.73; 16.50	12.19 [10.40; 13.67]*	<0.001
18:3n3	0.10 [0.06; 0.21]	0 [0; 0]*	0 [0; 0]*	<0.001
20:3n6	0.15 [0; 0.41]	0.35 [0; 1.78]	0 [0; 0.12]#	0.034
20:4n6	8.62 [7.92; 9.01]	8.50 [8.01; 8.88]	7.73 [6.99; 8.42]	0.146
22:5n3	0.04 [0; 0.09]	0.06 [0; 0.27]	0 [0; 0]#	0.005

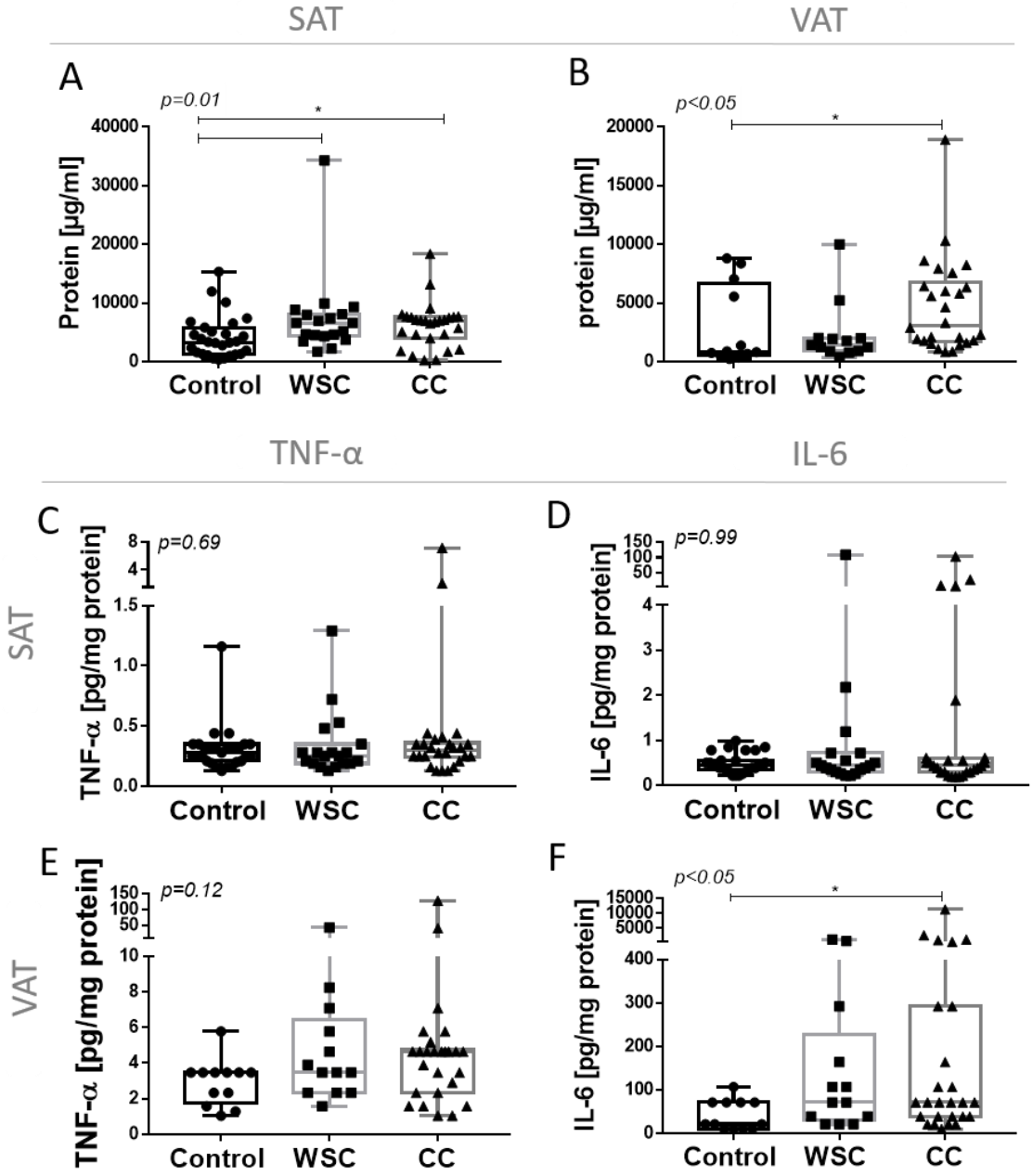
Data are expressed as median [1st quartile; 3rd quartile], p = significance level Kruskal-Wallis test. vs Control. *significant difference ($p < 0.05$); # Significant difference vs. WSC ($p < 0.05$).

Appendix 2 Groupings of fatty acids, data by Daniela Riccardi

%	N (n=14)	WSC (n=14)	CC (n=14)	*p*
SFA	39.67 [36.2; 42.59]	43.56 [40.71; 46.61]	44.2 [42.64; 47.46]*	**0.005**
MUFA	21.13 [20.13; 23.09]	28.44 [27.79; 30.68]*	29.85 [27.41; 30.88]*	**<0.001**
PUFA n-3 + n-6	32.14 [28.81; 34.89]	24.64 [22.37; 25.83]*	20.8 [17.29; 21.60]*	**<0.001**
PUFA n-3	0.13 [0.09; 0.28]	0.06 [0; 0.27]	0 [0; 0]*#	**<0.001**
PUFA n-6	32.04 [28.78; 34.62]	24.52 [21.84; 25.83]*	20.8 [17.29; 21.60]*	**<0.001**

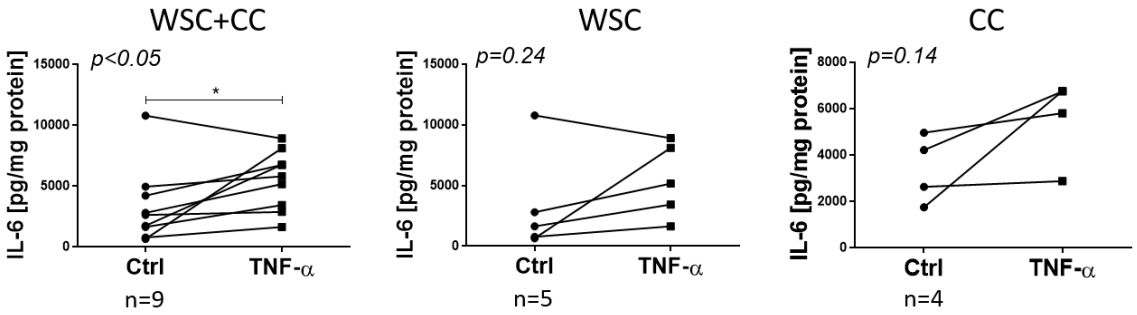
Data are expressed as median [1st quartile; 3rd quartile], p=significance level Kruskal-Wallis test. SFA: saturated fatty acids (groupings of fatty acids 14:0, 16:0, 17:0 and 18:0); MUFA: monounsaturated fatty acids (groupings of fatty acids 16:1, 18: 1C9); PUFA: polyunsaturated fatty acids (groupings of fatty acids 18:2n6, 18:3n3, 20:n6, 20:4n6 and 22:5n3); n-3: omega-PUFA (groupings of fatty acids 18:3n3 and 22 :5n3); n-6: omega-6 PUFA (groups of fatty acids 18:2n6, 20:n6 and 20:4n6) vs Control * significant difference ($p < 0.05$); # Significant difference vs. WSC ($p<0.05$)

APPENDIX B – ADIPOSE TISSUE CYTOKINES NOT NORMALIZED TO PROTEIN



Appendix 3: A,B Protein concentrations in SAT and VAT lysates ; C,D TNF-α, IL-6 concentration without normalization in SAT; E,F TNF-α, IL-6 concentration without normalization in VAT;

APPENDIX C – IL-6 IN VAT EXPLANTS

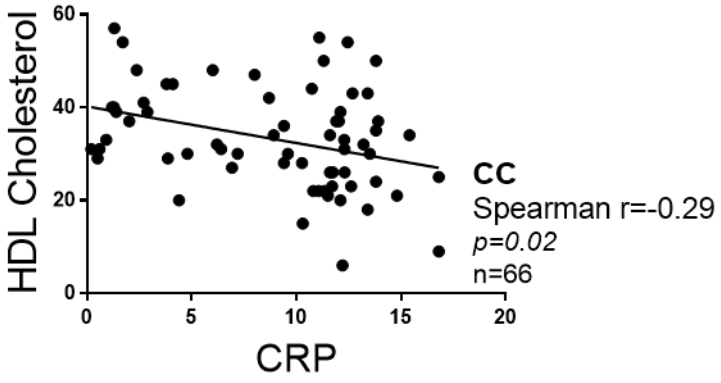


Appendix 4: IL-6 concentration in VAT explants after 24h incubation with TNF-α

APPENDIX D – HDL CORRELATION WITH CRP

HDL Cholesterol <> CRP

	all patients n=168	Control n=54	WSC n=48	CC n=66	WSC+CC n=114
Spearman r	**-0.30**	**-0.02**	**-0.30**	**-0.29**	**-0.36**
p-value	*<0.0001*	*0.89*	*<0.05*	*0.02*	*<0.0001*



Appendix 5: Spearman correlations of HDL-Cholesterol with CRP serum levels

APPENDIX E – CONSENT FORM

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

ESTUDO: Inflamação sistêmica em pacientes caquéticos com câncer: mecanismos e estratégias terapêuticas, uma abordagem da medicina translacional.

O sr(a) será internado na Divisão de Cirurgia do Hospital Universitário da USP.
Neste serviço além de cuidarmos dos pacientes, realizamos cirurgias e, também, estudamos as doenças com a finalidade de melhorar o atendimento aos pacientes.
Desta forma, o sr(a) está sendo convidado (a) a participar do Projeto de Pesquisa acima citado. O documento abaixo contém todas as informações necessárias sobre a pesquisa que estamos fazendo. Sua participação neste estudo será de muita importância para nós.
Caso o sr(a) não queira participar ou desistir a qualquer momento, isso não causará nenhum prejuízo ao seu tratamento, ou seja, sua participação é espontânea.

Estou ciente que:
O estudo é de importância para que possamos investigar as possíveis causas da caquexia (perda de peso, apetite e qualidade de vida), uma possível consequência da sua doença;

Caso aceite participar da pesquisa, no dia da sua internação no hospital, poderemos pedir para colher suas fezes. Durante a sua cirurgia, o cirurgião poderá retirar pequenos fragmentos (cerca de 1g, ou para melhor entendimento o tamanho de dois feijões) da gordura da sua barriga e de dentro dela, além de um pedaço semelhante do musculo que será cortado para a realização da cirurgia (músculo reto abdominal). Se na sua cirurgia for necessária a retirada de uma pequena porção de fígado (para a análise microscópica), será retirado um fragmento pequeno (cerca de 0,2g ou dois grãos de arroz) para o estudo. Se a cirurgia envolver a retirada de uma porção de intestino, parte do material que seria descartado poderá ser utilizado para a pesquisa. **Todos esses procedimentos podem aumentar o tempo de cirurgia em não mais que 5 minutos. Não será necessário prolongar a anestesia, por que os procedimentos realizados para a coleta são os mesmos da cirurgia. A coleta de amostras para esse estudo não modifica, de forma alguma, os procedimentos padrão da cirurgia a qual você será submetido.** Todo esse material servirá para entender melhor a doença chamada caquexia e, se você participar do estudo com exercício físico, o material também servirá para entender se o exercício melhora os sintomas da doença.

Mesmo que você concorde com a doação de todas as amostras, o cirurgião e a equipe médica poderão coletar apenas algumas delas ou nenhuma, conforme a condução da cirurgia, para que não haja aumento de riscos para você.

De acordo com os resultados dos exames laboratoriais e clínicos prévios à cirurgia, e após consentimento do médico, **você poderá ser convidado a participar do Protocolo de Exercício Físico, que terá duração de seis semanas**. Se você concordar em participar no estudo que envolve exercício físico, pediremos para realizar coletas de **20 mL de sangue** (quantidade mostrada na

seringa que o pesquisador está mostrando a você), nas primeira, terceira e sexta semanas de treinamento, para que os parâmetros plasmáticos e séricos possam ser aferidos e durante o Protocolo de Exercício Físico (isso permite avaliar como você está reagindo ao exercício). A coleta será realizada por um profissional da saúde devidamente habilitado e ocorrerá durante a sessão do treino no próprio hospital. Para aqueles que não participam do protocolo de exercício, será realizada uma coleta de sangue (**20 ml**) previamente à cirurgia, sem interferir no procedimento cirúrgico, por um profissional da saúde devidamente habilitado, também no próprio hospital, em condições de assepsia, ou seja limpeza total. O seu sangue será analisado para verificarmos grau de inflamação, que é uma medida utilizada pela equipe de saúde para entender as mudanças que ocorrem em seu organismo devido a doença e a associação desta inflamação com o que você sente. Se você também participar da pesquisa com exercício físico, avaliaremos se o protocolo causa melhora dos sintomas.

Antes da realização do exercício, um médico cardiologista do hospital avaliará sua condição física, para garantir sua segurança na realização do protocolo. Esse teste poderá ser repetido no fim do protocolo de exercício, para aferir seu grau de adaptação ao treinamento (o quanto o treinamento melhorou sua condição cardiorrespiratória – funcionamento do seu coração e do seu pulmão). Esses testes não causam dor e não têm risco associado.

Você pode ser convidado a realizar outro exame. Nesse exame o pesquisador estuda sua composição corporal (o quanto você tem de músculo, gordura e osso no corpo) e como a doença, ou o exercício físico, modificam esse parâmetro. O exame não causa desconforto e é solicitado pelo médico para completar o entendimento do que está acontecendo com o seu corpo e isto serve para o controle de sua doença.

As coletas realizadas, de tecidos, sangue e os exames de composição corporal e a avaliação cardiorrespiratória servirão para o entendimento e estudo da doença e poderão ajudar na busca de tratamento. A participação neste projeto não tem como objetivo, contudo, tratar sua doença, mas sim dar uma contribuição para que possamos definir as alterações que ocorrem no corpo devido a doença.

Você não terá qualquer despesa financeira com relação aos procedimentos médicos, clínicos e terapêuticos efetuados no estudo;

Você não receberá compensação financeira por participação do estudo, pois os procedimentos do estudo serão realizados nas visitas que você já deverá fazer ao hospital, dentro da indicação médica.

Você tem a liberdade de desistir ou de interromper a colaboração neste estudo no momento em que desejar, sem necessidade de qualquer explicação;

A desistência não causará nenhum prejuízo à sua saúde ou bem estar físico. Não virá a interferir no atendimento ou tratamento médico, ou no agendamentos necessários.

Os resultados obtidos durante este estudo serão mantidos em sigilo, mas você deve concordar que sejam divulgados em publicações científicas (resumos de congressos, livros e artigos de periódicos científicos), desde que seus dados pessoais não sejam mencionados. Isso é importante para garantir que a pesquisa possa ser conhecida e possivelmente melhorar o diagnóstico, o atendimento e o tratamento de pacientes com a mesma doença que a sua.

O material coletado poderá ser armazenado em soluções específicas para cada técnica, em freezer -80º, para manter a integridade das amostras e posterior utilização, sempre dentro da mesma linha de pesquisa;

Após a utilização do material coletado para a pesquisa, caso haja material remanescente, este será incinerado (queimado) em local para descarte de material biológico humano;

Caso você desejar, poderá, pessoalmente, tomar conhecimento dos resultados ao final desta pesquisa;

( ) Desejo conhecer os resultados desta pesquisa.
( ) Não desejo conhecer os resultados desta pesquisa.

Esse é um Projeto Temático, financiado pela Fundação de Amparo à Pesquisa do Estado de São Paulo (processo n. 12/50079-0), que será desenvolvido no Instituto de Ciências Biomédicas - ICB/USP/São Paulo numa colaboração que envolve os seguintes pesquisadores: Giorgio Trinchieri e Romina Goldzmid (National Institute of Health - NIH); Josép Argilés e Silvia Busquets (University of Barcelona); Alessandro Laviano e Maurizio Muscaritoli (Universíty La Sapienza UniRoma); Gerhard Püschel e Tiziana Magaria (University of Potsdam); Stephen Farmer (Boston University); Marília Cerqueira Leite Seelaender, Alison Colquhoun e José Cezar Rosa Neto (ICB/ USP); Paulo Sérgio Alcântara, Linda Maximiniano, Oscar Fujita, Claudio Campi e Emerson Muller (HU/ USP); José Pinhata Otoch e Geraldo Busatto Filho (FMUSP); Emerson Franchini (EEFE/ USP); Renata Wassermann (IME/ USP); Claudia Oller do Nascimento e Lila Missaie Oyama (UNIFESP); e Miguel Batista Junior (UMC – Universidade de Mogi das Cruzes).

A qualquer momento, você poderá entrar em contato com a Comissão de Ética em Experimentos com seres Humanos do Instituto de Ciências Biomédicas da USP, ou a Comissão de Ética em Pesquisa do Hospital Universitário, que são os órgãos que avaliam a realização de pesquisas com pessoas e garantem que a pesquisa da qual você participa seja de importância clínica e/ou científica e que está sendo conduzida de forma apropriada. O telefone, email e endereço dessas comissões estão no fim desse documento, que será emitido em 2 vias, para que você tenha uma cópia.

Caso você tenha, em algum momento, desconforto relacionado à sua doença (a participação na pesquisa só poderá causar desconforto adicional mínimo, relacionado à coleta de sangue), você deverá entrar em contato com o telefone fornecido ao final desse documento (Pronto-atendimento do HU).

Eu, (inserir o nome, profissão, residente e domiciliado na, telefone) _____ _____, portador da Cédula de identidade, RG _____, e inscrito no CPF/MF_____ nascido (a) em _____ / _____ / _____ , abaixo assinado (a), concordo de livre e espontânea vontade em participar como voluntário (a) do estudo

"***Inflamação sistêmica em participante da pesquisas caquéticos com câncer: mecanismos e estratégias terapêuticas, uma abordagem da medicina translacional****"*.

"CONCORDO, APÓS CONVENIENTEMENTE ESCLARECIDO PELO PESQUISADOR E TER ENTENDIDO O QUE ME FOI EXPLICADO, EM PARTICIPAR DA PRESENTE PESQUISA".

São Paulo, _____ de_____de 20__

( ) Participante da pesquisa _____

Testemunha 1: _____
Nome / RG / Telefone

Testemunha 2: _____
Nome / RG / Telefone

Responsável Médico_____
Prof. Dr. José Pinhata Otoch
Hospital Universitário USP
Tel 11 3091-9291 / 11 3091-9489

Responsável pelo Projeto: _____
Profª Dra. Marília Cerqueira Leite Seelaender
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Endereço: Av. Prof. Lineu Preste, 2565 – Cidade Universitária – Butantã, São Paulo – SP, CEP: 05508-000, telefone: 3091-9457, e-mail: cep@hu.usp.br (funcionamento - 8h às 12h e das 14h às 17h)

Identificação do CEESH-ICB/USP:
Endereço: Av. Prof. Lineu Prestes, 2415 - Cidade Universitária – Butantã, São Paulo – SP, CEP 05508-900, telefone: 3091-7733, e-mail: cep@icb.usp.br (funcionamento - 8h às 12h e das 14h às 17h)

Pronto-atendimento do HU: Endereço: Av. Prof. Lineu Preste, 2565 – Cidade Universitária – Butantã, São Paulo – SP, CEP: 05508-000, telefone: 3091-9344 (funcionamento 24h)

APPENDIX F – ANOREXIA QUESTIONAIRE

PONTUAÇÃO ANORÉXICA (FAACT-ESPEN)

	Nada	Um pouco	Moderadamente	Bastante	Muito
Meu apetite é bom?	0	1	2	3	4
A quantidade que eu como me satisfaz?	0	1	2	3	4
Me preocupo com meu peso?	0	1	2	3	4
Os alimentos têm gosto ruim?	4	3	2	1	0
Estou preocupado por parecer magro?	4	3	2	1	0
Meu apetite acaba no início das refeições?	4	3	2	1	0
Há dificuldade de comer alimentos pesados ou elaborados?	4	3	2	1	0
Minha família me induz a comer?	4	3	2	1	0
Vômitos?	4	3	2	1	0
Me sinto satisfeito após algumas mordidas?	4	3	2	1	0
Dores no estômago?	4	3	2	1	0
Minha saúde está melhorando?	0	1	2	3	4

APPENDIX G – QUALITY OF LIFE QUESTIONAIRE

QUESTIONÁRIO EORTC QLQ-C30

Gostaríamos de conhecer alguns pormenores sobre si e sua saúde. Responda você mesmo/a, por favor, a todas as perguntas fazendo um círculo à volta do número que melhor se aplica ao seu caso. Não há respostas certas ou erradas. A informação fornecida é estritamente confidencial.

Escreva as iniciais do seu nome: _____

A data de nascimento (dia/mês/ano): ___/___/___

A data de hoje (dia/mês/ano): ___/___/___

		Não	Um pouco	Bastante	Muito
1.	Custa-lhe fazer esforços mais violentos, por exemplo, carregar um saco de compras pesado ou uma mala?	1	2	3	4
2.	Custa-lhe percorrer uma grande distância a pé?	1	2	3	4
3.	Custa-lhe dar um pequeno passeio a pé, fora de casa?	1	2	3	4
4.	Precisa de ficar na cama ou numa cadeira durante o dia?	1	2	3	4
5.	Precisa que o ajudem a comer, a vestir-se, a lavar-se ou a ir à casa de banho?	1	2	3	4

Durante a última semana:		**Não**	**Um pouco**	**Bastante**	**Muito**
6.	Sentiu-se limitado/a no seu emprego ou no desempenho de suas atividades diárias?	1	2	3	4

7.	Sentiu-se limitado/a na ocupação habitual dos seus tempos livres ou noutras atividades de lazer	1	2	3	4
8.	Teve falta de ar?	1	2	3	4
9.	Teve dores?	1	2	3	4
10.	Precisou descansar?	1	2	3	4
11.	Teve dificuldade em dormir?	1	2	3	4
12.	Sentiu-se fraco?	1	2	3	4
13.	Teve falta de apetite?	1	2	3	4
14.	Teve enjoos?	1	2	3	4
15.	Vomitou?	1	2	3	4

Durante a última semana:		**Não**	**Um pouco**	**Bastante**	**Muito**
16.	Teve prisão de ventre?	1	2	3	4
17.	Teve diarréia?	1	2	3	4
18.	Sentiu-se cansado?	1	2	3	4
19.	As dores perturbaram suas atividades diárias?	1	2	3	4
20.	Teve dificuldade em concentrar-se, por exemplo, para ler o jornal ou ver televisão?	1	2	3	4
21.	Sentiu-se tenso(a)?	1	2	3	4
22.	Teve preocupações?	1	2	3	4
23.	Sentiu-se irritável?	1	2	3	4
24.	Sentiu-se deprimido?	1	2	3	4
25.	Teve dificuldade em lembrar-se das coisas?	1	2	3	4
26.	O seu estado físico ou tratamento médico interferiram na sua vida familiar?	1	2	3	4
27.	O seu estado físico ou tratamento médico interferiram na sua atividade social?	1	2	3	4

28.	O seu estado físico ou tratamento médico causaram-lhe problemas de ordem finaceira?	1	2	3	4

Nas perguntas que se seguem faça um círculo à volta do número, entre 1 e 7, que melhor se aplica ao seu caso

29. Como classificaria a sua saúde em geral durante a última semana?

1	2	3	4	5	6	7
péssima						ótima

30. Como classificaria a sua qualidade de vida global durante a última semana?

1	2	3	4	5	6	7
péssima						ótima

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