Inflammation and myosteatosis in pancreatic cancer cachexia

INFLAMMATION AND MYOSTEATOSIS IN PANCREATIC CANCER CACHEXIA

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TABLE OF CONTENTS

CHAPTER 1

General introduction, aims, and outline of the thesis

Pancreatic cancer

Pancreatic cancer is the twelfth most common cancer worldwide, accounting for approximately 2.6% (495,773) of new cancer cases and resulting in 466,003 deaths globally in 2020 (Figure 1) [1]. The global burden of pancreatic cancer has doubled over the past two decades and it was ranked as the seventh most common cause of cancer-related mortality worldwide in 2020. In the Netherlands, the age-standardized rates (ASR, per 100,000 persons-years) of incidence and mortality are 7.4 and 7.0, respectively (Figure 2). The risk factors for developing pancreatic cancer include modifiable factors such as smoking, alcohol, obesity, dietary factors, and exposure to toxic substances, and non-modifiable factors such as sex, age, ethnicity, diabetes mellitus, family history of pancreatic cancer, genetic factors, chronic infections, non-O blood group and chronic pancreatitis [2]. The incidence and mortality of pancreatic cancer rise dramatically with age. Given the aging population and lifespan increases, the incidence of pancreatic cancer is expected to grow remarkably worldwide, with an expected number of 801,634 new cases by 2040 (Figure 3A), more than 1.6 times the number of new cases in 2022.

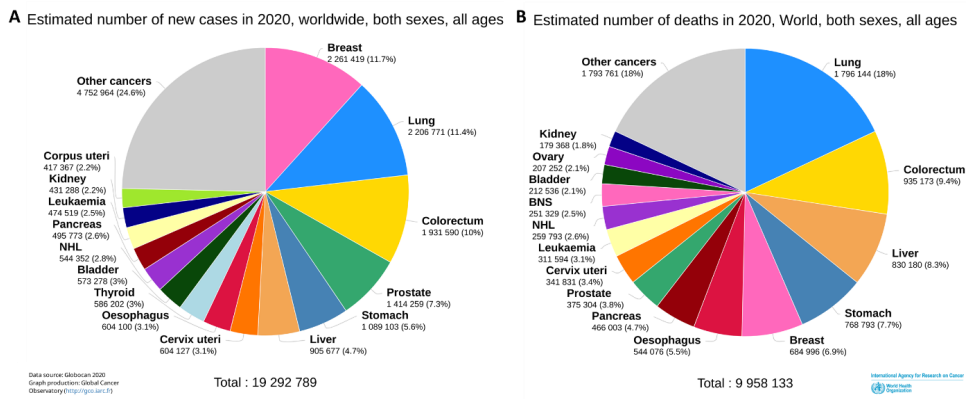

Figure 1: Incidence (A) and mortality (B) of different cancers worldwide in 2020.

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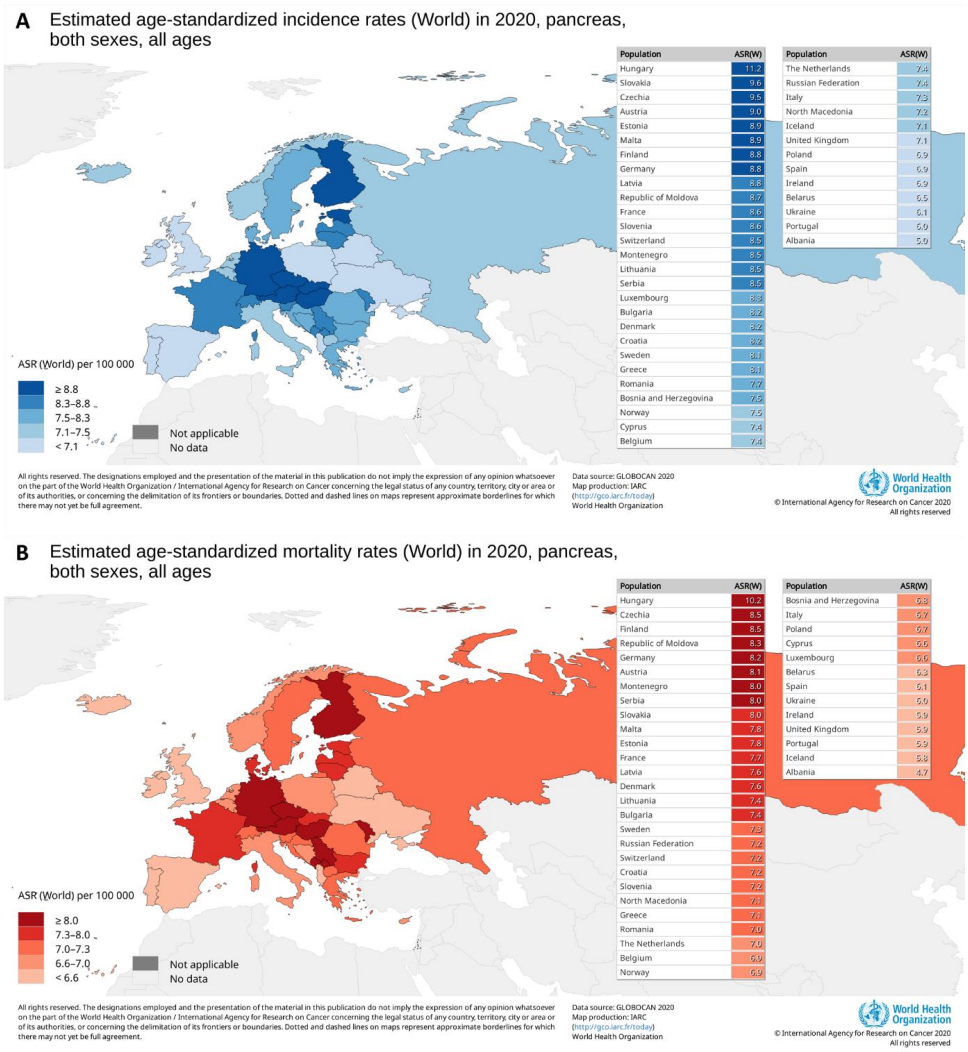

Figure 2: Incidence (A) and mortality (B) of pancreatic cancer in Europe in 2020. Age-standardized rates (ASR) (https://gco.iarc.fr/today/home)

In the past decades, several diagnostic strategies have been explored for the early screening of pancreatic cancer, including the use of blood-based biomarkers such as carbohydrate antigen 19-9 (CA19-9) [3, 4] carcinoembryonic antigen (CEA), and elastase 1 [5], imaging-based techniques such as magnetic resonance imaging (MRI), computed tomography (CT), positron emission tomography/computed tomography (PET-CT), and endoscopic ultrasonography (EUS), and DNA-based molecular techniques [6]. However, these strategies are still relatively

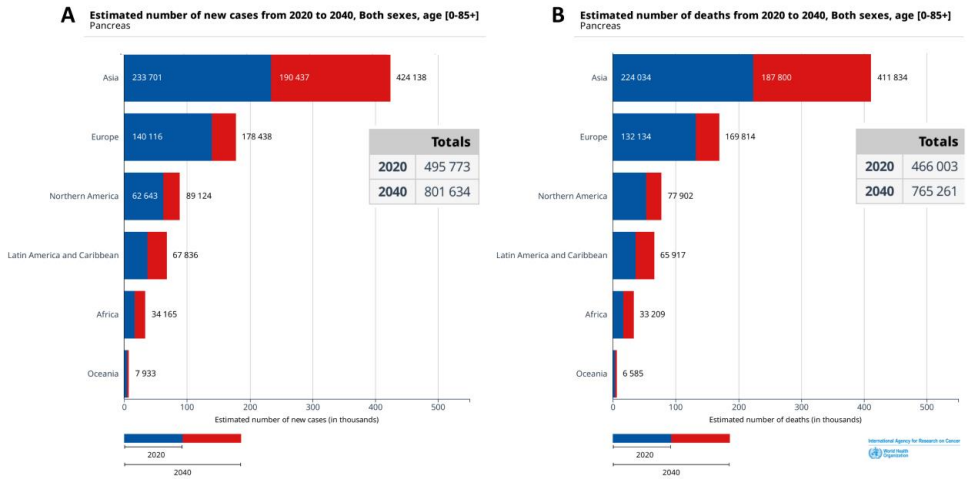

Figure 3: Estimated number of new pancreatic cancer cases (A) and number of deaths from pancreatic cancer (B) worldwide from 2020 to 2040. (https://gco.iarc.fr/today/home)

ineffective for early diagnosis, and also because of the lack of early disease-specific symptoms, more than 80% of the patients are still diagnosed only after developing advanced or metastatic disease. Currently, the first-line treatment for patients with resectable pancreatic tumors (10–20%) is surgery followed by adjuvant chemotherapy, with a 5-year survival rate of 30%. For patients with borderline resectable and/or locally advanced unresectable pancreatic cancer, neoadjuvant treatment is recommended in the guidelines. In the setting of metastatic pancreatic ductal adenocarcinoma (PDAC), which represents about 90% of pancreatic neoplasms, FOLFIRINOX and nab-paclitaxel plus gemcitabine are considered first-line treatment options in patients with good performance status. Furthermore, the use of nanoliposomal irinotecan, 5-fluorouracil and folinic acid combination therapy in second-line therapies has been approved to prolong survival after first-line treatment failure. Beyond traditional treatments, several alternative therapies such as immunotherapy and gene therapy are being explored in clinical studies. Despite the improvement in surgical and medical management, the overall 5-year survival rate of patients with pancreatic cancer is still lower than 10% worldwide.

Cancer cachexia

The term “cachexia” originates from the combination of two Greek words κακός (“bad” or “injurious”) and ἕξῐς (“act of having” or “state of body”) [7]. Cancer cachexia is a metabolic and multifactorial syndrome characterized by involuntary reduction of body weight (mainly skeletal muscle and fat mass), which ultimately leads to poor quality of life, resistance to anti-cancer treatment, and cancer-related morbidity and mortality [8, 9]. Before 2011, several definitions of cancer cachexia had been proposed considering weight loss, inflammation, anorexia, anemia, and fatigue, but no agreed consensus definition of cancer cachexia existed in the literature and clinical practice. In 2011, an international consensus statement specific to cancer cachexia was published, in which cancer cachexia was defined as “a multifactorial syndrome characterized by ongoing loss of skeletal muscle mass (with or without loss of fat mass), that is not fully reversible using conventional nutritional support and that eventually leads to functional impairment” [9]. More specifically, the cancer cachexia diagnosis was reserved for cancer patients who experienced more than 5% body weight loss in the previous 6 months without starvation or more than 2% weight loss in patients with either a body-mass index (BMI) <20 or sarcopenia. In addition, a framework for defining cachexia stages (pre-cachexia, cachexia, refractory cachexia) was introduced at the same conference and was evaluated in an international multicentre project (EPCRC-CSA) in 2014 [10]. Early metabolic changes, weight loss (≤5%), and anorexia may already happen pre-diagnosis of cachexia, in a stage referred to as pre-cachexia. Although nutritional interventions may help in alleviating symptoms of pre-cachexia, most patients continue to lose body weight and develop cachexia. Cachectic patients usually progress to refractory cachexia when anticancer treatments are no longer effective to control tumor growth. Once patients enter the refractory cachexia stage, it is generally considered irreversible, and death occurs within three months [9].

Cancer cachexia affects up to 80% of patients with advanced or metastatic cancer and has been estimated to account for 20% of all cancer-related deaths [11]. The prevalence of cancer cachexia varies depending on the cancer site, cancer stage, genotype, body mass index (BMI), and age. The prevalence of cancer cachexia is approximately 67% in pancreatic cancer, 60% in gastro-oesophageal cancer, and 49% in head/neck cancer, with corresponding weight loss percentages of 13.7%, 13.4%, and 9.5%, respectively [12]. In the past few years, several therapeutic strategies for cancer cachexia have been proposed, including multimodal care, pharmacologic treatment, nutrition, and exercise. From all these different strategies, the

provision of nutritional supplementation is still a mainstay of treatment. However, its therapeutic effect varies, with some clinical studies reporting clear benefits while others reporting little to no amelioration in body weight or quality of life [13]. To date, clinically meaningful care guidelines and effective treatment for patients with cancer cachexia remain lacking.

Inter-tissue communication in cancer cachexia

It is generally accepted that cancer cachexia is a multiorgan disorder. Except for skeletal muscle and adipose tissues, the immune system as well as multiple organs including the brain, pancreas, liver, heart, bone, and gut are also affected during cachexia progression. Several interactions between tumor and host-organs are thought to occur earlier than the clinical manifestation of cancer cachexia. As shown in the hypothetical Figure 4, the immune system acts as a line of defense in response to tumor growth, which may lead to systemic inflammation [14]. In the early stages of cancer cachexia, an increase in gluconeogenesis and acute phase protein synthesis by hepatocytes has been reported, the latter of which could lead to decomposition of muscle proteins resulting in muscle wasting. The pancreas has the dual function of releasing digestive enzymes via exocrine ducts to digest lipids, carbohydrates, and protein [15] and secreting hormones like insulin and glucagon to control glucose homeostasis [16]. Pancreatic exocrine insufficiency was found in up to 90% of patients with pancreatic head tumors, leading to poor nutrient uptake and malnutrition [16], the latter of which often occurs in cachectic patients. Furthermore, 75% of patients with pancreatic cancer have compromised endocrine function [17], leading to insulin resistance that, in general, has been reported to gradually increase with the development of cancer cachexia [18, 19]. Loss of appetite (anorexia) is another early event in cachexia. Studies in mouse models of cancer cachexia have revealed that tumor-derived mediators such as growth differentiation factor 15 (GDF-15), insulin-like peptide 3 (INSL3) as well as bone-derived LCN-2 can induce anorexia by activating their receptors in hypothalamus of the brain that regulates feeding behaviors. In addition, tumor-derived catabolic factors such as interleukin 6 (IL-6), tumor necrosis factor-alpha (TNF-α), and interleukin 1 beta (IL-1β) promote lipolysis of adipose tissue resulting in the release of free fatty acids into the bloodstream, which benefits tumor growth and metastasis. Moreover, a switch from white to brown adipose tissue has been reported in a

mouse model of cancer cachexia [20], which is also an early event in the pathophysiology of cachexia and triggered by systemic inflammation/IL-6 [20], and tumor-derived parathyroid hormone-related protein (PTHrP) [21]. The cross-talk between adipose tissue and muscle further aggravates muscle wasting. Finally, gut barrier dysfunction has also been associated with cancer cachexia. For instance, increased gut permeability and gut microbiota dysbiosis are often observed during the development of cachexia. Although a recent study suggests that gut barrier dysfunction occurs at the onset of cachexia [22], further investigations are required to confirm this.

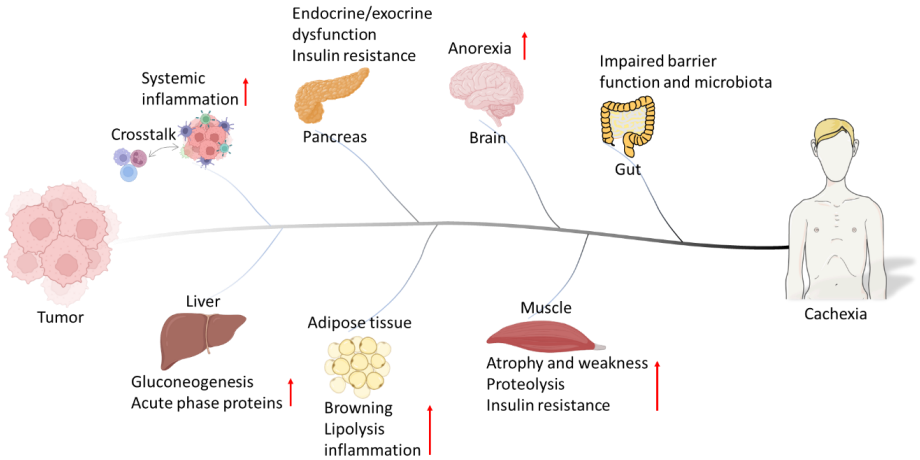

Figure 4: Multiple organs are involved in the development of cancer cachexia. The crosstalk between tumor cells and host immune cells releases different factors, including but not limited to pro-inflammatory cytokines, leading to systemic inflammation. This influences many organs such as liver, pancreas, adipose tissue, brain, skeletal muscle, and gut, which are involved in the progression of cancer cachexia. In liver, the secretion of hepatic acute-phase proteins can be driven by systemic inflammation related molecules such as IL-6 and TNF-α [23, 24], leading to an acute phase response and increased energy expenditure. Furthermore, impaired insulin signaling [19] and increased plasma amino acids concentrations from protein degradation in muscle [25] can promote hepatic gluconeogenesis, thereby contributing to cachexia. Impairments of the endocrine/exocrine pancreatic functions have been seen in many cancer patients, especially pancreatic cancer, resulting in decreased insulin sensitivity associated with the cachectic process [26]. Adipose tissue and skeletal muscle are the most investigated tissues in cachexia; tumor-derived molecules can directly elicit catabolism in

these tissues, resulting in lipolysis of white adipose tissue and proteolysis and atrophy of skeletal muscle. Increased circulating tumor-released mediators such as TNF-α, IL-6, and IL-1 are also able to induce loss of appetite (anorexia) by impairing hypothalamus functions [27, 28]. In the gut, barrier dysfunction and changes of gut microbiota are often seen in cancer patients due to cancer treatment, giving rise to further systemic inflammation and muscle atrophy via the activation of the NF-κB pathway [29-31].

Inflammation in cancer cachexia

Systemic inflammation is a hallmark of cancer cachexia and plays a central role during its progression. The systemic inflammation may originate from the tumor cells, tumor tissue infiltrating immune cells, and/or native host tissue infiltrating immune cells. Currently, tumor-derived pro-inflammatory cytokines have been mostly explored as the main culprits leading to systemic inflammation in cachexia. In the context of cachexia, previous studies have shown systemic inflammation with the ability to affect the function of several tissues including skeletal muscle, adipose tissue, liver, gut, and brain [32, 33]. Pro-inflammatory cytokines such as IL-6, TNF-α, and IL-1β are often observed in cachexia, and elevated circulating levels of these cytokines have been associated with body weight loss and muscle wasting. For instance, Baltgalvis and colleagues have demonstrated that IL-6 is important for the development of cachexia in the $Apc^{Min/+}$ mouse cancer model as evidenced by the fact that genetic deletion of IL-6 rescued muscle and fat loss in this model characterized by high levels of circulating IL-6 [34]. Furthermore, both *in vivo* and *in vitro* studies have revealed that IL-6 induces muscle atrophy through the activation of nuclear factor-κB (NF-κB) and Janus kinase (JAK)-signal transducer and activator of transcription (JAK/STAT) pathways. Except for their metabolic functions in regulating muscle mass, systemic inflammation and IL-6 are also involved in regulating white adipose tissue browning in cachexia. For example, Petruzzelli and colleagues showed a significantly higher mRNA expression of browning markers such as uncoupling protein 1 (UCP1), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), peroxisome proliferator-activated receptor gamma (PPARG), and cell death inducing DFFA like effector A (CIDEA) in axillary and inguinal white adipose tissue from cachectic mice as compared with controls. In the same study, silencing tumor-derived IL-6 or

pharmacological inhibition of IL-6 blocked the increased UCP1 protein expression in subcutaneous fat and rescued the cachectic phenotype of the mice [20].

C reactive protein (CRP), an acute phase protein, is routinely measured in the clinical setting as a marker of systemic inflammation. In the past decades, emerging evidence revealed that elevated circulating CRP is associated with poor prognosis in patients with various types of cancer including lung, liver, melanoma, lymphoma, ovarian, colorectal, and pancreatic tumors. Furthermore, increased circulating CRP levels have been consistently reported to be associated with weight loss, anorexia, and fatigue in patients with chronic disease, suggesting a close relationship between circulating CRP levels and cancer cachexia [35, 36]. Interestingly, a well-recognized regulator of CRP is IL-6, which is known as a pro-cachectic factor, as described above. IL-6 initially activates the STAT3 pathway, leading to an increase in STAT3 protein. Together with CCAAT/enhancer binding proteins (C/EBP), STAT3 binds to the proximal region of the CRP promoter, resulting in increased CRP expression [37]. Although circulating CRP has been proposed as a diagnostic biomarker of cancer cachexia [35, 38], no direct effect of CRP on muscle wasting has been reported and the role of CRP in the development of cancer cachexia is incompletely understood.

Innate immune system

It is widely recognized that the immune system can mobilize immune cells in response to pathogenic infections or tumorigenesis. At present, the link between immune system activation and cancer cachexia has yet to be fully explored, however, a body of studies has shown that multiple immune cell populations such as macrophages, neutrophils, and myeloid-derived suppressor cells are involved in the progression of cancer cachexia [39, 40]. Macrophages are highly plastic cells that can polarize into either pro-inflammatory macrophages (M1 phenotype) or anti-inflammatory macrophages (M2 phenotype) in response to their micro-environment. M1 macrophage polarization is induced by exposure to T-helper type-1 cytokines such as interferon-γ (IFN-γ), TNF-α, as well as by exposure to lipopolysaccharide (LPS) [41]. Macrophages with this phenotype promote a proinflammatory Th1 response by secreting TNF-α, IL-1β, interleukin 12, and interleukin 23 (IL-12 and IL-23)

[42], and play a vital role in antitumor activity [43, 44]. In contrast, M2 macrophage polarization is driven by the stimulation with Th2 cytokines such as interleukin 4 (IL-4), interleukin 10 (IL-10), interleukin 13 (IL-13), or immune complexes [45] and is associated with immunosuppression, tumorigenesis, wound healing, and elimination of parasites [46]. As a key component of the innate immune system, macrophage polarization serves a crucial role in controlling the balance between pro-inflammatory and anti-inflammatory reactions. In the PDAC microenvironment, tumor-associated macrophages are particularly abundant immune cells that create an immunosuppressive environment by producing cytokines, growth factors, and secreting chemokines. A study in human PDAC patients has shown an inverse correlation between CD163 positive (M2) macrophages and muscle-fiber cross-sectional area [47]. In the same study, reduced myotube thickness and protein content after exposure to a combination of conditioned medium from M2-polarized macrophages and C57BL/6J-congenic KPC mouse-derived pancreatic tumor cells were observed [47]. Of note, a study in a mouse model of hepatocellular carcinoma showed a protective effect of macrophages against the loss of adipose tissue during cancer cachexia [48], highlighting the complex role of macrophage biology in cachexia-associated metabolic alterations.

Neutrophils, also known as polymorphonuclear leukocytes or neutrophilic granulocytes, are the most common type of white blood cells in the circulation of humans (up to 70% of all white blood cells). Like macrophages, neutrophils also belong to the innate immune system and play an important role in the host defense against invading pathogens. In the tumor microenvironment, neutrophils can display considerable plasticity in response to environmental signals. Previous studies have suggested two distinct phenotypes of neutrophils in the tumor microenvironment, i.e. an immunostimulatory (N1-like) and an immunosuppressive (N2-like) phenotype. Transforming growth factor-beta (TGF-β) plays a key role in regulating the direction of this neutrophil polarization. Neutrophils polarize to the N2 phenotype upon continuous stimulation of TGF-β, whereas a shift towards the pro-inflammatory phenotype (N1) occurs following blockage of TGF-β. N1 neutrophils exhibit highly cytotoxic activities to kill tumor cells and release pro-inflammatory cytokines, such as IL-12, TNF-α, and C-X-C motif chemokine ligand 10 (CXCL10) [49], which enhance the effect of neutrophils killing tumor cells. In contrast, N2 neutrophils exhibit a tumor-promoting activity and produce various factors such as reactive oxygen species (ROS) [50], neutrophil elastase

(NE) [51], and matrix metalloproteinase (MMPs) [52], the latter of which plays a vital role during tumor invasion and metastasis. Although neutrophils were initially considered to have a defensive function against tumor cells [53-56], accumulating evidence revealed the prominent role of neutrophils in infiltrating tumor tissues to promote their growth, invasion, angiogenesis, and metastasis in various types of cancers. For instance, elevated neutrophil extracellular traps (NETs) were observed in metastatic liver tumors compared with benign liver tumors [57]. In the same study, neutrophils co-cultured with pancreatic cancer cells (PANC-1, MIAPaCa-2) induced high levels of NETs, which enhanced cancer migration and invasion [57].

Another key player of the innate immune system is the complement system. It was discovered more than 100 years ago and known as an ancient defense mechanism against invading pathogens. In addition to the classic complement factors C1-C9, the complement cascade comprises over 50 plasma and cell surface proteins [58-60]. The complement system can be activated through the 'canonical' pathways (classical, lectin, or alternative) that converge at the cleavage of C3 and lead to the generation of diverse immune effectors [61]. The classical pathway is initiated by immunoglobulin G (IgG) and Immunoglobulin M (IgM) -containing immune complexes binding to complement component 1q (C1q), which results in the activation of complement component 1r (C1r) and complement component 1s (C1s). A subsequent cascade in the cleavage of C2 and C4 leads to the formation of C4b2a (a C3 convertase). The lectin pathway is triggered after the recognition by collectins (mannose-binding lectin (MBL), collectin 11 (CL-K1), or ficolins) of carbohydrate ligands on the surface of pathogens or damaged or necrotic cells [62]. Different from the classical and lectin pathways, activation of the alternative pathway does not require specific pathogen recognition. Given a low level of spontaneous hydrolysis of C3, the alternative pathway is constitutively active at low levels to amplify the deposit of C3 activation fragments [63-65]. To prevent damage to healthy tissue, activation of the complement system is strictly regulated. Indeed, aberrant complement activation has been linked to the pathogenesis of a wide range of disorders including Crohn's disease, periodontal diseases, autoimmune diseases, Alzheimer's syndrome, and schizophrenia atypical hemolytic-uremic syndrome [66]. Besides the functions of the complement system in the maintenance of host homeostasis and host defense against pathogens, it is also involved in regulating tumor growth and metastasis. Although activation

of the complement system has long been implicated in tumor immunosurveillance and anti-tumor immunity, recent evidence has demonstrated a role of complement activation in malignancy progression [67]. For instance, elevated complement proteins such as C3/C3a/C3aR were found in several common human malignancies including colon cancer, melanoma, lung cancer, gastric cancer as well as pancreatic cancer [68]. A study in a mouse model of PDAC also demonstrated that complement contributes to cancer progression through the C3a-C3aR axis [69]. Furthermore, complement activation can promote neutrophil recruitment and neutrophil chemotaxis within malignancies [70, 71]. While it is known that cachexia is highly associated with advanced stage malignancies and that complement system activation contributes to cancer progression and malignant progression, the role of the complement system in the progression of cachexia is still unexplored.

Anorexia–cachexia syndrome

Anorexia, also known as loss of appetite, affects up to 90% of patients with advanced cancer and is common in patients with cachexia [72-75]. In general, the causes of anorexia can be categorized as central mechanisms including pain, depression, and hypothalamic inflammation, and peripheral mechanisms including chemotherapy-induced nausea, tumor-induced dysphagia, and tumor-released cytokines. To date, the exact cause of anorexia is still incompletely understood, but multiple factors have been considered to contribute to this phenomenon. For example, several studies in experimental animals have revealed that cytokines released from both cancer cells and host immune cells such as IL-1, IL-6, TNF-α, LCN-2, and GDF-15 alter the activity of appetite regulating-related neurons in the brain resulting in appetite suppression [76, 77]. Elevated circulating IL-1, IL-6, TNF-α, LCN-2, and GDF-15 were also associated with muscle or fat loss [78, 79]. Among these cytokines, GDF-15 and LCN-2 represent promising intervention targets for the treatment of the anorexia–cachexia syndrome. In 2017, several groups simultaneously identified glial cell-derived neurotrophic factor receptor α-like (GFRAL) specifically expressed in the hindbrain as the receptor of GDF-15. Administration of GDF-15 to mice with obesity induces rapid neuronal activation at the hindbrain and hypothalamus, resulting in weight loss and anorexia. Conversely, anorexia–cachexia syndrome was reversed by a GDF-15 neutralizing antibody in mice bearing tumors [80]. Furthermore, studies in mice showed that osteoblast-derived LCN-2 induces appetite loss

by crossing the blood-brain barrier and binding to the melanocortin 4 receptor (MC4R) in the hypothalamus [81]. In the context of cancer cachexia, elevated circulating LCN-2 levels were reported in a mouse model of pancreatic cancer cachexia and were associated with reduced food consumption; LCN-2 gene deletion mitigated pancreatic cancer cachexia–anorexia [82]. In the same study, a significant inverse correlation between circulating LCN-2 levels and visceral fat and skeletal muscle mass loss was reported in patients with pancreatic cancer, implying a potential role of LCN-2 in regulating appetite in cancer patients with cachexia. So far, several GDF-15 antibodies including NGM120, CTL-002, and PF-06946860 are being tested in clinical trials of patients with advanced cancer, the latter of which is specific for the treatment of cachexia-anorexia symptoms [76]. Several MC4R agonists have also been tested in clinical trials for obesity therapy. However, whether pharmacologic suppression of LCN-2/MC4R signaling prevents cachexia-anorexia symptoms remains to be explored in humans.

Cancer cachexia-associated muscle wasting and myosteatosis

Skeletal muscle loss is a key feature of cancer cachexia which is associated with muscle weakness and fatigue. Accumulating evidence revealed that metabolic alterations, including decreased protein synthesis, excessive protein degradation, and abnormal amino acid metabolism contribute to muscle wasting [83]. In a small study, Fearon et al. reported a staggering 75% reduction in muscle protein of patients with cachexia as compared to normal subjects [84]. Insulin growth factor-1 (IGF-1), one of the most important mediators in the regulation of protein turnover, can stimulate skeletal muscle protein synthesis via activation of phosphoinositide−3−kinase (PI3K)–Akt/(mammalian target of rapamycin (mTOR) and glycogen synthase kinase 3 (GSK-3)) pathways, counteracting muscle atrophy. In animal models of cancer cachexia, decreased circulating IGF-1 has been reported [85, 86]. Similarly, patients with heart failure and cardiac cachexia have been reported to have low circulating concentrations of IGF-1 in comparison with patients with heart failure but without cachexia [87]. Interestingly, pancreatic tumors overexpress insulin-like growth factor binding protein 3 (IGFBP3), which can bind to the insulin-like growth factor IGF-1 and lead to inhibition of insulin and IGF-1 signaling. Furthermore, a recent study using matrix-assisted laser desorption/ionization mass spectrometry imaging demonstrated metabolic derangements, especially in certain amino acids, in skeletal muscle from both mouse models of cachexia and

patients with cancer [88]. A significant increase in lysine, arginine, proline, and tyrosine was reported, which were mostly released by the breakdown of proteins involved in oxidative phosphorylation [88].

In addition, multiple factors such as tumor-derived catabolic proteins (activin and myostatin) and pro-inflammatory cytokines arising from tumor-immune system crosstalk (TNF-α, IL-1β, GDF-15, and IL-6) also contribute to cancer-associated muscle loss. These factors directly elicit catabolism in target tissues such as skeletal and cardiac muscle. In skeletal muscle, pro-inflammatory factors and tumor-derived catabolic factors promote muscle wasting via two well-known intracellular signaling pathways, the NF-κB and p38 mitogen-activated protein kinases (MAPK) pathways. In the NF-κB pathway, pro-inflammatory factors TNF-α and IL-1 induce upregulation of the expression of E3 ligases muscle RING finger-containing protein 1 (MURF1, also known as TRIM63) and Atrogin 1 (also known as FBXO32). These ligases mediate proteolysis of myofibrillar protein. [89]. For instance, previous studies have shown that exposure of C2C12 myotubes to TNF-α leads to activation of NF-κB signaling [90-92]. Accordingly, inhibition of NF-κB activation by pharmacologic and muscle-specific genetic deletion of NF-κB both alleviated muscle wasting in tumor-bearing mice [93, 94]. p38 MAPK is a member of the MAPK family that plays a critical role in many biological processes, including cell proliferation, differentiation, and apoptosis. Upon inflammatory stimuli (e.g. TNF-α, IL-1), the p38 MAPK cascade gets activated and results in the upregulation of MuRF1 and Atrogin-1 [95].

Fat infiltration in skeletal muscle is frequently referred to as myosteatosis. It is defined by the ectopic accumulation of fat in skeletal muscle which may occur as intermuscular adipose tissue, intramuscular adipose tissue, as well as intramyocellular lipids. Although myosteatosis is often associated with sarcopenia (low skeletal muscle mass), they represent different aspects of skeletal muscle status. In particular, sarcopenia reflects muscle quantity, and myosteatosis reflects muscle quality. Stretch and colleagues described that sarcopenia and myosteatosis are two different muscle-wasting diseases accompanied by distinct biological profiles (body compositions, gene expression, and metabolites) in patients with pancreatic cancer [96]. Sarcopenia and myosteatosis are also associated with several other diseases including type 2 diabetes, cirrhosis [97], and non-alcoholic fatty liver disease (NAFLD) [98, 99],

as well as other types of cancer [100]. In clinical studies, a large body of evidence showed that myosteatosis was prognostic for poor overall survival in patients with cancer, as reviewed by Aleixo et al. [101]. Myosteatosis can be determined either by invasive techniques like muscle biopsy or by noninvasive techniques such as cross-sectional magnetic resonance imaging (MRI) or computed tomography (CT). Given the noninvasive nature of CT, it has been widely used for the diagnosis of myosteatosis by measuring skeletal muscle radiation attenuation (MRA) expressed in Hounsfield units (HU). However, there is no consensus cutoff value for the diagnosis of myosteatosis in patients with cancer, and various cutoff values have been well-reviewed by Aleixo et al. In the same review, it was reported that 20 out of 40 studies used the cutoff value suggested by Martin et al. in 2013 [102], proposing that cancer patients with MRA below 41 HU and BMI < 25 kg/m^2 or MRA <33 HU and BMI >25 kg/m^2 should be diagnosed with myosteatosis. In the context of cancer cachexia, Stephen and colleagues reported that lipid accumulation in skeletal muscle increases with the development of cancer cachexia. In particular, the numbers and size of intracellular lipid droplets were positively correlated with weight loss in patients with upper gastrointestinal cancer [103]. Furthermore, a recent study revealed that the proton density fat fraction of the psoas muscle was negatively correlated with the severity of cancer cachexia [104]. To date, several mechanisms have been proposed to be responsible for lipid accumulation in skeletal muscle, such as the dedifferentiation of skeletal muscle precursor stem cells, mitochondrial dysfunction, as well as disturbed expression of perilipin-2, a lipid droplet protein [105-107]. However, further studies are needed to uncover the mechanisms underlying myosteatosis in patients with cancer.

Organoids model

In the past decade, our understanding of the mechanisms underlying cancer cachexia-associated muscle wasting has been improved by using cancer cell lines and mouse models of cancer cachexia. Studies using tumor cell lines or mouse models of cancer cachexia have shown that tumor-derived factors can directly induce muscle wasting. For example, exposure of C2C12 myotubes to conditioned medium from Capan-1 pancreatic cancer cells leads to myotube wasting, and IGF binding protein-3 (IGFBP-3) gene deletion in Capan-1 cells or IGFBP-3 antibody treatment ameliorated conditioned medium from Capan-1 pancreatic cancer cells-induced muscle-wasting [108]. Likewise, human pancreatic tumor cell-derived interleukin-8

(IL-8) was identified to induce muscle atrophy through the CXC motif chemokine receptor 2 - extracellular signal-regulated kinases 1 and 2 (CXCR2-ERK1/2) axis [109]. Furthermore, elevated tumorkines IL-6 and activin A have been associated with weight loss in experimental models [110-112]. Although two-dimensional cancer cell cultures are widely used to identify novel tumor-derived factors contributing to cachexia-associated muscle wasting, several limitations of these conventional *in vitro* systems should be noted. First, monolayer cell cultures do not display the natural structures of the tumor and are limited by the absence of extracellular matrix (ECM), cell-ECM interactions, and oxygen and nutrient gradients, which leads to insufficient modeling of the complex biological tumor characteristics observed *in vivo* [113-115]. Second, whereas immortalized cancer cells are commonly thought to be stable, morphological changes and genetic mutations may happen during long-term culture, resulting in limited representation of the original tumor from which the cell lines were established [116, 117]. For instance, 2D culturing of human PDAC cells has been reported to result in an irreversible epithelial-mesenchymal transition [118]. In the context of cancer cachexia research, the Colon26 (C26) and Lewis Lung Cancer (LLC) mouse models generated by subcutaneously implanting tumor cells (C26 colorectal cancer cells or Lewis Lung Cancer cells) into syngeneic mice are widely used [119-124]. In these mouse models, cancer cell growth results in significant and aggressive loss of body weight and skeletal muscle mass within 1-2 weeks [119, 121, 125, 126], and the tumor mass commonly represents more than 10% of whole body weight [123, 124], which differs vastly from human patients with cancer cachexia. Furthermore, a recent study showed that the skeletal muscle of C26 and LLC mouse models does not reflect the muscle gene expression patterns in PDAC patients [127]. Therefore, new models with the ability to better recapitulate the original tumor and its downstream impact on metabolic target tissues are urgently needed.

Recently, organoids have attracted attention because of their ability to mimic tissue architectures and their representation of key biological properties of parent tissues *in vitro*. The term organoids was first introduced in 1946 by Simith and Cochrane [128], but was re-defined in the last decade by the laboratory of Hans Clevers in the Netherlands [129]. Genuine organoids should comply with the following criteria: “(1) a 3D structure containing cells that establish or retain the identity of the organ being modeled; (2) the presence of multiple cell types, as in the organ itself; (3) the tissue exhibits some aspect of the specialized function of

the organ; and (4) self-organization according to the same intrinsic organizing principles as in the organ itself" [130]. Organoids can be established from pluripotent stem cells such as embryonic stem cells and induced PSCs, but can also be generated from adult stem cells [131]. To date, various normal and tumor organoids have been successfully developed from organs including liver [132-134], colon [135], kidney [136], ovary [137], prostate [138], esophagus [139], breast [140], as well as pancreas [141-143]. Given that patient-derived organoid cultures recapitulate tissue properties in a dish, they hold great promise for drug screening, personalized medicine, studying cancer progression, and modeling cancer cachexia. In our lab, Vaes et al. have previously introduced eight pancreatic tumor organoids established from three non-cachectic PDAC patients and five cachectic PDAC patients. Typical malignancy features that were in line with the primary tumor characteristics were observed in these tumor organoids. Furthermore, a set of cachexia-associated proteins including IL-6, TNF-α, IL-1β, and GDF-15 were expressed by these tumor organoids [141].

Aims of this thesis

In the past decade, our understanding of the importance of inflammation-related mechanisms for the pathogenesis of cancer cachexia has been vastly improved. However, the role of the innate immune system during cancer cachexia development is still relatively unexplored. Therefore, our first aim was to investigate the association between key innate immunity players, i.e. the complement system and neutrophils, and various aspects of cachexia in PDAC patients. Our second aim relates to myosteatosis. Despite the strong negative predictive power of myosteatosis for the survival of patients with cancer, the mechanisms underlying its development during cancer cachexia are unclear. Several studies have suggested that tumor-derived factors and inflammation contribute to lipolysis of adipose tissue and skeletal muscle mitochondrial dysfunction resulting in insulin resistance and muscle atrophy in cancer patients with cachexia, but whether tumor-derived factors directly promote lipid accumulation in skeletal muscle remains unknown, as are the mechanisms involved. Thus, we aim to 1) better characterize myosteatosis in cancer patients with cachexia with and without inflammation, and 2) to explore the underlying mechanisms by which tumor-derived factors contribute to lipid accumulation in the skeletal muscle of cancer patients with cachexia.

Outline of this thesis

In **Chapter 2**, the levels of key complement factors in pancreatic cancer patients with and without cachexia and their correlation with systemic inflammation are reported. **Chapter 3** examines the relationship between human pancreatic cancer cachexia and neutrophil activation. It focuses on LCN-2, addressing its association with patient appetite and nutritional status. Furthermore, the link between neutrophil activation markers, systemic inflammation and complement activation, as well as specific cachexia features is described. In **Chapter 4**, the intramyocellular lipid content of pancreatic cancer patients with and without cachexia and inflammation is investigated. Innovative mass-spectrometry imaging approaches are used to assess the nature and distribution of intramyocellular lipids in these patients, and their link with cachexia and inflammation. The mechanisms underlying lipid accumulation in the skeletal muscle of pancreatic cancer patients with cachexia are investigated in **Chapter 5** using pancreatic tumor organoids and an *in vitro* skeletal muscle cell model. Finally, in **Chapter 6**, the findings of this thesis are summarized and discussed, and future perspectives are provided.

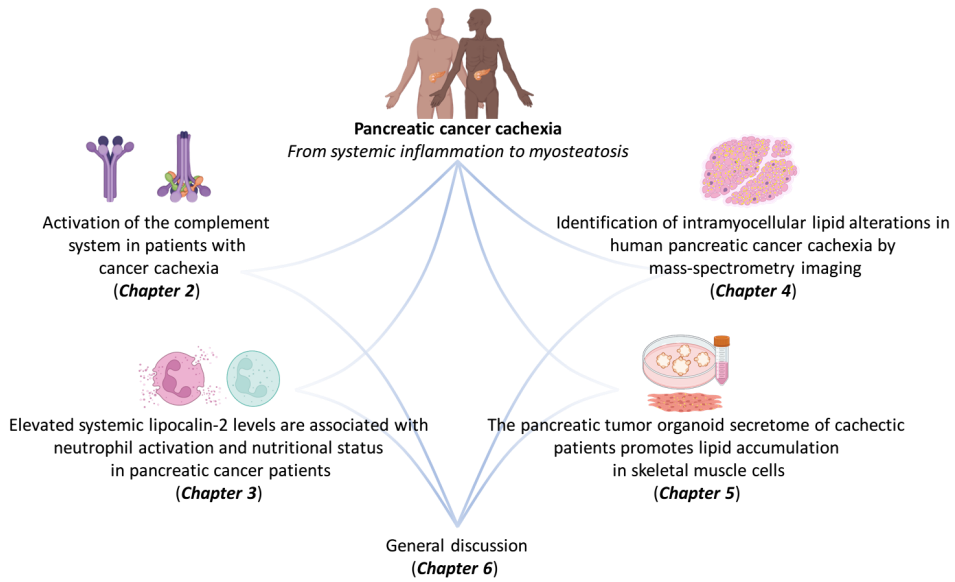

Figure 5: Thesis outline

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CHAPTER 2

Activation of the complement system in patients with cancer cachexia

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Abstract

Background: Systemic inflammation is thought to underlie many of the metabolic manifestations of cachexia in cancer patients. The complement system is an important component of innate immunity that has been shown to contribute to metabolic inflammation. We hypothesized that systemic inflammation in patients with cancer cachexia is associated with complement activation.

Methods: Based on plasma levels of C-reactive protein (CRP) and the consensus definition of cachexia, pancreatic cancer patients (*n*=62) were categorized into no cachexia (*n*=13), cachexia with (*n*=23, CRP≥10 mg/L) or without (*n*=26, CRP<10 mg/L) inflammation groups. The concentration of plasma C1q, mannose-binding lectin (MBL), C3a, and terminal complement complex (TCC) were measured by ELISA.

Results: Systemic C3a levels were higher in cachectic patients with inflammation as compared to patients without inflammation or without cachexia (median 102.4 (IQR 89.4-158.0) vs. 81.4 (47.9-124.0) vs. 61.6 (46.8-86.8) ng/mL, *p*=0.0186). In line, terminal complement complex (TCC) concentrations gradually increased in these patient groups (medians 2298 (IQR 2022-3058) vs. 1939 (IQR 1725-2311) vs. 1805 (IQR 1552-2569) mAU/ml, respectively, *p*=0.0511). C3a and TCC concentrations showed a strong correlation (r_s=0.468, p=0.0005). Whereas concentrations of C1q and mannose-binding lectin did not differ between groups, C1q levels correlated with both C3a and TCC concentrations (r_s=0.394, *p*=0.0042 and r_s=0.300, *p*=0.0188, respectively).

Conclusion: systemic inflammation in patients with cancer cachexia is associated with activation of key effector complement factors. The correlations between C1q and C3a/TCC suggest that the classical complement pathway could play a role in complement activation in patients with pancreatic cancer.

Introduction

Cachexia is a severe complication of many types of cancer with a particularly high prevalence in pancreatic cancer [1]. It is a multifactorial metabolic syndrome that has a substantial detrimental impact on the survival and quality of life of patients [2]. Cancer cachexia is characterized by loss of body weight, negatively impacting both adipose tissue and skeletal muscle mass [3].

Although its precise underlying mechanisms remain unclear, many lines of evidence point towards an important role of pro-inflammatory factors released by tumor cells, immune cells, or metabolic target tissues in the pathophysiology of cancer cachexia. In particular, inflammation has been shown to be associated with both muscle wasting and fat depletion in patients with cancer cachexia [4, 5]. Interactions between tumor and host cells leading to systemic inflammation result in an acute phase response characterized by the production of C-reactive protein (CRP) in the liver, and increases in circulating proinflammatory cytokines [6, 7]. In recent years, research mainly focused on the role of pro-inflammatory cytokines such as interleukin-6 (IL-6), interleukin-1 (IL-1), tumor necrosis factor alpha (TNFα), and interferon gamma (IFN-γ), and confirmed their involvement in the pathogenesis of cachexia [8-11].

Despite these studies, the characterization of the systemic inflammatory response in patients with cancer cachexia is still incomplete. For example, there is no literature on the potential involvement of the complement system, a cornerstone of innate immunity that plays a key role in cancer-associated cachexia. The complement system comprises over 50 circulating, cell surface-bound, or intracellular proteins that collectively promote the recognition and removal of both infectious agents and apoptotic, necrotic, or malignant cells [12, 13], the latter of which could be highly relevant in the context of tissue wasting in cancer cachexia. Complement molecules may be expressed by activated immune cells including macrophages, dendritic cells, natural killer cells, B cells, and T cells. They can also be produced by other cell types involved in the metabolic aberrations seen in cachexia, such as hepatocytes and adipocytes [14, 15]. Various studies have reported elevated levels of complement factors in cancers with a high prevalence of cachexia, including colorectal cancer and lung cancer [16],

further supporting a role for complement system activation in cachexia-associated inflammation.

There are three different ways to activate the complement system, referred to as the classical pathway, the lectin pathway, and the alternative pathway. The classical pathway of complement activation is initiated by binding of the C1q molecule to antigen-antibody complexes, CRP, or apoptotic cells [17]. Lectin pathway activation is dependent on recognition of aberrant carbohydrate patterns by the mannose-binding lectin (MBL) protein, collectins, or ficolins [18]. Activation of the alternative pathway can be initiated by spontaneous activation of the central complement C3 molecule on 'activating surfaces'; this pathway also serves to amplify complement activation as a result of classical or lectin complement pathway activation [19].

Though the three pathways of complement activation differ with respect to their way of target recognition, they all converge at the level of the C3 molecule, which is activated through cleavage by C3 convertases generated by the respective pathways. Upon its activation, several effector complement molecules with different functions are generated: the opsonin C3b, the anaphylatoxins C3a and C5a, and the membrane attack-complex (MAC). Deposition of the C3b opsonin on complement activating surfaces enhances the phagocytosis potential of macrophages, and promotes their secretion of pro-inflammatory cytokines [20]. Anaphylatoxins like C3a are potent chemo-attractants that recruit monocytes and granulocytes to the site of activation and can stimulate the production of additional inflammatory mediators. C3a has been shown to enhance pro-inflammatory cytokine secretion by various immune cells [21]. For example, Elvington et al. have reported that C3a increases IL-6 production by CD4+ T cells, contributing to lipolysis [22]. C3a also stimulates the production of cachexia-associated inflammatory mediators such as TNFα and IL-1 [23, 24]. Assembly of the MAC is the ultimate consequence of complement activation and leads to effective lysis of target cells by forming a pore in the membrane, disrupting cellular integrity and function [25]. When the MAC is formed on membranes, a soluble counterpart sMAC, also referred to as the Terminal Complement Complex (TCC), can be formed. TCC levels are increased in various acute and chronic inflammatory diseases [25].

In view of the many ways by which complement activation can promote inflammation in various disease settings, we hypothesized that systemic inflammation in patients with cancer cachexia could be associated with complement activation. We set out to investigate the plasma levels of the initiating complement factors C1q and MBL and the effector complement factors C3a and TCC in the blood of a cohort of pancreatic cancer patients with and without cachexia and inflammation. We report that cachectic patients with inflammation have higher systemic C3a and TCC concentrations than patients without inflammation or cachexia, and that these concentrations are strongly correlated overall. C3a and TCC concentrations also correlate with C1q levels even though C1q concentrations did not differ between groups. These data may imply that activation of the classical pathway of the complement system contributes to the inflammation seen in patients with cancer cachexia.

Materials and Methods

Patients

Patients undergoing pancreaticoduodenectomy at the Maastricht University Medical Centre (MUMC+) for suspected adenocarcinoma of the pancreas were enrolled in this study. Exclusion criteria included the use of systemic glucocorticoids in the past four weeks, neoadjuvant chemo- and/or radiotherapy, and the presence of another malignancy. This study was approved by the Medical Ethical Board of the Maastricht University Medical Center in line with the ethical guidelines of the 1975 Declaration of Helsinki, and written informed consent was obtained from each subject (METC 13-4-107 and 2019-0977).

Diagnosis of cancer cachexia and screening of cachexia status

Cachexia was defined according to the international consensus definition as 1) weight loss >5% over the past 6 months in the absence of starvation, and/or 2) BMI <20 kg/m2 and >2% ongoing weight loss, and/or 3) sarcopenia and >2% ongoing weight loss [3]. Patients were diagnosed with cancer cachexia if ≥1 of the criteria were met. The cachexia status of the patients was assessed by a trained physician in the outpatient clinic. The screening included measurements of body weight and height and patient-reported weight loss in the last six months.

To assess the presence of sarcopenia, body composition was investigated by analysis of computed tomography (CT) imaging scans and sliceOmatic 5.0 software (TomoVision, Magog, Canada). Skeletal muscle mass was quantified on a cross-sectional CT-image at the third lumbar (L3) vertebra that was preoperatively acquired for diagnostic purposes. Using predefined Hounsfield Unit (HU) ranges, the total cross-sectional area (cm^2) of skeletal muscle tissue (-29 to 150 HU) was determined. In addition, the total areas of visceral adipose tissue (VAT, -150 to -50 HU) and subcutaneous adipose tissue (SAT) as well as intramuscular adipose tissue (IMAT) (-190 to -30 HU) were assessed. Tissue areas (cm^2) were adjusted for height to calculate the respective L3-indices (L3-SMI, L3-VAT, L3-SAT) in cm^2/m^2, which correspond well with total body muscle and adipose tissue mass [26]. Previously published validated sex-specific cut-off values (SMI, 45.1 cm^2/m^2 for men and 36.9 cm^2/m^2 for women) that were established from a cohort including pancreatic cancer patients were used for the CT-derived body composition analysis [27]. In addition, the skeletal muscle radiation attenuation (M-RA) was calculated as the average HU value of the total tissue area for muscle (i.e. within the specified range of -29 to 150 HU).

Systemic inflammation was assessed by measuring plasma C-reactive protein (CRP) levels preoperatively (routine in-hospital laboratory test, MUMC+). CRP values were considered to be elevated when they exceeded 10 mg/L)[28].

Collection of plasma samples

Prior to the start of surgery, after a minimum of eight hours of fasting, venous blood was collected in EDTA tubes and stored on ice until further processing. The blood was centrifuged at 1150 x g at 4°C for 12 min without brake. Plasma aliquots were stored at -80°C until analysis.

ELISAs

Plasma C1q, MBL, C3a, TCC, and IL-6 concentrations were measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Hycult Biotech, Uden, the Netherlands for the complement factors, U-CyTech biosciences, Utrecht, the Netherlands for IL-6). All plasma samples were analyzed in duplicate in the same run. The

intra- and inter-assay coefficients of variance of the various assays were < 10%. TCC concentrations are given in 'milli Arbitrary Units' (mAU)/ml values.

Statistical analysis

Statistical analysis was performed using Prism 7.0 for Windows (GraphPad Software Inc., San Diego, CA), RStudio (v. 1.2.5033, Affero General Public License, Boston, MA, USA). Data are presented as median and interquartile range. Differences between groups were analyzed by the Kruskal-Wallis test followed by Dunn's post-testing. Correlations were calculated using Spearman's correlation coefficient. *P*-values <0.05 were considered statistically significant.

Results

Patient characteristics

Sixty-two patients with pancreatic cancer were included in this study. They had a median age of 68.3 years, and a median BMI of 24.4 kg/m^2. In total, 35 males and 27 females were studied. The median weight loss percentage in the cohort was 7.2. CT-scan-based body composition analysis showed that 45.2% of patients were sarcopenic, with a median L3_SMI of 46.7 cm^2/m^2 for males and of 36.1 cm^2/m^2 for females. The median M_RA value was 35.5 HU for the whole cohort. L3_VAT and L3_SAT indices were 63.9, 43.6 cm^2/m^2 and 28.9, 67.6 cm^2/m^2 for males and females, respectively. CRP concentrations were assessed as a measure of systemic inflammation, and found to have a median value of 9.0 mg/L. Additional descriptive patient characteristics can be found in table 1.

Based on the consensus definition of cancer cachexia, we next subdivided the group into patients with and without cachexia. In line with the literature on the prevalence of cachexia in pancreatic cancer patients, we found that 49 patients (79%) were cachectic and 13 patients (21%) were not. Patients with cachexia had a significantly lower BMI as compared to patients without cachexia. Since cachexia is associated with inflammation, and because activation of the complement system is a key feature of many inflammatory conditions, we further subdivided the cachexia group into patients with and without inflammation as evidenced by elevated systemic CRP levels. The median CRP concentration in the group with cachexia but

without inflammation was 4.0 versus 22.0 in the group with cachexia with inflammation. Body weight loss was similar between the groups with and without inflammation (median 9.5% IQR 7.3-13.9 versus 9.7% IQR 5.7-12.7).

Table 1. General characteristics of patients with pancreatic tumor according to cachectic state.

	Overall	**No cachexia**	**Cachexia**	**Cachexia**	***p***
			Without inflammation	***With inflammation***	
n	62	13	26	23	
Male/Female (%)	35/27 (56.5/43.5)	6/7 (46.2/53.8)	14/12 (53.8/46.2)	15/8 (65.2/34.8)	0.511
Age (years)	68.3 [63.1, 75.2]	67.6 [61.4, 76.3]	70.2 [63.2, 74.8]	67.8 [64.9, 74.2]	0.997
Weight (kg)	68.5 [58.7, 81.5]	76.5 [68.0, 82.2]	67.1 [56.5, 78.1]	67.0 [59.0, 82.3]	0.150
BMI (kg/m^2)	24.4 [21.7, 26.8]	26.9 [25.6, 28.3]	23.5 [21.6, 26.1]†	23.0 [20.7, 25.7]†	0.015
Weight loss percentage (%)	7.2 [4,0, 12.2]	1.9 [0.0, 3.0]	9.7 [5.7, 12.7]†	9.5 [7.3, 13.9]†	<0.001
L3_IMAT (cm^2)					
Male	14.6 [7.2, 19.5]	18.0 [10.3, 21.6]	12.2 [6.3, 19.7]	13.5 [7.4, 17.6]	0.581
Female	9.7[7.6, 19.2]	13.9 [10.7, 18.4]	8.1 [7.7, 22.3]	7.7 [7.2, 13.6]	0.292
M_RA (HU)					
Male	35.3[30.8, 42.8]	35.5 [33.7, 38.3]	32.5 [29.4, 41.2]	35.3 [32.5, 44.2]	0.757
Female	37.4 [30.0, 40.7]	37.4 [31.4, 40.0]	35.6 [28.5, 40.9]	36.8 [33.1, 41.6]	0.843
L3_SMI (cm^2/m^2)					
Male	46.7 [41.7, 50.3]	47.0 [45.6, 48.3]	47.6 [43.2, 51.3]	43.0 [40.3, 48.8]	0.564
Female	36.1 [34.2, 42.5]	38.9 [35.8, 42.7]	36.7 [34.9, 42.5]	35.1 [33.5, 39.4]	0.665
L3_VATI (cm^2/m^2)					
Male	63.9 [33.4, 79.7]	70.6 [66.3, 79.7]	64.6 [31.8, 85.1]	44.4 [30.5, 71.6]	0.221
Female	28.9 [12.4, 47.7]	39.7 [25.1, 48.8]	29.0 [10.2, 49.4]	28.7 [4.8, 37.3]	0.446
L3_SATI (cm^2/m^2)					
Male	43.6 [31.6, 57.3]	52.1 [39.8, 59.2]	47.1 [34.8, 56.0]	39.7 [27.1, 45.6]	0.349
Female	67.6 [49.7, 87.8]	87.1 [63.8, 88.8]	59.3 [46.7, 77.2]	63.1 [30.0, 85.3]	0.413
C3a (ng/mL)	90,0 [52.3, 120.8]	61.6 [46.8, 86.8]	81.4 [47.9, 124.0]	102.4 [89.4, 158.0]†	0.019
TCC (mAU/mL)	2005.5 [1718.6, 2602.9]	1805.1 [1552.1, 2569.4]	1938.5 [1724.6, 2310.6]	2297.5 [2021.5, 3057.8]	0.051
C1q (µg/mL)	10.5 [8.2, 13.0]	10.2 [9.4, 12.6]	10.3 [8.3, 13.2]	11.3 [7.3, 13.7]	0.882
MBL (ng/mL)	437.3 [167.8, 1498.9]	694.4 [385.8, 1503.0]	333.7 [191.0, 1185.8]	406.4 [138.2, 1564.7]	0.730
IL-6 (pg/mL)	5.1 [2.9, 15.0]	4.4 [1.3, 9.8]	4.1 [1.9, 6.5]	11.2 [6.0, 31.7]‡	0.042
CRP (mg/L)	9.0 [4.0, 20.8]	12.5 [10.3, 31.8]	4.0 [2.0, 5.3]†	22.0 [12.8, 32.5]‡	<0.001

The data are presented as median and interquartile range. Groups were compared using the Kruskal-Wallis test. †Significant differences in comparison to the no cachexia group. ‡Significant differences in comparison to the cachexia without inflammation group. BMI: body mass index; HU: Hounsfield unit; L3_IMAT: L3-intermuscular adipose tissue; M_RA: muscle radiation attenuation; L3_SMI: L3-muscle index; L3_VATI: L3-visceral adipose tissue index; L3_SATI: L3-subcutaneous adipose tissue index; TCC: terminal complement complex; C1q: complement component 1q; MBL: mannose binding lectin; IL-6: interleukin 6; CRP: C-reactive protein. mAU/ml: milli arbitrary units/ml.

Concentrations of complement factors in the blood of patients with and without weight loss and inflammation

We next assessed if we could detect activated complement factors with a key effector role in the blood of pancreatic cancer patients. Out of the 62 patients of the full cohort, we were able to assess levels of TCC, the ultimate result of activation of the complement system, for 61 patients. The median systemic concentration of TCC for the whole cohort was 2006 mAU/ml (IQR 1719-2603). Interestingly, TCC concentrations differed markedly among the subgroups (see Figure 1A), with the lowest values in the group without cachexia (1805 mAU/ml, IQR 1552-2569), intermediate values in the group with cachexia but without inflammation (1939 mAU/ml , IQR 1725-2311), and the highest values in the group with both cachexia and systemic inflammation (2298 mAU/ml, IQR 2022-3058, p=0.0511).

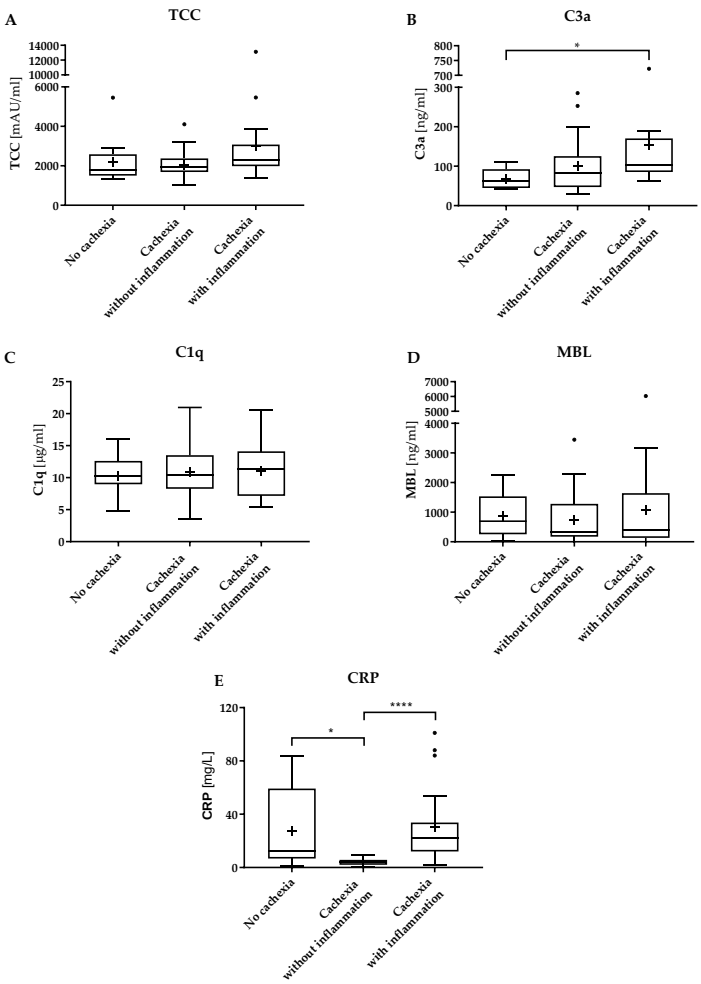

Figure 1. Plasma concentrations of C3a, TCC, C1q, MBL and CRP in patients with pancreatic cancer. Tukey box and whiskers plots are displayed showing significantly elevated C3a concentrations in the blood of patients with cachexia and inflammation (panel A, $p = 0.0186$). TCC concentrations are higher in this group as well, with a trend towards significance (panel B, $p = 0.0511$). C1q (panel C) and MBL (panel D) concentrations do not differ between the groups. CRP concentrations were lower in cachectic patients without inflammation as compared to cachectic patients with inflammation or patients without cachexia (panel E, $p < 0.0001$, $p = 0.0137$, respectively). The line reflects the median, the hinges of the boxes are drawn at the 25th and 75th percentile. The dots reflect the outliers as defined by the Tukey method. The mean concentrations are indicated by the 'plus' sign. * $p \leq 0.05$.

A similar pattern was observed when we analyzed the concentrations of C3a, a split product of the central complement component C3 that indicates activation of the complement system, which we could analyze for 51 patients (see Figure 1B). C3a levels differed significantly across the groups (p=0.0186), with higher values in the group with cachexia and inflammation (median 102.4 ng/ml, IQR 89.4-158.0) in comparison to the group with cachexia without inflammation (median 81.4 ng/ml, IQR 47.9-124.0) and the group without cachexia (median 61.6 ng/ml, IQR 46.8-86.8). Post-testing revealed that the significance was driven by the difference between the non-cachectic group and the group with cachexia and inflammation (p=0.0167), whereas the group without inflammation showed a p-value of 0.1720 versus this latter group.

To gain more insight into initiating complement factors that could be responsible for complement activation in patients with cachexia and inflammation, we next assessed the systemic concentrations of C1q and MBL, the initiating factors of the classical and the lectin pathway, respectively. Whereas we could detect both C1q and MBL in the plasma of 60 patients, we did not observe any significant differences in their concentrations between the different groups. For C1q, median concentrations were 10.2 µg/ml (IQR 9.4-12.6) versus 10.3 µg/ml (IQR 8.3-13.3) versus 11.3 µg/ml (IQR 7.3-13.7) in the groups without cachexia, with cachexia but without inflammation, and with cachexia and inflammation, respectively (p=0.882, see Figure 1C). For MBL, median concentrations were 694.4 ng/ml (IQR 385.8-

1503.0) versus 333.7 ng/ml (IQR 191.0-1185.8) versus 406.4 ng/ml (IQR 138.2-1564.7) for these respective groups (p=0.730, see Figure 1D).

Correlation analysis of complement factors and cachexia parameters

Because the generation of TCC can only occur when the preceding complement factors in the complement cascade have been activated, we next investigated the assocation between concentrations of TCC and C3a, one of the upstream activated complement factors, in the blood of the patients across the entire cohort. Importantly, we found a strong positive correlation between TCC and C3a concentrations (r_s = 0.4684, p = 0.0005, see Figure 2), supporting that patients with cachexia and inflammation display systemic complement activation. Additional correlation analyses revealed that C1q concentrations were also correlated to both TCC and C3a (r_s = 0.3002, p = 0.0188, and 0.3942, p = 0.0042, respectively, see Figure 2). MBL concentrations were not associated with any of the other complement factors analyzed in Spearman correlation analyses.

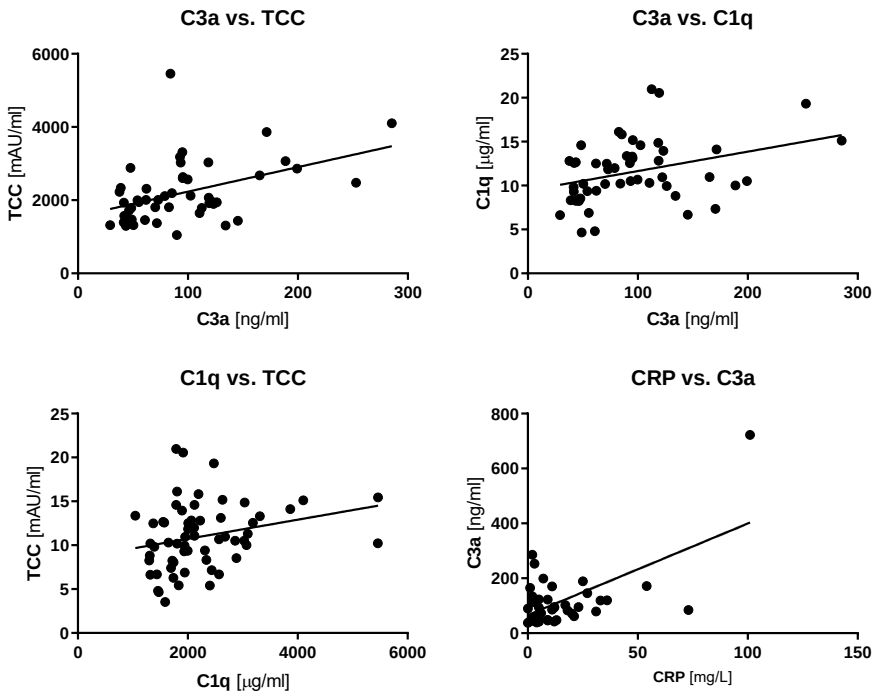

Figure 2. Associations between C3a, TCC, C1q, and CRP. x- and y-plots showing significant correlations between C3a and TCC as well as between C3a and C1q, and TCC and C1q. No significant correlation between CRP and C3a.

To study potential interactions between complement activation and hallmarks of cachexia such as weight loss, body composition features (e.g. skeletal muscle index and adipose tissue indices, skeletal muscle radiation attenuation), and BMI, we extended the correlation analyses (see Figure 3). Whereas BMI and body composition features showed the expected strong correlations, neither of these cachexia-related parameters was correlated to any of the complement factors assessed in this study.

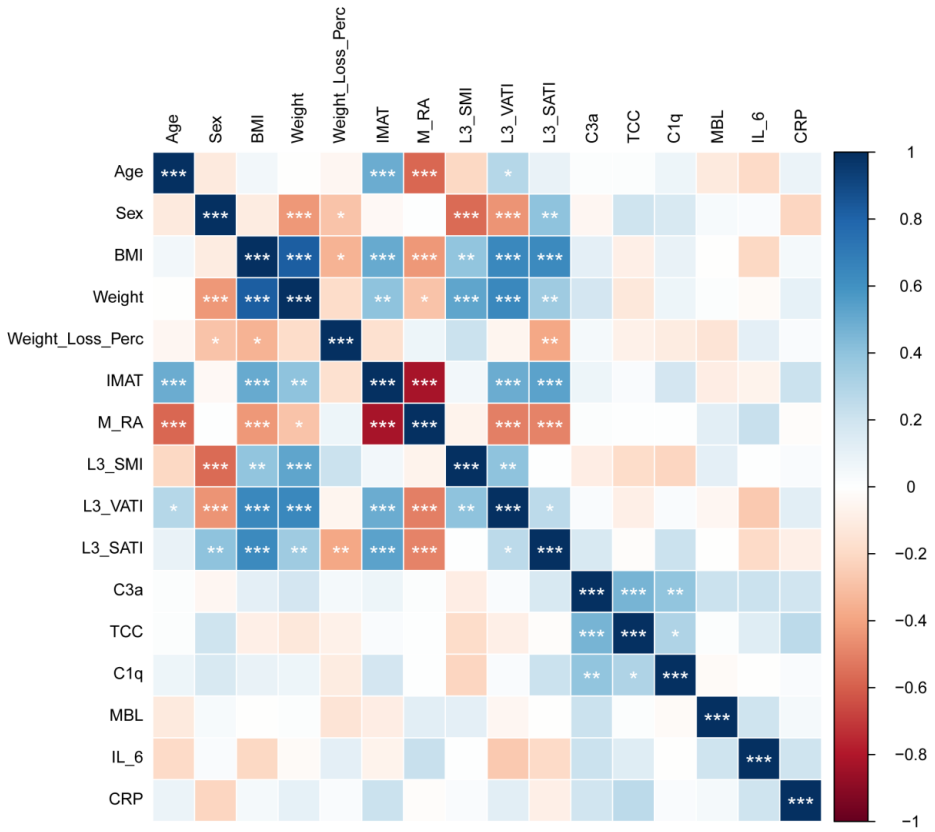

Figure 3. Correlation plot showing Spearman correlations between indicated cachexia-related variables and complement factors. Positive correlations are shown in blue, negative

correlations in red. The color intensity indicates the strength of the correlation. The asterisks indicate the level of statistical significance (* ≤ 0.05, ** ≤ 0.01, ***≤ 0.001).

Discussion

In the present study, we describe that systemic activation of the complement system is observed in patients with pancreatic cancer who are cachectic and who display a systemic inflammatory response as evidenced by elevated CRP levels. Both C3a, a cleavage product of the central complement C3 component with potent pro-inflammatory functions, and TCC, an end product of complement activation, are present at higher levels in these patients as compared to those who are not cachectic, even though the latter group also displayed an elevation in CRP concentrations. The association between C1q and C3a as well as TCC in these patients might indicate that the classical pathway of the complement system is involved in complement activation in patients with pancreatic cancer.

It is well known that inflammation plays a pivotal role in the development of cachexia in cancer patients [29, 30]. As such, cachexia is generally associated with increased levels of inflammatory molecules like CRP, TNF-alpha, IL-6, and IL-8. In the current study, we confirmed that cachectic patients with inflammation as defined by elevated CRP levels display higher circulating IL-6 concentrations. These cytokines promote tissue wasting in cachexia through the activation of transcription factors such as NF-kappa B [5, 31]. Analogous to these pro-inflammatory cytokines, complement factors may be produced by either cancer cells, immune cells, or metabolic target cells such as hepatocytes or adipocytes [32-35].

In addition to their classical pro-inflammatory functions, various complement factors have been shown to have a tumor-promoting role in several cancer types [36]. They may promote cellular proliferation, invasion, and migration, and mediate the development of an immunosuppressive microenvironment. In view of these contributions of complement to cancer progression, it is not surprising that we found increased complement activation products in the plasma of patients with cachexia and inflammation, which are associated with more advanced cancer [37].

An important next step will be to identify the cellular and/or tissue sources of complement activation, and their relative contributions to systemic active complement levels. One clue in this context may come from the association between the levels of C1q, the initiating factor of the classical complement pathway, and the levels of complement effectors C3a and TCC that we observed. C1q can instigate complement activation after direct binding to apoptotic cancer cells. It can also activate the complement cascade after binding to IgM or IgG antibody-antigen complexes, which may occur in pancreatic tumors as a result of neo-antigen expression by tumor cells. Both processes lead to clearance of cells through macrophage phagocytosis which could promote chronic inflammation as seen in cachexia.

Alternatively, the compromised gut barrier integrity that has been observed in models of cancer cachexia [38, 39] and cachectic cancer patients [40] may promote complement activation through the classical pathway as a result of the formation of antibodies complexed to translocated bacteria. Furthermore, we have previously shown that both C1q, C3a, and MAC are deposited on steatotic hepatocytes in obese patients with nonalcoholic fatty liver disease [41]. Since it is known that cancer cachexia is also associated with hepatic steatosis, it should be investigated if lipid accumulation in the liver of cachectic patients leads to deposition of activated complement factors as well. In addition, older studies have shown that several complement factors including C1, C2, C4, and C3 can be synthesized by skeletal muscle cells *in vitro* [42, 43], and that they are upregulated by pro-inflammatory cytokines including IL-1 [42]. Proteomic and transcriptomic analyses support a key role for complement C3 in myogenesis [44].

Another important question that should be addressed concerns the potential effects of complement activation in the context of cancer cachexia. Given that C3a and TCC were specifically increased in those cachectic patients who displayed systemic inflammation, it is tempting to assume that complement activation in cachexia may propagate or even initiate the many pro-inflammatory events that play a central role in its pathogenesis. Furthermore, it is well known that the complement system can affect tissue homeostasis and regeneration in various conditions. For example, complement factors play a role in removing cellular debris during skeletal muscle remodeling after injury [45]. In particular, complement C3 has been shown to be essential for physiological tissue regeneration as evident from the inability of

damaged cells to be cleared from C3-deficient mice and the subsequent fibrosis that develops [46, 47]. At the same time, complement is known to participate in muscle destruction in inflammatory myopathies including myasthenia gravis, dermatomyositis, and dysferlinopathies [48]. In view of the accumulating evidence for the impact of inflammatory events on the skeletal muscle microenvironment in the setting of cancer cachexia [49], complement may also contribute to muscle breakdown in cachexia. In line with this, it was recently found that complement is activated in the skeletal muscle of pancreatic cancer patients, and that C3 deficient mice display attenuated muscle atrophy in the KPC model of pancreatic cancer cachexia (Dr. AR Judge, University of Florida Health Science Center, personal communication). C1q has also been reported to reduce muscle regeneration in mice [50]. All in all, it remains to be established to what side the regenerative and destructive effects of complement in muscle are balanced in the context of cancer cachexia.

This balance is also dependent on the level of expression of regulatory molecules that inactivate complement factors at various steps of the complement cascade, which should be investigated in metabolic tissues of patients with cancer cachexia. It has been shown that myoblasts are protected from complement-mediated destruction and cell lysis by their abundant expression of the regulatory molecules CD46, CD59, and C4BP [51], but the expression of these complement inhibitory factors by mature myofibers *in vivo* is unknown. Similarly, human hepatocytes are characterized by high expression of a battery of complement inhibitors, including CD46, CD55, CD59, and factor H [52, 53], although it is not known whether their expression is stable in metabolic disorders such as obesity or cachexia.

Next to the functions of complement factors in inflammation and tissue regeneration, it is noteworthy that adipocyte-derived C3a and its desarginated form, also known as acylation-stimulating protein, have been shown to affect lipid metabolism in adipocytes by stimulating triglyceride synthesis through inhibition of hormone-sensitive lipase and by increasing glucose uptake [14]. In addition, systemic levels of complement C3 and C4 have been shown to be higher in patients with metabolic syndrome [54], and C1q expression has been reported to be dysregulated in adipocytes during insulin resistance [35], which also occurs in cachexia [55]. Complement factor D, also referred to as adipsin, is another complement protein produced by adipocytes that appears to affect metabolism by triggering C3a-dependent adipogenic

signaling in adipose tissue as well as insulin secretion by pancreatic beta cells [14]. It would be interesting to investigate if the aberrations in lipid metabolism in cancer cachexia are associated with changes in these complement factors, even when the current consensus is that expansion, not atrophy, of adipose tissue leads to complement activation.

Even though we showed evidence for complement activation at multiple levels, some potential limitations of this study should be mentioned. First of all, the study population was relatively small, with a particularly minor fraction of patients displaying no cachexia, as is to expected in pancreatic cancer. It will be important to expand this study to other cancer types with a lower cachexia prevalence to address this concern. In such a follow-up study, additional inflammation parameters such as TNF-α, IFN-γ, and/or IL-1 next to CRP should be included to get more insight into potential mechanistic links between complement activation and pro-inflammatory processes in subjects with cancer cachexia. In particular, analysis of other complement factors that have been implicated in inflammation but also in metabolism and regeneration, including C5a, properdin, factor D, and factor H, will be informative, next to analysis of complement regulatory proteins, as discussed above. Such a study should keep the strengths of the current study design, with analysis of cleavage products of complement factors reflecting complement activation instead of detection of intact complement fragments (as is frequently seen in the literature), and with a thorough characterization of cachexia according to the consensus definition [3]. Finally, it is important to note that the 'no cachexia group' had elevated systemic CRP levels, indicating that this group had inflammation. The underlying causes of inflammation-related CRP increases might vary for each patient. However, since we first stratified the patients according to their cachexia status before subdividing the cachexia group according to CRP levels, we consider that CRP elevations in the 'no cachexia' group are not related to the metabolic and inflammatory alterations that come with cachexia. Our data support this notion since the higher CRP levels of the 'no cachexia' group do not translate into increases in other inflammatory molecules known to be elevated in cachexia such as IL-6 as well as the complement factors C3a, TCC, and C1q.

Given that several complement-targeting therapeutics are already in clinical use [56], more evidence for complement activation as an important inflammatory process in cachexia could be the lead-up for intervention studies directed at complement inhibition to treat cachexia.

However, first, more studies should be done to confirm a role for complement activation in the pathogenesis of cancer cachexia.

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CHAPTER 3

Elevated systemic lipocalin-2 levels are associated with neutrophil activation and nutritional status in pancreatic cancer patients

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Submitted

Abstract

Background: Cancer cachexia is a multifactorial syndrome characterized by body weight loss and systemic inflammation, resulting in reduced quality of life and poor survival. Lipocalin-2 has recently been implicated in the development of appetite suppression in pancreatic cancer cachexia. However, the source of the elevated lipocalin-2 levels in cachectic patients is unknown. We hypothesized that elevated lipocalin-2 in cancer cachexia could be associated with neutrophil activation and nutritional status of pancreatic ductal adenocarcinoma (PDAC) patients.

Methods: Plasma levels of neutrophil activation markers calprotectin, myeloperoxidase (MPO), elastase, and bactericidal/permeability-increasing protein (BPI) were compared between non-cachectic PDAC patients (n=13) and cachectic PDAC patients with high (≥26.9 ng/mL, *n*=34) or low (<26.9 ng/mL, *n*=34) circulating lipocalin-2 levels. Patients' nutritional status was assessed by the patient-generated subjective global assessment (PG-SGA). Body composition was assessed by analyses of computed tomography slides at the L3 level. Correlations between circulating lipocalin-2 levels and markers of neutrophil activation as well as cachexia features were analyzed.

Results: Nevertheless, no difference in circulating lipocalin-2 levels were observed between cachectic and non-cachectic PDAC patients (median 26.7 (IQR 19.7-34.8) vs. 24.8 (16.6-29.4) ng/mL, *p*=0.141). Cachectic patients with high systemic lipocalin-2 levels had higher concentrations of calprotectin, myeloperoxidase, and elastase than non-cachectic patients or cachectic patients with low lipocalin-2 levels (calprotectin: median 542.3 (IQR 355.8-724.9) vs. 457.5 (213.3-606.9), *p*=0.448 vs. 366.5 (294.5-478.5) ng/mL, *p*=0.009; myeloperoxidase: 30.3 (22.1-37.9) vs. 16.3 (12.0-27.5), *p*=0.021 vs. 20.2 (15.0-29.2) ng/mL, *p*=0.011; elastase: 137.1 (90.8-253.2) vs. 97.2 (28.8-215.7), *p*=0.410 vs. 95.0 (72.2-113.6) ng/mL, *p*=0.006; respectively). The CRP/albumin ratio was also higher in cachectic patients with high lipocalin-2 levels (median 2.3 (IQR 1.3-6.0) as compared to non-cachectic patients (1.0 (0.7-4.2), *p*=0.041). Lipocalin-2 concentrations correlated with those of calprotectin (r_s=0.36, *p*=0.0009), myeloperoxidase (r_s=0.48, *p*<0.001), elastase (r_s=0.50, *p*<0.001), and BPI (r_s=0.22, *p*=0.048).

Lipocalin-2 levels of patients with normal versus reduced food intake were not different (median 26.1 (IQR 24.1-32.7) vs. 25.7 (16.7-31.0) ng/mL, p=0.320). However, lipocalin-2 tended to be elevated in severely malnourished patients compared with well-nourished patients (median 27.2 (IQR 20.3-37.2) vs. 19.9 (13.4-26.4) ng/mL, p=0.058). Whereas no significant correlations with weight loss, BMI, or L3 skeletal muscle index were observed, lipocalin-2 concentrations were associated with subcutaneous adipose tissue index (r_S=-0.25, p=0.034).

Conclusions: These data suggest that elevated lipocalin-2 levels in pancreatic cancer cachexia are associated with neutrophil activation and contribute to poor nutritional status of patients.

Abbreviations:

BMI	Body mass index
BPI	Bactericidal/permeability-increasing protein
CCR2	C-C motif chemokine receptor 2
CRP	C-reactive protein
ELISA	Enzyme-linked immunosorbent assay
GDF-15	Growth/differentiation factor 15
HU	Hounsfield unit
IL-6	Interleukin-6
L3-IMAT	L3-intermuscular adipose tissue
L3-SATI	L3-subcutaneous adipose tissue index
L3-SMI	L3-skeletal muscle index
L3-VATI	L3-visceral adipose tissue index
LCN-2	Lipocalin-2
MC4R	Melanocortin 4 receptor
MPO	Myeloperoxidase
NE	Neutrophil elastase
NETs	Neutrophil extracellular traps
NLR	Neutrophil to lymphocyte ratio
PDAC	Pancreatic ductal adenocarcinoma
PG-SGA	Patient-generated subjective global assessment
PVN	Paraventricular nucleus
SMRA	Skeletal muscle radiation attenuation
TCC	Terminal complement complex
TNF-α	Tumor necrosis factor-alpha

Introduction

Cancer cachexia is a multifactorial syndrome characterized by ongoing body weight loss that results in reduced quality of life, low tolerance for anti-cancer treatment, and poor survival [1]. It is highly prevalent in many types of cancers but is most common in pancreatic cancer, where it affects up to 80% of patients with frequently more than 10% body weight loss [2]. The molecular mechanisms underlying the development of cancer cachexia remain poorly defined, although tumor-derived catabolic factors such as activins, myostatin, and pro-inflammatory cytokines arising from tumor-immune system crosstalk are thought to contribute to its progression [3, 4]. For example, elevated circulating TNF-α, IL-6, and GDF-15 have been reported to be associated with the severity of cachexia in cancer patients and mouse models [5, 6]. Furthermore, functional data support the participation of pro-inflammatory factors in tumor progression and cachectic features such as adipose tissue lipolysis and muscle wasting [7, 8].

Neutrophils are the most abundant immune cells in the circulation of humans (up to 70% of the total white blood cell count) and form an essential part of the innate immune response against infection and various other inflammatory cues. They have also been implicated in pancreatic cancer. For instance, Pratt et al. have shown that gene signatures associated with neutrophil recruitment are increased in pancreatic ductal adenocarcinoma (PDAC) tissue as compared to normal pancreatic tissue [9]. Furthermore, high levels of circulating and intratumoral neutrophils have been shown to correlate with poor survival in patients with pancreatic cancer [10]. Additionally, neutrophils can promote pancreatic tumor metastasis by the formation of so-called neutrophil extracellular traps (NETs). Pancreatic cancer cells can induce the release of NETs in vitro [11], and NETs are elevated in the blood of mice and patients with PDAC [12, 13]. In the context of cancer cachexia, emerging investigations revealed increased circulating neutrophils both in patients with cancer cachexia and in different mouse models of cancer cachexia [1, 14, 15]. Furthermore, blocking C-C motif chemokine receptor 2 (CCR2) signaling by neutrophils infiltrated in the velum interpositum region of the brain has been shown to ameliorate cachexia in mouse models of pancreatic cancer cachexia [16].

Upon activation by inflammatory stimuli, neutrophils can secrete a plethora of cytotoxic proteins, including neutrophil elastase (NE), myeloperoxidase (MPO), calprotectin, bactericidal/permeability-increasing protein (BPI), and lipocalin 2 (LCN-2, also known as neutrophil gelatinase-associated lipocalin or NGAL). LCN-2 can also be released by other cell types including macrophages, adipocytes, and hepatocytes [17]. This protein has been associated with several diseases such as obesity, type 2 diabetes, breast cancer, and pancreatic cancer [17, 18]. The biological functions of LCN-2 are diverse and include antibacterial, anti-inflammatory, as well as pro-metastatic actions [17]. Recently, LCN-2 was identified as a bone-derived hormone with central metabolic regulatory effects which suppresses appetite by binding to the melanocortin 4 receptor (MC4R) [19]. Furthermore, a study in a mouse model of pancreatic cancer cachexia revealed that circulating LCN-2 levels were increased in cachectic mice and correlated with anorexia and muscle loss; genetic deletion of LCN-2 ameliorated cachexia-associated anorexia [20]. In the same study, a significant increase in LCN-2 mRNA was found in circulating neutrophils of cachectic mice and it was suggested that together with the bone marrow compartment, neutrophils are a predominant source of circulating LCN-2 during cancer cachexia development. Using IL6- and Myd88- knockout mice, it was shown that LCN-2 is an inflammation-induced factor in cancer cachexia [20]. Although it is clear that neutrophil and bone marrow derived-LCN-2 contributes to cancer cachexia development by suppressing appetite in mice, whether the same mechanism applies in PDAC patients with cachexia remains unknown.

Given that systemic inflammation is a hallmark of cancer cachexia, and since neutrophils release cytotoxic proteins and LCN-2 upon activation by inflammatory stimuli, we hypothesized that neutrophils contribute to systemic inflammation and the release of LCN-2 in cachectic patients with pancreatic cancer. We aimed to 1) investigate the association between circulating levels of LCN-2 and neutrophil activation markers as well as features of cachexia in PDAC patients; 2) determine whether there is a link between LCN-2 levels and appetite in pancreatic cancer patients with cachexia.

Materials and Methods

Patients

81 patients undergoing pancreaticoduodenectomy for suspected adenocarcinoma of the pancreas at the Maastricht University Medical Centre (MUMC+) or University Hospital RWTH Aachen were enrolled in this study. Exclusion criteria were neoadjuvant chemo- and/or radiotherapy and the presence of another malignancy. This study was approved by the Medical Ethical Board of the MUMC+ in line with the ethical guidelines of the 1975 Declaration of Helsinki, and written informed consent was obtained from each subject (METC 13-4-107 and 2019-0977 for patients from MUMC+, EK 172/17 for patients from Uniklink Aachen).

Diagnosis of cancer cachexia and screening of cachexia status

Cachexia was defined according to the international consensus definition as 1) weight loss >5% over the past 6 months in the absence of starvation, and/or 2) BMI <20 kg/m^2 and >2% ongoing weight loss, and/or 3) sarcopenia and >2% ongoing weight loss. Patients were diagnosed with cancer cachexia if ≥1 of the criteria were met [1]. Body weight loss was reported by the patient and body weight data were retrieved from the medical record.

Body composition parameters were quantified by analyzing a cross-sectional CT image at the third lumbar (L3) vertebra that was acquired preoperatively for diagnostic purposes, using sliceOmatic 5.0 software (TomoVision, Magog, Canada) for Windows. Using predefined Hounsfield Unit (HU) ranges, the total cross-sectional area (cm^2) of skeletal muscle (SM) tissue (-29 to 150 HU) was determined. In addition, the total areas of visceral adipose tissue (VAT, -150 to -50 HU) and subcutaneous adipose tissue (SAT) as well as intramuscular adipose tissue (IMAT) (-190 to -30 HU) were assessed. Tissue areas (cm^2) were adjusted for patient height to calculate the respective L3-indices (L3-SMI, L3-VATI, L3-SATI) in cm^2/m^2, which correspond well with total body muscle and adipose tissue mass [21]. Previously published validated sex-specific cut-off values (SMI, 45.1 cm^2/m^2 for men and 36.9 cm^2/m^2 for women) that were established from a local MUMC+ cohort including pancreatic cancer patients [22] were used for the CT-derived body composition analysis. In addition, the skeletal muscle radiation attenuation (SMRA) was calculated as the average HU value of the total tissue area for muscle

(i.e. within the specified range of -29 to 150 HU). A total of 80 patients were included for body composition analysis (one patient had no CT-scan available). L3-SATI could not be accurately measured in 8 patients due to their higher BMI.

Assessment of patient's nutritional status and appetite

Patients' nutritional status was assessed by using the patient-generated subjective global assessment, a nutritional screening tool: (PG-SGA, category A: well-nourished, category B: moderate malnutrition, category C: severe malnutrition). Patient's appetite was assessed according to the question in box 2 of the PG-SGA questionnaire and rated as normal food intake (unchanged or more than usual) or less than usual food intake.

Plasma preparation

To avoid artefactual neutrophil activation during plasma preparation, venous blood was collected in EDTA tubes and gently centrifuged at 1500xg at 4°C for 15 min without brake, after which plasma aliquots were stored at -80°C until analysis.

ELISAs

Levels of circulating neutrophil activation markers calprotectin, myeloperoxidase (MPO), elastase, bactericidal permeability increasing protein (BPI), and LCN-2 were measured by solid-phase enzyme-linked immunosorbent assays (ELISA) based on the sandwich principle, according to the manufacturer's instructions (Hycult Biotech, Uden, The Netherlands; Human calprotectin, Catalog #HK379; Human MPO, Catalog # HK324; Human elastase, Catalog # HK319; Human BPI, Catalog # HK314; Human LCN-2, Catalog # HK330). All plasma samples were analyzed in duplicate in the same run. The intra- and inter-assay coefficients of variance of the various assays were < 10%. Clinical laboratory data including circulating C-reactive protein (CRP), albumin, neutrophils (%), and lymphocytes (%) were measured in the clinical setting. For some of the patients, these clinical data were not available, the exact number of the studied patients for these data is indicated in each figure legend.

Statistical analysis

Statistical analysis was performed using Prism 7.0 for Windows (GraphPad Software Inc., San Diego, CA) and R (R-4.2.0 for windows system). Data are presented as the median with interquartile range (IQR). Non-parametric tests were used for statistical analysis (Mann-Whitney U test for analysis of two groups; Kruskal-Wallis test followed by Dunn's post-testing for analysis of multiple groups). Correlations were calculated using Spearman's correlation coefficient (r_s), and Spearman's correlation matrix was generated by a Corrplot R package [23]. *P* values <0.05 were considered statistically significant.

Results

Characteristics of the Study Cohort

A total of 81 patients with PDAC were enrolled in this study (31 females and 50 males). The median age of the patients was 69.0 years. CT scan-based body composition analysis showed that 63.7% (n=51) of patients were sarcopenic, with a median L3-SMI of 47.5 (42.8-51.2) cm^2/m^2 for males and 37.5 (35.1-40.5) cm^2/m^2 for females. The median L3-VAT and L3-SAT indices were 40.5 (25.3-74.5) cm^2/m^2 and 46.7 (34.7-58.5) cm^2/m^2.

Given that LCN-2 levels have been reported to correlate with fat and lean mass wasting (two key features of cachexia) in patients with pancreatic cancer [20], we subdivided cachectic patients into groups with high or low LCN-2 using a median cut-off value of 26.9 ng/mL (see Table 1). The median weight loss of the non-cachectic patients was 3.1 (0.7-3.6) %, which was significantly less than the weight loss of the cachectic patients with high LCN-2 (median 11.5 (7.8-14.1) %, $p<0.001$) and the cachectic patients with low LCN-2 (median 8.4 (6.5-14.2) %, $p<0.001$). According to the PG-SGA, 95% (n=18) of patients with cachexia and high LCN-2 were malnourished (42% moderate malnutrition (category B) + 53% severe malnutrition (category C)), which was higher than the prevalence of malnutrition in patients without cachexia (50%; 40% category B + 10% category C) and in patients with cachexia with low LCN-2 (74%; 53% category B + 21% category C). Further patient characteristics are presented in Table 1, and Spearman correlations between studied variables are shown in Figure 1.

Table 1 General characteristics of included patients

	Overall	No cachexia	Cachexia with low LCN-2	Cachexia with high LCN-2	*p-value**
n	81	13	34	34	
Age (years)	69.0 (62.0, 75.0)	67.0 (58.0, 72.0)	71.9 (64.1, 75.8)	68.7 (61.5, 75.9)	0.463
Sex = M/F (%)	50/31 (61.7/38.3)	7/6 (53.8/46.2)	17/17 (50.0/50.0)	26/8 (76.5/23.5)	0.066
BMI (kg/m^2)	23.8 (21.9, 26.5)	25.0 (23.5, 26.2)	23.0 (21.7, 25.9)	24.0 (22.0, 26.7)	0.327
Weight loss percentage (%)	8.3 (5.2, 13.9)	3.1 (0.7, 3.6)	8.4 (6.5, 14.2)*	11.5 (7.8, 14.1)*	**<0.001**
Sarcopenia = Yes/No (%)	51/29 (63.7/36.2)	9/4 (69.2/30.8)	20/13 (60.6/39.4)	22/12 (64.7/35.3)	0.863
PG-SGA *n* (%)					**0.029**
A	11 (22.9)	5 (50.0)	5 (26.3)	1 (5.3)	
B	22 (45.8)	4 (40.0)	10 (52.6)	8 (42.1)	
C	15 (31.2)	1 (10.0)	4 (21.1)	10 (52.6)	
Normal/Less food intake (%)	24/38 (38.7/61.3)	7/6 (53.8/46.2)	8/17 (32.0/68.0)	9/15 (37.5/62.5)	0.448
SMRA (HU)	35.4 (30.4, 42.8)	37.5 (35.3, 38.9)	33.7 (28.6, 43.5)	35.4 (30.3, 41.5)	0.664
Male	36.2 (33.1, 43.2)	36.6 (35.9, 38.9)	34.9 (32.5, 44.0)	36.1 (31.7, 42.9)	0.787
Female	30.6 (27.5, 39.2)	37.5 (26.7, 38.5)	30.6 (27.4, 42.6)	30.0 (29.0, 35.5)	0.806
IMAT (cm^2)	7.9 (4.3, 14.0)	7.1 (3.9, 12.5)	7.7 (4.5, 13.2)	8.9 (4.6, 15.0)	0.782
Male	7.6 (4.2, 13.9)	4.6 (3.9, 9.4)	6.3 (4.4, 12.6)	9.0 (4.1, 15.5)	0.516
Female	8.4 (6.1, 13.7)	10.7 (5.1, 13.7)	8.8 (6.1, 11.9)	7.2 (6.5, 11.1)	0.942
L3-SMI (cm^2/m^2)	43.3 (38.0, 49.2)	42.9 (37.2, 47.2)	42.5 (38.0, 46.2)	46.2 (39.4, 51.1)	0.173
Male	47.5 (42.8, 51.2)	47.2 (45.0, 48.5)	46.5 (42.2, 50.7)	48.0 (44.0, 51.4)	0.857
Female	37.5 (35.1, 40.5)	37.2 (36.9, 37.3)	38.9 (34.9, 42.5)	38.2 (34.5, 39.7)	0.750
L3-VATI (cm^2/m^2)	40.5 (25.3, 74.5)	43.5 (25.4, 50.0)	35.4 (25.0, 63.8)	51.0 (26.1, 85.2)	0.291
Male	59.7 (32.0, 87.4)	50.0 (35.2, 74.3)	49.8 (29.7, 73.9)	66.3 (36.9, 91.2)	0.436
Female	26.4 (19.6, 39.1)	28.9 (22.2, 40.7)	33.1 (20.6, 37.2)	23.0 (16.7, 29.9)	0.662
L3-SATI (cm^2/m^2)	46.7 (34.7, 58.5)	55.0 (39.8, 87.1)	48.8 (41.9, 67.3)	41.4 (31.5, 51.7)	0.065
Male	42.5 (32.1, 54.5)	39.8 (27.4, 52.6)	51.9 (46.2, 67.3)	40.6 (30.1, 45.5)	0.071
Female	49.7 (42.3, 81.5)	87.2 (67.7, 88.1)	47.4 (40.8, 65.7)	47.6 (42.0, 58.7)	0.093
CRP/Albumin ratio	1.6 (0.7, 4.2)	1.0 (0.4, 1.4)	1.2 (0.3, 6.2)	2.3 (1.3, 6.0)*	**0.043**

The data are presented as median + IQR. Groups were compared using the Kruskal–Wallis test. * Significant difference in comparison to the no cachexia group. BMI: body mass index; PG-SGA: patient-generated subjective global assessment; HU: Hounsfield unit; L3-IMAT: L3-intermuscular adipose tissue; SMRA: skeletal muscle radiation attenuation; L3-SMI: L3-muscle index; L3-VATI: L3-visceral adipose tissue index; L3-SATI: L3-subcutaneous adipose tissue index; CRP: C-reactive protein.

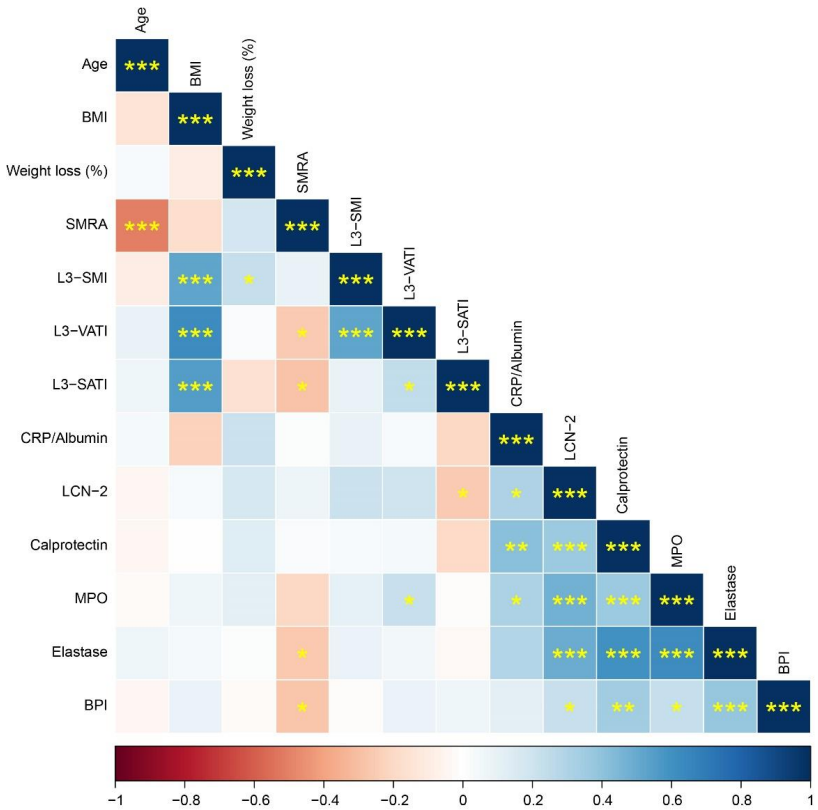

Figure 1. Correlation matrix showing Spearman correlations between patient characteristics and circulating factors. Positive correlations are shown in blue, negative correlations in red. The color intensity indicates the Spearman's correlation coefficient (bottom legend). The asterisks indicate the level of statistical significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Circulating LCN-2 is higher in males and correlates with systemic inflammation

To assess whether LCN-2 is altered in cancer cachexia, we determined the levels of circulating LCN-2 by ELISA. Whereas higher LCN-2 levels were observed in cachectic patients (median 26.7 (19.7-34.8) ng/mL) as compared to non-cachectic patients (median 24.8 (16.6-29.4) ng/mL), the difference was not significant (p=0.141, Figure 2A). In line with other studies [24-26], circulating LCN-2 levels showed a sex-specific difference, being higher in males than in females (median 27.8 (23.8-37.8) ng/mL vs. 21.6 (15.9-29.0) ng/mL, p<0.005, Figure 2B).

Since systemic inflammation is a hallmark of cancer cachexia, and LCN-2 release is associated with inflammation, we determined the degree of systemic inflammation as expressed by the CRP to albumin ratio in non-cachectic patients and cachectic patients with low or high LCN-2 levels. As expected, a significantly higher CRP/Albumin ratio was observed in cachectic PDAC patients with high LCN-2 levels as compared with patients without cachexia (2.3 (1.3-6.0) vs. 1.0 (0.4-1.4), p=0.041, Figure 2C). Furthermore, circulating LCN-2 levels correlated positively with the CRP/Albumin ratio (r_s=0.30, p=0.047, Figure 2D).

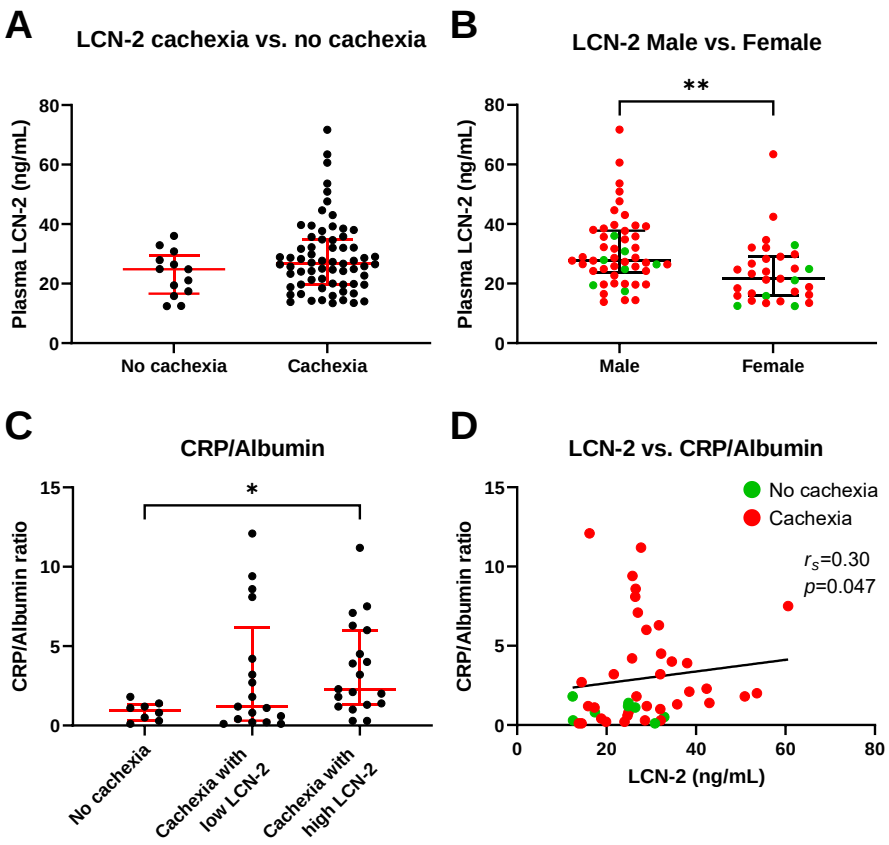

Figure 2. Circulating LCN-2 levels of PDAC patients differ according to sex and correlate with systemic inflammation. Comparison of circulating LCN-2 levels in PDAC patients with and without cachexia (A). Comparison of circulating LCN-2 levels between male and female PDAC patients (B). CRP/Albumin ratio in PDAC patients within the indicated study groups (n=44) (C). Relationship between circulating LCN-2 levels and CRP/Albumin ratio in PDAC patients (n=44)

(D). Scatter plots (A-C) show the median + IQR and individual data points in each group. Mann-Whitney U test for analysis of two groups; Kruskal-Wallis test followed by Dunn's post-testing for analysis of multiple groups. Spearman's rank correlation coefficient (r_s) was used to test for the relationship between variables. Significant differences among the groups are signified by asterisks (* $p < 0.05$, ** $p < 0.01$).

Systemic lipocalin 2 levels correlate with levels of neutrophil activation markers

LCN-2 can be produced by many different cell types, including cells relevant to cachexia such as adipocytes and hepatocytes [27, 28]. However, it was previously shown that in experimental cachexia in mice, neutrophils were the main source of LCN-2 [20]. To investigate the contribution of neutrophils to the systemic LCN-2 pool in pancreatic cancer patients with and without cachexia, we quantified circulating levels of reliable neutrophil activation markers calprotectin, MPO, elastase, and BPI in relation to levels of LCN-2. We observed consistent significant positive correlations between the concentrations of LCN-2 and all tested neutrophil activation markers (calprotectin: r_s=0.36, p<0.001; Figure 3A; MPO: r_s=0.48, p<0.001; Figure 3B; neutrophil elastase: r_s=0.50, p<0.001; Figure 3C; BPI: r_s=0.22, p=0.048; Figure 3D). Moreover, levels of calprotectin, MPO, elastase, and BPI were also strongly positively correlated to each other (Figure 1).

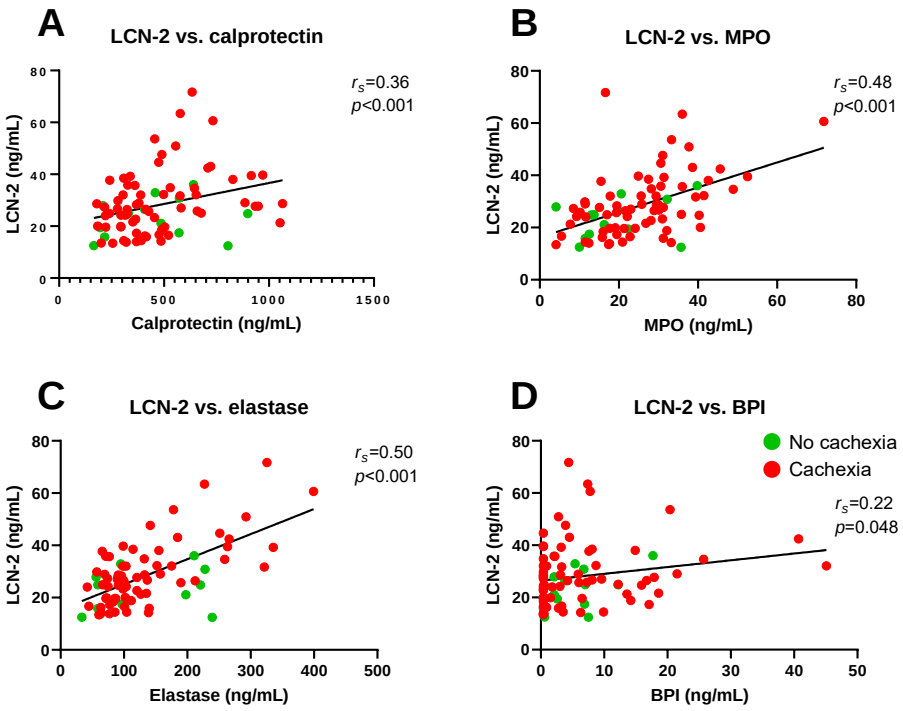

Figure 3. Correlation analysis of circulating LCN-2 and neutrophil activation markers in PDAC patients. Systemic levels of LCN-2 were positively correlated with levels of calprotectin (**A**), MPO (**B**), elastase (**C**), and BPI (**D**). Spearman's rank correlation coefficient (r_s) and level of significance are indicated in the respective plots. N=81.

In addition, when comparing cachectic patients with high versus low levels of LCN-2, the median MPO levels of cachectic patients in the high LCN-2 group were significantly higher than those of cachectic patients with low LCN-2 or those found in patients without cachexia (median 30.3 (22.1-37.9) ng/mL vs. 20.2 (15.0-29.2) ng/mL, p=0.011; vs. 16.3 (12.0-27.5) ng/mL, p=0.021, respectively) (Figure 4A). Similarly, cachectic patients with high LCN-2 levels had significantly higher concentrations of calprotectin and elastase than cachectic patients with low LCN-2 levels (calprotectin: median 542.3 (355.8-724.9) ng/mL vs. 366.5 (294.5-478.5) ng/mL, p=0.009, elastase: 137.1 (90.8-253.2) ng/mL vs. 95.0 (72.2-113.6) ng/mL, p=0.006) (Figures 4B, 4C). However, no significant differences in calprotectin and elastase levels were observed between patients without cachexia and patients with cachexia with either high or

low LCN-2 levels (Figures 4B, 4C). For BPI, no significant differences were observed between cachectic patients with high LCN-2 (median 3.6 (0.4-8.9) ng/mL) and patients without cachexia (5.4 (2.1-7.3) ng/mL, *p*=0.584) or cachectic patients with low LCN-2 levels (2.9 (0.4-8.6) ng/mL, *p*=0.931) (Figure 4D). Taken together, these data strongly indicate that elevated LCN-2 levels in PDAC patients are associated with neutrophil activation.

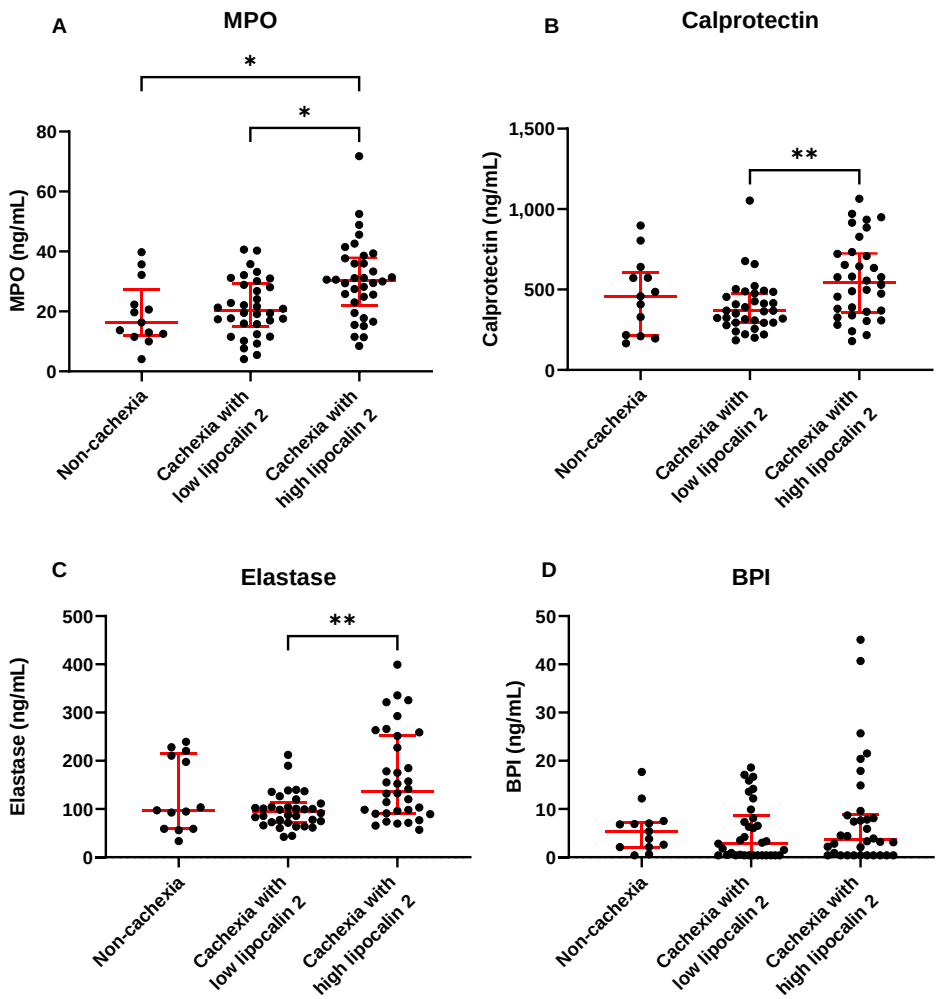

Figure 4. Circulating levels of neutrophil activation markers in PDAC patients without cachexia and in cachectic patients with high or low LCN-2 levels. Comparison of systemic levels of calprotectin (**A**), MPO (**B**), elastase (**C**), and BPI (**D**) in PDAC patients without cachexia and in cachectic patients with high or low LCN-2 levels. Scatter plots show the median + IQR

and individual data points in each group. For statistical analysis, the Kruskal-Wallis test followed by Dunn's multiple comparisons test was used. Significant differences among the groups are signified by asterisks (* $p < 0.05$, ** $p < 0.01$).

Circulating LCN-2 does not correlate with neutrophil abundance or cachexia features

To investigate whether circulating LCN-2 levels correlate with neutrophil abundance and cachexia features in PDAC patients, we performed correlation analyses. As shown in Figure 5A-C, circulating LCN-2 levels did not correlate with either neutrophil content (%) or neutrophil to lymphocyte ratio (r_s=0.35, p=0.215, r_s=0.04, p=0.916, respectively), albeit this information was only available for 14 and 12 patients, respectively. Likewise, no correlation between circulating LCN-2 levels and cachexia features such as weight loss (%) (r_s=0.17, p=0.120, Figure 5D), body mass index (r_s=0.04, p=0.748, Figure 5E), or skeletal muscle index (r_s=0.22, p=0.054, Figure 5F) were observed. However, a negative correlation between plasma LCN-2 and subcutaneous fat index (SATI) was found (r_s=-0.25, p= 0.034) (Figure 5G).

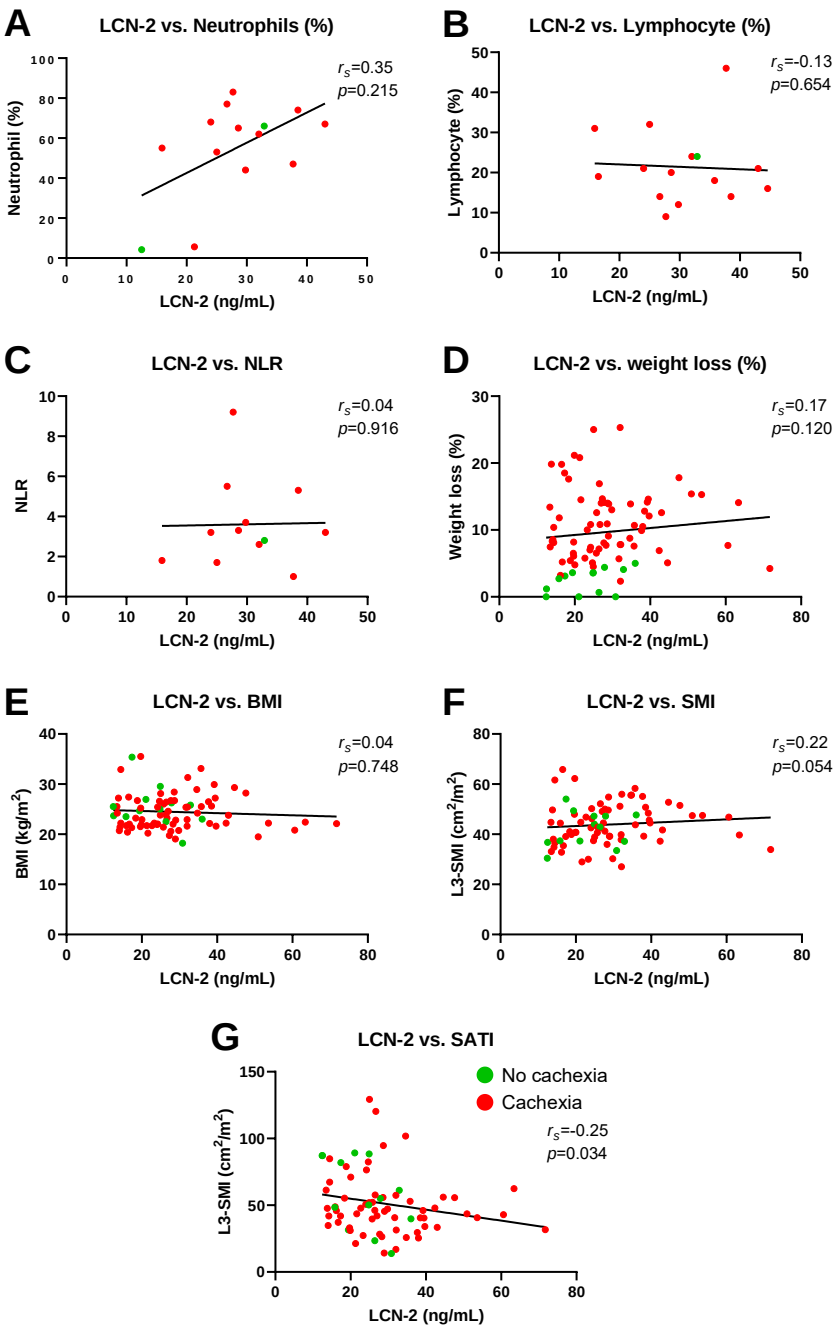
A
LCN-2 vs. Neutrophils (%)
r_S=0.35
p=0.215
Neutrophil (%)
LCN-2 (ng/mL)
B
LCN-2 vs. Lymphocyte (%)
r_S=-0.13
p=0.654
Lymphocyte (%)
LCN-2 (ng/mL)
C
LCN-2 vs. NLR
r_S=0.04
p=0.916
NLR
LCN-2 (ng/mL)
D
LCN-2 vs. weight loss (%)
r_S=0.17
p=0.120
Weight loss (%)
LCN-2 (ng/mL)
E
LCN-2 vs. BMI
r_S=0.04
p=0.748
BMI (kg/m^2)
LCN-2 (ng/mL)
F
LCN-2 vs. SMI
r_S=0.22
p=0.054
L3-SMI (cm^2/m^2)
LCN-2 (ng/mL)
G
LCN-2 vs. SATI
No cachexia
Cachexia
r_S=-0.25
p=0.034
L3-SMI (cm^2/m^2)
LCN-2 (ng/mL)

Figure 5. Circulating LCN-2 levels do not correlate to neutrophil abundance and cachexia features in pancreatic cancer patients. Correlation analysis between circulating LCN-2 levels and circulating neutrophil percentage (n=14) (A), circulating lymphocyte percentage (n=15) (B), and neutrophil to lymphocyte ratio (NLR) (n=14) (C). Relationship between circulating LCN-2 levels and cachexia features weight loss (%) (D), body mass index (E), skeletal muscle index (n=80) (F), as well as subcutaneous fat index (n=72) (G). Spearman's rank correlation coefficient (r_s) was used for the relationship between variables.

Neutrophil activation is associated with complement system activation in PDAC patients

Next, we focused on potential causes of neutrophil activation. Since it is well-known that complement factors promote neutrophil activation [29] and because we previously reported complement system activation in patients with cancer cachexia [30], we investigated whether neutrophil activation was associated with complement system activation in a subgroup of patients in the current cohort that overlapped with the cohort reported on in [30] (n=16). Patient characteristics of this subgroup are shown in Supplementary Table 1. Interestingly, both C3a, a cleavage product of the central complement C3 component, and terminal complement complex (TCC), an end product of complement activation, were strongly positively correlated with the studied neutrophil activation markers calprotectin (C3a: r_s=0.51, p=0.046; TCC: r_s=0.47, p=0.066; Figures 6A, 6B), MPO (C3a: r_s=0.80, p=<0.001; TCC: r_s=0.52, p=0.041; Figures 6A, 6B), elastase (C3a: r_s=0.52, p=0.040; TCC: r_s=0.53, p=0.036; Figures 6A, 6B), and BPI (C3a: r_s=0.43, p=0.095; TCC: r_s=0.28, p=0.292; Figures 6A, 6B). This suggests that complement activation may contribute to neutrophil activation in patients with pancreatic cancer.

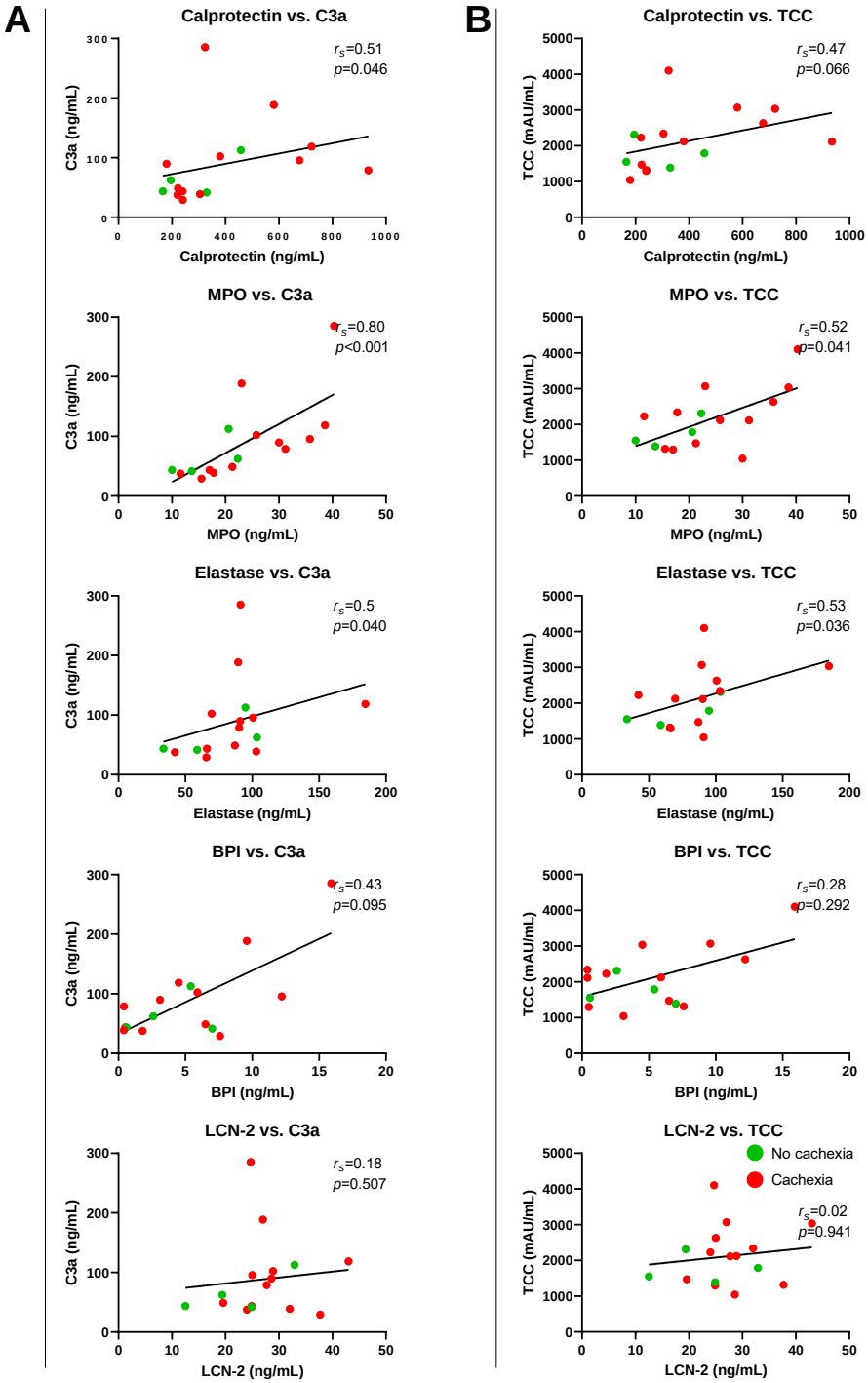
A
Calprotectin vs. C3a
r_s=0.51
p=0.046
C3a (ng/mL)
Calprotectin (ng/mL)
MPO vs. C3a
r_s=0.80
p<0.001
C3a (ng/mL)
MPO (ng/mL)
Elastase vs. C3a
r_s=0.5
p=0.040
C3a (ng/mL)
Elastase (ng/mL)
BPI vs. C3a
r_s=0.43
p=0.095
C3a (ng/mL)
BPI (ng/mL)
LCN-2 vs. C3a
r_s=0.18
p=0.507
C3a (ng/mL)
LCN-2 (ng/mL)
B
Calprotectin vs. TCC
r_s=0.47
p=0.066
TCC (mAU/mL)
Calprotectin (ng/mL)
MPO vs. TCC
r_s=0.52
p=0.041
TCC (mAU/mL)
MPO (ng/mL)
Elastase vs. TCC
r_s=0.53
p=0.036
TCC (mAU/mL)
Elastase (ng/mL)
BPI vs. TCC
r_s=0.28
p=0.292
TCC (mAU/mL)
BPI (ng/mL)
LCN-2 vs. TCC
No cachexia
Cachexia
r_s=0.02
p=0.941
TCC (mAU/mL)
LCN-2 (ng/mL)

Figure 6. Correlation analysis of neutrophil activation markers and complement system activation markers. Relationship between systemic levels of C3a and calprotectin, MPO, elastase, BPI and LCN-2 (**A**). Relationship between systemic levels of TCC and calprotectin, MPO, elastase, BPI and LCN-2 (**B**). n=16 for each graph. Spearman's rank correlation coefficient (r_s) and level of significance are indicated in the respective plots.

LCN-2 levels in severely malnourished patients with pancreatic cancer

Given that administration of LCN-2 has been shown to suppress appetite in mouse models of pancreatic cancer cachexia [20], we next examined the link between LCN-2 levels and the nutritional status of patients using the validated PG-SGA questionnaire, which contains questions about food intake. Whereas patients with cachexia had a higher prevalence of poor appetite than non-cachectic patients, the difference was not significant (65.3% (32/49) vs. 46.2% (6/13), Table 1, p=0.448). Moreover, no significant difference was observed between PDAC patients with normal food intake and PDAC patients with less food intake in terms of circulating LCN-2 (median 26.1 (24.1-32.7) ng/mL vs. 25.7 (16.7-31.0) ng/mL, p=0.320, Figure 7A). However, we found that LCN-2 levels tended to be higher in patients with poor nutritional status (PG-SGA category A vs. category B vs. category C, median 19.9 (13.4-26.4) ng/mL vs. 27.2 (19.3-32.1) ng/mL vs. 27.2 (20.3-37.2) ng/mL, p=0.058, Figure 7B).

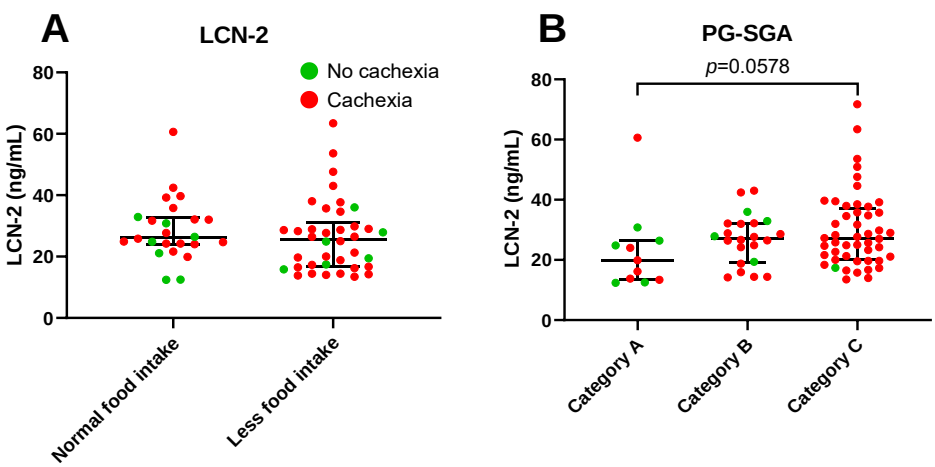

Figure 7. Circulating LCN-2 levels in PDAC patients according to food intake and nutritional status. Comparison of systemic levels of LCN-2 in PDAC patients with normal versus reduced

food intake (A). LCN-2 levels in plasma from well-nourished (category A), moderately malnourished (category B) and severely malnourished (category C) PDAC patients (B). Scatter plots showing the median + IQR and individual data points in each group. For statistical analysis, the Mann–Whitney U test was used for two groups and Kruskal-Wallis test followed by Dunn's post-testing for analysis of multiple groups. Significant differences among the groups are signified by asterisks (* $p < 0.05$).

Discussion

It was previously reported that LCN-2 increases in pancreatic cancer patients correlate with loss of fat and muscle, two key features of cachexia [20]. Based on relatively weak correlations to neutrophil abundance and the neutrophil/lymphocyte ratio, the LCN-2 elevations were attributed to neutrophil expansion [20]. The current study provides several lines of evidence for a contribution of neutrophil activation to the elevated LCN-2 levels in patients with pancreatic cancer. We showed strong correlations between circulating levels of LCN-2 and the degree of systemic inflammation (CRP/albumin ratio) as well as a set of four different neutrophil activation markers and demonstrated that cachectic patients with high systemic LCN-2 levels have significantly higher levels of the neutrophil activation markers calprotectin, MPO, and elastase than patients with low LCN-2 levels. Furthermore, consistent correlations between these neutrophil activation markers and activated complement factors C3a and TCC were observed in these patients, suggesting that systemic complement activation may contribute to neutrophil activation in pancreatic cancer. Of note, although circulating LCN-2 levels were not related to cachexia and food intake, higher LCN-2 levels were associated with worse nutritional status of patients. Taken together, these results suggest that elevated LCN-2 in cachectic patients with pancreatic cancer is related to neutrophil activation and complement activation.

LCN-2 is a polypeptide released by several cell types including adipocytes, hepatocytes, epithelial cells, and neutrophils. Elevated circulating LCN-2 has been found in many types of cancer and promotes malignant development in cancer patients [18, 31]. The functional roles of LCN-2 include regulating body fat mass and lipid metabolism as well as immune responses to inflammatory stimuli. As a biomarker of inflammation, LCN-2 has been associated with

chronic inflammatory disorders such as inflammatory bowel disease, obesity, and pancreatic cancer [18, 32, 33]. In line with this, we found a positive correlation between circulating LCN-2 levels and systemic inflammation. However, LCN-2 levels were not associated with cachexia status of the patients. Of note, the mean concentration of LCN-2 in our cohort was 28.2 ng/mL, which is much lower than the levels of 150.3 and 217.5 ng/mL reported by Olson et al. in two different patient cohorts [20]. A possible explanation for this discrepancy is that different methods were used for plasma preparation. To avoid neutrophil activation during plasma sample preparation, we applied careful centrifugation, which is unlikely to be performed in retrospective studies where collected blood undergoes routine processing procedures at clinical chemistry departments.

Recently, emerging evidence revealed that LCN-2 suppresses appetite in mice. For example, Mosialou and colleagues demonstrated that LCN-2 suppresses food intake in mice by crossing the blood-brain barrier and binding to its receptor MC4R in the hypothalamic paraventricular nucleus (PVN) [19]. Similar appetite suppression by LCN-2 was observed in primates who received daily administration of recombinant human LCN-2 which resulted in a 21% decrease in food intake [34]. In the context of cancer cachexia, a more recent study showed that administration of LCN-2 to mice reduced food intake and decreased body weight, while deletion of LCN-2 restored appetite in a mouse model of pancreatic cancer cachexia [20]. To explore the relevance of this finding in human pancreatic cancer cachexia, we compared LCN-2 levels between PDAC patients with normal or reduced food intake and investigated the relationship between circulating LCN-2 and several features of cachexia including weight loss, body composition, and nutritional status. While LCN-2 is able to suppress appetite in mice with pancreatic cancer cachexia, we did not observe a relationship between food intake and LCN-2 levels in our patient cohort. Moreover, circulating LCN-2 levels did not correlate with weight loss and body mass index, although a significant negative association between circulating LCN-2 and the subcutaneous adipose tissue volume was observed. Also, LCN-2 levels were higher in patients that were malnourished according to the PG-SGA. Thus, although our data do not provide evidence for a direct link between LCN-2 and appetite in the context of human pancreatic cancer cachexia, LCN-2 may still indirectly affect cachexia-related nutritional factors.

Previously, we reported complement system activation in pancreatic cancer patients with cachexia [30]. To gain an understanding of the potential relationship between neutrophil activation and complement activation in PDAC patients with cachexia, we performed correlation analysis. Intriguingly, we found strong correlations between all neutrophil activation markers studied and the central complement system activation markers C3a and TCC. A previous *in vitro* study showed that neutrophils activate the alternative pathway of complement and release C5 fragments that further enhance neutrophil activation [29], which is in line with our current observations. Furthermore, several studies have shown that the treatment of human neutrophils with C3a leads to neutrophil degranulation, aggregation, and chemotaxis [35, 36]. Thus, complement and neutrophil activation in these patients may result from a positive feedback loop.

In obesity and type 2 diabetes, neutrophilic inflammation has been shown to be involved in the development of insulin resistance and other metabolic aberrations [37, 38]. In line with this, we found a correlation between SMRA and levels of neutrophil activation markers elastase and BP (see Figure 1), which could suggest that neutrophil activation also promotes inflammation in skeletal muscle tissue of pancreatic cancer patients leading to insulin resistance and lipid accumulation. In addition, we could corroborate the previously described differences in LCN-2 levels between males and females [24-26], with higher levels in males. It would be interesting to explore if this contributes to the recently reported sex differences in the progression of cancer cachexia [39, 40], also given that we identified a correlation between LCN-2 and SATI, which is higher in females.

Certain limitations of this first clinical study on circulating LCN-2 levels in association with neutrophil activation in pancreatic cancer patients should be acknowledged. First, the study population was relatively small, and our results should be validated in a larger patient cohort. Second, the applied cut-off value for LCN-2 was based on the median value in cachectic patients which should be optimized by generating ROC curves in future large cohort studies. Third, although sarcopenia (defined by low SMI) is usually strongly associated with cachexia, cachectic patients in our study did not have a lower SMI than non-cachectic patients. Since self-reported unintentional weight loss of >5% is the central diagnostic criterion for cachexia, this “subjective” value could obscure actual differences in the SMI of each group. Finally,

although a strong positive correlation between circulating LCN-2 and neutrophil activation markers was observed which is in line with activated neutrophils as the main source of LCN-2, we cannot exclude the contribution of bone-derived LCN-2 to circulating levels in the patients studied.

In conclusion, the present study shows that circulating LCN-2 is associated with neutrophil activation in pancreatic cancer patients, irrespective of their cachexia status. Generalized activation of the innate immune system seems to contribute to the production of circulating LCN-2 as indicated by the correlations between neutrophil activation markers and activated complement components. Follow-up studies investigating the potential of LCN-2 as a therapeutic target in cancer cachexia are warranted given the association between its levels and the nutritional status of PDAC patients.

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Supplementary Table 1

Patient characteristics

	Overall	No cachexia	Cachexia	*p*-value
n	16	4	12	
Age (year)	71.0 (59.0, 75.0)	64.0 (54.5, 74.2)	71.0 (63.0, 75.0)	0.715
BMI (kg/m^2)	24.2 (22.6, 26.5)	25.2 (24.4, 26.8)	23.8 (21.8, 26.5)	0.275
Weight loss (%)	7.4 (4.4, 11.3)	3.6 (3.0, 3.7)	9.5 (6.9, 12.9)*	**0.004**
SMRA (HU)	36.9 (29.8, 40.1)	38.8 (34.9, 41.2)	35.7 (29.8, 39.3)	0.396
L3-SMI (cm^2/m^2)	42.2 (39.2, 48.3)	40.6 (37.1, 45.4)	42.2 (39.8, 48.3)	0.467
L3-VATI (cm^2/m^2)	54.9 (30.6, 74.7)	39.0 (30.5, 47.0)	64.0 (32.2, 78.7)	0.182
L3-SATI (cm^2/m^2)	51.9 (32.5, 71.8)	74.2 (53.8, 87.4)	50.6 (31.4, 56.7)	0.192
CRP/Albumin	0.9 (0.4, 2.5)	0.5 (0.4, 0.9)	1.0 (0.6, 6.0)	0.404
LCN-2 (ng/mL)	26.0 (24.5, 29.7)	22.1 (17.7, 26.9)	27.4 (24.8, 29.7)	0.203
Calprotectin (ng/mL)	314.2 (221.9, 488.4)	262.4 (187.9, 361.5)	314.2 (235.2, 605.1)	0.332
MPO (ng/mL)	21.8 (16.6, 30.3)	17.1 (12.8, 21.0)	24.4 (17.6, 32.3)	0.090
Elastase (ng/mL)	89.9 (66.1, 96.3)	76.8 (52.5, 97.1)	89.9 (68.8, 93.6)	0.716
BPI (ng/mL)	5.0 (1.5, 7.2)	4.0 (2.1, 5.8)	5.2 (1.5, 8.1)	0.716
C3a (ng/mL)	70.6 (43.0, 104.9)	53.0 (43.1, 74.8)	84.4 (42.4, 106.4)	0.716
TCC (mAU/mL)	2117.2 (1452.2, 2412.0)	1670.6 (1511.9, 1919.4)	2173.1 (1434.2, 2731.6)	0.467

The data are presented as median + IQR. Groups were compared using the Mann–Whitney U test. * Significant difference in comparison to the no cachexia group. BMI: body mass index; HU: Hounsfield unit; L3-IMAT: L3-intermuscular adipose tissue; SMRA: skeletal muscle radiation attenuation; L3-SMI: L3-muscle index; L3-VATI: L3-visceral adipose tissue index; L3-SATI: L3-subcutaneous adipose tissue index; CRP: C-reactive protein; LCN-2: lipocalin 2; MPO: myeloperoxidase: BPI: bactericidal permeability increasing protein (BPI); TCC: terminal complement complex.

CHAPTER 4

Identification of intramyocellular lipid alterations in human pancreatic cancer cachexia by mass spectrometry imaging

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Manuscript in preparation

CHAPTER 5

The pancreatic tumor organoid secretome of cachectic patients promotes lipid accumulation in skeletal muscle cells

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CHAPTER 6

General discussion

Introduction

Cancer cachexia is a multifactorial and devastating syndrome characterized by substantial body weight loss, involving, in particular, loss of skeletal muscle and adipose tissue that cannot be fully reversed by nutritional supplementation. Currently, no effective interventions or treatments are available for patients with cancer cachexia. Cancer patients with cachexia usually display systemic inflammation, anorexia, muscle weakness, lower responsiveness to anticancer therapies, and poor survival. The morbidity of cancer cachexia varies depending on tumor type, location, and stage. Pancreatic cancer has the highest prevalence of cachexia (up to 80%), followed by gastro-oesophageal cancer and head/neck cancer. Cachexia affects 50-80% of patients with advanced-stage cancer and is thought to be directly responsible for 20% of cancer-related deaths.

It is well documented that systemic inflammation plays a vital role in the pathogenesis of cancer cachexia. Many studies have therefore focused on identifying the catabolic cytokines released by tumor cells or innate and adaptive immune cells inside and outside of the tumor microenvironment. However, the characterization of the immune system in the context of cancer cachexia is still rather incomplete. For example, the role of the complement system in the inflammatory response seen in pancreatic cancer patients with cachexia is unknown, even though the complement system is a central component of the innate immune system that is closely related with inflammatory conditions and generalized as well as local immune responses. Furthermore, the potential impact of neutrophil activation in the setting of cancer cachexia has only recently gotten some attention. As a main player in the innate immune system, neutrophils are involved in the host defense against micro-organisms by mounting acute inflammatory responses, for example by their phagocytic functions, degranulation of cytotoxic compounds, and formation of NETs. Besides their functions mentioned above, neutrophils have recently been reported to be involved in cachexia-associated anorexia in mice by releasing LCN-2 (also known as neutrophil gelatinase-associated lipocalin), which crosses the blood-brain barrier, binds to the melanocortin 4 receptor (MC4R) in the hypothalamus, and stimulates an MC4R-dependent anorexigenic pathway. However, whether the same mechanism is operational in PDAC patients with cachexia remains unknown.

Sarcopenia or loss of muscle mass is one of the criteria for the diagnosis of cancer cachexia and is widely studied in cancer patients with cachexia. In contrast, myosteatosis, also known as fat infiltration in skeletal muscle, has received much less attention in the context of cancer cachexia even though it is also associated with muscle wasting and has a strong negative impact on patients' prognosis.

In this thesis, several factors reflecting the role of complement and neutrophil activation as well as myosteatosis in pancreatic cancer patients with or without cachexia were broadly studied. In this chapter, several of these pancreatic cancer cachexia-related aspects will be discussed: 1) complement system activation in patients with cancer cachexia; 2) neutrophil activation and the role of circulating LCN-2 in pancreatic cancer cachexia; 3) myosteatosis in pancreatic cancer patients with cachexia and the potential mechanisms underlying it.

Complement system activation in patients with cancer cachexia

Complement system activation is an ancient defense mechanism against invading pathogens which is initiated by C3 activation through so-called classical, lectin, and/or alternative pathways. Inappropriate complement system activation has been associated with various diseases such as nonalcoholic fatty liver disease, systemic lupus erythematosus, systemic autoimmune disease, carcinogenesis as well as cancer progression. In the context of cancer cachexia, complement system activation had not yet been explored. In **Chapter 2,** therefore, we investigated whether systemic activation of the complement system occurred in pancreatic cancer patients with cachexia and if it correlated with the canonical marker of systemic inflammation, CRP. The results showed that complement system activation was present in pancreatic cancer patients with cachexia, particularly in those with a systemic inflammatory response as evidenced by elevated plasma CRP levels. These patients displayed higher levels of C3a, a cleavage product of the central complement C3 component with potent pro-inflammatory functions, and TCC, an end product of complement activation, as compared to those who were not cachectic, even though the latter group also displayed an elevation in CRP concentrations. Furthermore, a strong correlation between C1q and C3a, as well as TCC, was observed in these patients, which may indicate that the classical pathway of the complement system is involved in complement activation in patients with pancreatic cancer.

The complement system is closely associated with inflammation. For example, stimulation of human astrocytomas, epithelial cells, monocytes, or monocyte-derived macrophages with C3a or C5a induces the production of proinflammatory mediators such as IL-1b, TNF-a, IL-6, and IL-8 [1-4]. Furthermore, a recent study revealed that C3 was expressed in synovial sub-lining fibroblasts from patients with rheumatoid arthritis and that its levels increased with repeated TNF-α stimulation *in vitro* [5]. Systemic inflammation is one of the key cachexia features. As such, cachexia is generally associated with increased levels of inflammatory molecules such as CRP, TNF-α, IL-6, and IL-8. In the current study, we confirmed that cachectic patients with inflammation as defined by elevated CRP levels displayed higher circulating IL-6 concentrations. Besides their classical pro-inflammatory functions, various complement factors have been shown to be involved in carcinogenesis in several types of cancer [6]. They can promote cellular proliferation, invasion, and migration, and mediate the development of an immunosuppressive microenvironment [7]. For instance, using a TC-1 syngeneic mouse model of cervical cancer, Markiewski and colleagues revealed that C5a deposition in the tumor microenvironment promotes tumor growth, and that this effect was inhibited by pharmacological blockade of C5a receptor [8]. Furthermore, previous studies have shown that C3a/C3aR and C5a/C5aR binding results in activation of P38/ERK MAPK and phosphatidylinositol 3-kinase-AKT-mammalian target of rapamycin (PI3K-AKT-mTOR) signaling pathways, which are strongly linked to oncogenesis [9-13]. In view of these contributions of the complement system to cancer progression, it is not surprising that we found increased complement activation products in the plasma of patients with cachexia and inflammation, which are characteristics associated with more advanced cancer [14].

In vitro, neo-antigen expression by tumor cells induces IgM or IgG antibodies, which can bind to C1q resulting in the activation of the classical complement pathway. Furthermore, the classical complement pathway can also be activated by the binding of C1q to apoptotic cells. Both processes lead to the clearance of cells through macrophage phagocytosis, which could promote chronic inflammation as seen in cachexia. C1q is an initiator molecule in the classical complement system activation pathway that has been associated with many pathological disorders including autoimmunity, glomerulonephritis, arterial stiffness, and cancer [15, 16]. Unlike other complement proteins that are mainly produced by the liver, C1q can be synthesized by various cell types including macrophages [17, 18], epithelial cells [19],

mesenchymal cells [20], dendritic cells [21], mast cells [22], fibroblasts [23], endothelial cells [24], as well as pancreatic cancer cells [25]. Any of the cells mentioned above could contribute to systemic active complement by stimulating C1q-C3a signaling. Further studies are needed to identify the cellular and/or tissue sources of cancer related complement activation. The direction in this context may focus on the classical complement pathway-related complement factors such as C1r/s, C2, and C4 given the association between the levels of C1q, the initiating factor of the classical complement pathway, and the levels of complement effectors C3a and TCC that we observed.

Emerging investigations have revealed that gut barrier dysfunction is correlated with the development of cancer cachexia in mouse models as well as patients [26, 27]. It could therefore be hypothesized that the complement system might be activated through the classical pathway in these patients as a result of the formation of antibodies complexed to translocated bacteria. Furthermore, a study in mice showed that complement C3 contributed to alcohol-induced liver steatosis [28]. In line, our lab previously showed that both C1q, C3a, and MAC are deposited on steatotic hepatocytes in obese patients with nonalcoholic fatty liver disease [29]. Since cancer cachexia is associated with hepatic steatosis, it should be investigated if lipid accumulation in the liver of cachectic patients leads to the deposition of activated complement factors as well. In skeletal muscle, activation of the classical pathway of complement in patients with immune-mediated necrotizing myopathies has been reported and correlated with muscle fiber necrosis [30]. In addition, older studies have also shown that several complement factors, including C1, C2, C4, and C3, can be synthesized by skeletal muscle cells *in vitro* [31, 32], and that they are upregulated by pro-inflammatory cytokines including IL-1 [32]. Given the accumulating evidence for the impact of inflammatory events on the skeletal muscle microenvironment in the setting of cancer cachexia [33], complement activation may also contribute to muscle breakdown in cachexia. In line with this, it was recently found that complement is activated in the skeletal muscle of pancreatic cancer patients and that C3-deficient mice displayed attenuated muscle atrophy in the KPC model of pancreatic cancer cachexia (Dr. AR Judge, University of Florida Health Science Center, USA, personal communication). Of note, complement C3 also seems to benefit skeletal muscle regeneration by activating the C3a–C3aR signaling pathway after injury [34]. Furthermore, proteomic and transcriptomic analyses support a key role for complement C3 in myogenesis

[35]. All in all, it remains to be established to what side the regenerative and destructive effects of complement in muscle are balanced in the context of cancer cachexia.

Besides the functions of complement factors in inflammation and tissue regeneration, complement proteins are also known to be involved in regulating adipose tissue lipid metabolism. On the one hand, adipose tissue can be a target organ of complement activation that potentially promotes atrophy of fat compartments in the body. On the other hand, adipose tissue releases a variety of complement components, including C1, C3, factor B, factor D, factor H, and properdin. Among these complement components, C3 fragment C3a-desArg (also known as acylation-stimulating protein), has been shown to affect lipid metabolism in adipocytes by stimulating triglyceride synthesis through the inhibition of hormone-sensitive lipase and by increasing glucose uptake [36]. In addition, systemic levels of complement C3 and C4 are higher in patients with metabolic syndrome [37], and C1q, complement factor B and factor H expression have been reported to be dysregulated in adipocytes during insulin resistance [38], which also occurs in cachexia [39]. Since complement activation (especially through C3a-C3aR and C5a-C5aR signaling) in adipose tissue thus may support adipose expansion instead of wasting/atrophy, it would be interesting to investigate if the aberrations in lipid metabolism in cancer cachexia are associated with changes in these complement factors.

Neutrophil activation and the role of circulating LCN-2 in pancreatic cancer cachexia

Neutrophils are the most abundant immune cells in the circulation of humans (up to 70% of the total white blood cell count) and form an essential part of the innate immune response against infection and various other inflammatory cues. In the context of cancer, neutrophil infiltration in the tumor microenvironment has been shown to be involved in promoting tumor growth, invasion, and metastasis. In addition, a more recent study has shown that neutrophil-derived LCN-2, also known as neutrophil gelatinase-associated lipocalin, participates in the development of cancer cachexia by mediating appetite suppression in mouse models of pancreatic cancer cachexia. This study highlighted that besides neutrophils' function in the

host defense against micro-organisms, tumorigenesis, and cancer progression, they also play a role in regulating metabolic processes.

In **Chapter 3**, we determined the circulating LCN-2 levels in pancreatic cancer patients with or without cachexia and assessed their relationship with neutrophil activation markers and systemic inflammation as well as specific cancer cachexia features. Our results revealed that elevated circulating LCN-2 levels in cachectic patients with pancreatic cancer were related to neutrophil activation, potentially as a result of complement activation, and that they may contribute to a poor nutritional status. This conclusion is based on several lines of evidence: 1) A strong correlation between circulating levels of LCN-2 and markers of systemic inflammation (CRP/Albumin ratio) or neutrophil activation; 2) Cachectic patients with high systemic LCN-2 levels have significantly higher levels of neutrophil activation markers; 3) Consistent and strong correlations between neutrophil activation markers and activated complement factors C3a and TCC were observed in these patients; 4) Pancreatic cancer patients with malnutrition showed a trend toward increased circulating LCN-2 levels.

LCN-2 is an innate immune protein and has been used as a biomarker in inflammatory and metabolic diseases such as obesity, heart failure, and kidney damage. LCN-2 can be synthesized by many types of cells including hepatocytes, adipocytes, macrophages, epithelial cells, endothelial cells, astrocytes, renal cells, osteoblasts, and neutrophils upon stress conditions and inflammatory stimuli. Several pro-inflammatory mediators such as TNF-α, IFNγ, IL-1β, and IL-6 have been shown to be involved in LCN-2 production. For example, 3T3-L1 adipocytes treated with TNF-α, IL-1β, or IL-6 responded with a significant LCN-2 expression and secretion [40]. In neutrophils, LPS and TNF-α are known as two strong inducers of LCN-2 expression [41]. Previous studies have also shown that the promoter of LCN-2 includes transcription factor binding sites for NF-κB and C/EBP [40], which have been implicated in the development of cancer cachexia, like the pro-inflammatory factors TNF-α, IL-1β, and IL-6 [42-44]. In **Chapter 3**, we showed that cachectic PDAC patients with higher systemic LCN-2 levels had significantly higher systemic inflammation (CRP/Albumin) as compared to non-cachectic patients and a strong correlation between circulating LCN-2 levels and systemic inflammation.

It would be interesting to investigate if circulating LCN-2 also correlates with levels of pro-inflammatory cytokines in cancer patients with cachexia. Furthermore, a study in mice showed that overexpression of LCN-2 in adipose tissue promotes the beiging of inguinal white adipose tissue [45]. Given that a switch from white adipose tissue to brown adipose tissue is being recognized as a characteristic of cancer cachexia [46, 47], and a strong correlation between circulating LCN-2 levels and subcutaneous fat mass was observed in our study cohort, further studies exploring the effect of LCN-2 on adipose tissue lipolysis and browning should be encouraged.

LCN-2 was first purified and identified in neutrophils by Kjeldsen et al. in 1993 [48]. Upon activation by inflammatory stimuli, neutrophils can release NETsand secrete a plethora of cytotoxic proteins, including neutrophil elastase, myeloperoxidase, calprotectin, and bactericidal/permeability-increasing protein [49]. Using immunohistochemical analyses, Li and colleagues demonstrated that LCN-2 protein is present in NETs from activated neutrophils in both mice and humans [50]. Given that a higher level of neutrophil activation markers was observed in cachectic patients with high LCN-2 levels, it would be interesting to investigate neutrophil derived-NETs and cytotoxic protein levels in skeletal muscle, adipose tissue as well as tumor tissue of cachectic patients. Our results showed a strong positive correlation between neutrophil activation and complement activation in pancreatic cancer patients, the latter of which was reported in **Chapter 2**. Interestingly, neutrophil exposure to complement fragment C5a has been reported to promote NETs formation [51]. In addition, complement fragment C5a has also been reported to stimulate neutrophil activity such as glucose uptake, phagocytosis, and reactive oxygen species generation [52]. Thus, a potential mechanism underlying elevated circulating LCN-2 in pancreatic cancer patients with cachexia is that neutrophils release LCN-2 as a result of activation triggered by elevated systemic inflammation (CRP to albumin ratio) and/or complement system activation.

Besides the role of LCN-2 in innate immunity, it is also involved in tumor invasion and metastasis. Several studies have reported high levels of LCN-2 in patients with cancer such as cervical cancer, breast cancer, and endometrial cancer [53-55], where its levels correlate with

the development of metastasis. For example, a study revealed that the monomeric form of LCN-2, matrix metalloproteinase-9 (MMP-9), and the MMP-9/LCN-2 complex were significantly increased in breast cancer patients as compared with healthy controls. The binding of LCN-2 to MMP-9 generates an MMP-9/LCN-2 complex that protects MMP-9 from autodegradation and upregulates MMP-9 activity *in vitro* [56, 57]. In the same study, significant correlations between serum MMP-9 and LCN-2 and breast disease severity scores were observed [58]. As an epithelial-mesenchymal transition-associated protein, MMP-9 can degrade basement membranes and the extracellular matrix, promoting tumor invasion and metastasis [59]. Cancer cachexia is highly prevalent in patients with advanced cancer. Since many pancreatic cancer patients in our studies were already at the advanced stage at the time of diagnosis, we expected to find elevated circulating LCN-2 levels in pancreatic cancer patients with cachexia. However, even though circulating LCN-2 levels trended towards an increase in pancreatic cancer patients, the difference was not significant. In addition, no correlation between circulating LCN-2 levels and BMI, body weight loss, or skeletal muscle mass was observed. These results may suggest that circulating LCN-2 does not associate with the development of cancer cachexia in humans. In this context, it is noteworthy that contradictory results regarding the role of LCN-2 in promoting tumor invasion and metastasis have also been reported. In particular, Lu and colleagues demonstrated that overexpression of LCN-2 in HOS osteosarcoma cells inhibits their motility, invasion, and migration via activation of the MEK–ERK pathway, and LCN-2 silencing promotes motility and migration [60]. To address the question of whether LCN-2 promotes pancreatic cancer invasion and metastasis, further studies are needed. These studies should include analyses of MMP-9 and the MMP-9/LCN-2 complex.

Like GDF-15, LCN-2 has the ability to regulate appetite. In 2017, Mosialou and colleagues demonstrated that bone-derived LCN-2 can cross the blood-brain barrier and bind to the melanocortin 4 receptor (MC4R) in the paraventricular and ventromedial neurons of the hypothalamus, which results in appetite suppression [61]. More recently, a study in a mouse model of pancreatic cancer cachexia demonstrated that a high level of LCN-2 is associated with anorexia, reduced food intake, and muscle loss, and that genetic deletion of LCN-2 or pharmacologic inhibition of MC4R ameliorates pancreatic cancer cachexia associated-

anorexia [62]. In the same study, it was shown that there was an inverse correlation between increasing circulating LCN-2 levels and loss of visceral fat and skeletal muscle in patients with pancreatic cancer. In our study, no correlation between circulating LCN-2 and fat or muscle mass was observed. However, we were not able to analyze the correlation regarding changes in circulating LCN-2 levels and muscle and fat mass because we did not have access to follow-up samples. To investigate whether circulating LCN-2 contributes to cancer cachexia by influencing appetite, we determined the level of circulating LCN-2 in pancreatic cancer patients with normal food intake versus reduced food intake as indicated by the PG-SGA questionnaire. Our result showed no significant difference in circulating LCN-2 levels between pancreatic cancer patients with normal food intake and reduced food intake. This result does not support the notion that circulating LCN-2 contributes to cancer cachexia by suppressing appetite. Thus, although overexpression of LCN-2 has been linked to appetite suppression in mouse models of pancreatic cancer cachexia, this might not be the case in patients.

Myosteatosis in pancreatic cancer patients with cachexia and its potential underlying mechanism

Ectopic fat deposition in skeletal muscle is linked to metabolic disorders such as obesity, type 2 diabetes, and cancer. In the past decade, a large body of studies has focused on studying the clinical impact of myosteatosis in cancer patients, and most of them showed consistent evidence that myosteatosis is associated with poor survival outcomes. Although myosteatosis (generally considered to reflect poor muscle quality) contributes to muscle wasting, this has received comparatively little attention in the context of cancer cachexia. With the advancement of body composition analysis and lipidomics, in **Chapter 4**, we investigated lipid accumulation in skeletal muscle from pancreatic cancer patients with or without cachexia and analyzed the composition and distribution of intramyocellular lipids in these patients. Furthermore, in **Chapter 5**, using patient derived pancreatic tumor organoids and RNA-sequencing, we explored the mechanisms underlying lipid accumulation in skeletal muscle. In this section, I will discuss lipid accumulation in skeletal muscle from pancreatic cancer patients with or without cachexia, from the measurement to the underlying mechanism.

As aforementioned in **Chapter 1**, lipid accumulation in skeletal muscle can be measured by invasive muscle biopsy, or through noninvasive techniques such as cross-sectional MRI and CT. With muscle biopsy, lipid content as well as lipid composition can be studied. With noninvasive CT images, the degree of lipid accumulation can be assessed by measuring SMRA expressed in Hounsfield units (HU). However, CT-based analyses are not able to identify the nature of intracellular lipids (content and species). In Chapter 4, a combination of muscle biopsy and CT-derived body composition analysis was used for assessing lipid accumulation in skeletal muscle from pancreatic cancer patients with or without cachexia. We observed high content of intramyocellular lipids in muscle biopsies from pancreatic cancer patients with cachexia as compared to those without cachexia. CT-derived body composition analysis revealed a significantly lower SMRA (e.g increased ectopic fat deposition) in cachectic PDAC patients with inflammation as compared to non cachectic patients. Consistent with previous data [63], we also observed a significant positive correlation between increasing intramyocellular lipid content and loss of body weight.

Importantly, differences in SMRA were observed between cachectic patients with inflammation vs. cachectic patients without inflammation or patients without cachexia, but not between cachectic patients without inflammation vs. patients without cachexia. In addition, a positive correlation between intramyocellular lipid content and CRP levels was observed. These results suggest that systemic inflammation could play a role in lipid accumulation in skeletal muscle. Indeed, several studies have already highlighted the close relationship between muscle fat infiltration and systemic inflammation in patients with colorectal cancer or pancreatic cancer [64]. For example, Rollins and colleagues revealed that PDAC patients with myosteatosis had significantly higher levels of markers of systemic inflammation including white blood cell count, neutrophil-lymphocyte ratio, and CRP than those without myosteatosis [65]. Intriguingly, intramyocellular lipid content also positively correlated with local inflammation as reflected by expression of IL-6 in skeletal muscle, indicating that lipid accumulation in skeletal muscle of cachectic patients may be promoted by both systemic inflammation and local inflammation.

Skeletal muscle is a major site with regard to insulin-stimulated glucose uptake. Impaired insulin metabolic signaling in skeletal muscle plays a key role in the development of insulin resistance. In healthy skeletal muscle, insulin promotes glucose uptake through the activation of the IRS-1-PI3K-GLUT4 pathway. In particular, insulin binds and activates its receptor in the membrane of skeletal muscle, leading to phosphorylation of insulin receptor substrate-1 (IRS-1) which results in downstream phosphatidylinositol 3-kinase (PI3K) activation. Subsequent translocation of glucose transporter type 4 (GLUT4) to the plasma membrane promotes glucose uptake. Accumulation of lipids such as diacylglycerol and ceramide in skeletal muscle is thought to have a negative effect on its insulin sensitivity. For example, a study in healthy male subjects revealed that insulin sensitivity was negatively correlated with muscle total ceramide content [66]. Furthermore, ceramide was reported to have the ability to decrease the translocation of GLUT4 [67]. Importantly, we observed altered intramyocellular ceramides and genes coding key enzymes involved in the *de novo* ceramides synthesis pathway in skeletal muscle from cachectic PDAC patients (**Chapter 4 and Chapter 5**). Since cachectic PDAC patients had a higher intramyocellular lipid content than non-cachectic patients (**Chapter 4**) and considering that the pancreatic tumor organoid secretome suppressed the expression of SLC2A4 (encoding GLUT4) in mature C2C12 myotubes in the presence of fatty acids (**Chapter 5**), tumor factors may induce lipid accumulation in skeletal muscle by impairing metabolic insulin-IRS-1-PI3K-GLUT4 signaling.

In general, intracellular lipid accumulation in skeletal muscle is caused by an imbalance between lipid uptake and oxidation, either increased lipid uptake or decreased lipid oxidation, or both. One of the cancer cachexia-associated features is adipose tissue depletion, which occurs through lipolysis of adipose tissue. As a consequence, the circulating free fatty acids (FFA) flux is increased [68]. It is reasonable to assume that excess circulating FFA “spill over” to skeletal muscle results in more lipid uptake by muscle. In **Chapter 5**, two genes coding for FFA transport proteins (CD36 and FAB4) were slightly albeit non-significantly increased in C2C12 myotubes after exposure to conditioned medium from pancreatic tumor organoids of cachectic patients. Since CD36 promotes FFA transport by rapidly translocating from an intracellular protein pool to the plasma membrane [69], the lack of overt changes in CD36 mRNA expression does not imply that FFA transport is not affected by tumor organoid factors.

Indeed, using real-time fluorescence measurements, a previous study revealed that oleic acid translocation into HEK cells took place in a couple of minutes even without overexpression of CD36 [70]. In general, fatty acid oxidation in the cell is regulated by a set of genes including fatty acid CoA ligase (FACL1/ACSL1), carnitine palmitoyl CoA transferase (CPT1A, CPT2), carnitine acylcarnitine translocase (CACT/SLC25A20), as well as by the peroxisome proliferator-activated receptors (PPARα, PPARGC1A/PGC1A) [71-73]. In normal conditions, cytosolic fatty acids are converted from long-chain fatty acetyl CoA to long-chain fatty acyl-CoA (LCFACoA) by fatty acid CoA ligase (also known as acetyl-CoA synthetase). Subsequently, LCFACoA is transferred to the mitochondrial complex by carnitine shuttle involving 1) Cpt-1, which catalyzes the transesterification of fatty acyl-CoA to acylcarnitine, 2) CACT, which mediates acylcarnitine transports into the mitochondrial matrix, and 3) CPT2, which converts acylcarnitine to acyl-CoA in the inner mitochondrial membrane (Figure 1). Finally, acyl-CoA enters β-oxidation in the mitochondrial matrix [73]. In our study, several genes related to fatty acid oxidation were suppressed in myotubes after exposure to CM from pancreatic tumor organoids of cachectic patients (**Chapter 5**). This suggests that the pancreatic tumor organoid secretome decreases fatty acid oxidation, thereby contributing to lipid accumulation in myotubes. A previous *in vitro* study also showed that conditioned medium from pancreatic cancer cells inhibits oleic acid oxidation [74], which strengthens our observation. In addition, decreased mRNA expression of PPARα and PPARGC1A was found in the C2C12 myotubes after exposure to conditioned medium from pancreatic tumor organoids of cachectic patients. These two transcription factors are known to regulate the expression of fatty acid oxidation genes and are involved in mitochondrial biogenesis [75, 76]. Taken together, these data indicate that factors released by pancreatic tumor organoids derived from cachectic patients may impair fatty acid oxidation and mitochondrial biogenesis resulting in lipid accumulation in skeletal muscle.

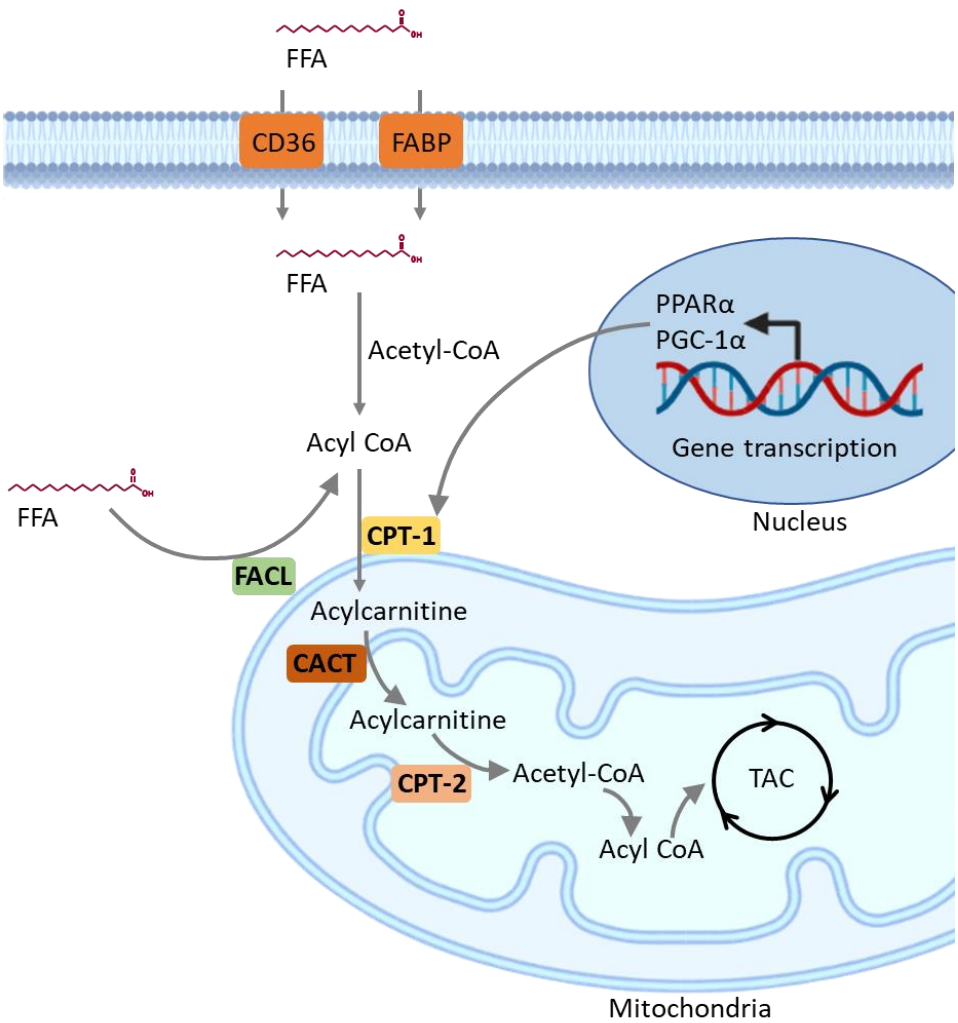

Figure 1: Schematic representaion of fatty acid oxidation in skeletal muscle

Concluding remarks and future perspectives

To date, the underlying mechanisms of cancer cachexia remain to be elucidated. In this thesis, we investigated several potential mechanistic factors in pancreatic cancer patients with or without cachexia, focusing on complement system activation, neutrophil activation, and myosteatosis. Our results showed that 1) systemic inflammation in pancreatic cancer patients is associated with complement system activation; 2) elevated circulating LCN-2 in pancreatic cancer patients with cachexia is associated with neutrophil activation. Systemic inflammation

in these patients might be related to a positive feedback loop involving both complement system and neutrophil activation; 3) neutrophil-released LCN-2 may contribute to cancer cachexia by affecting patients' nutritional status; 4) pancreatic tumor organoid factors promote lipid accumulation in myotubes. Taken together, this thesis expanded our understanding of the mechanisms underlying cancer-associated cachexia, moving towards the development of novel treatment strategies for cancer patients with cachexia.

Some specific suggestions for follow-up studies will now follow. In our study, an increased circulating complement fragment C3a was observed in cachectic patients with inflammation, but not in cachectic patients without inflammation, as compared to non-cachectic patients. Future studies investigating the source of activated complement fragments in cachectic patients with inflammation are needed. Gut barrier dysfunction is often seen in models of cancer cachexia and cachectic cancer patients [77, 78], leading to intestinal permeability. Increased intestinal permeability allows the entrance of microbiota-derived pathogen-associated molecular patterns into the bloodstream, which are related to activation of innate immunity and the complement system [79, 80]. Therefore, gut barrier dysfunction could contribute to the increased circulating activated complement fragments in cachectic patients with inflammation. Alternatively, adipose tissue is a rich source of complement proteins. Therefore, future studies to determine complement protein levels in fat and their metabolic effects in adipose tissue in cancer cachexia should be encouraged. In addition to their function in the innate immune response against foreign pathogens, complement proteins have been shown to promote cancer metastasis [81]. Direct assessment of complement protein levels and their activation state in the tumor biopsy from cachectic patients may provide deeper insights into the contribution of complement proteins to the process of cachexia in patients with advanced cancer. Though a close relationship between systemic inflammation, complement system activation, and neutrophil activation was observed in PDAC patients, which is cause or consequence remains unclear. *In vitro* and *in vivo* loss- and gain-of function studies may answer this vital question.

Neutrophil-derived LCN-2 has been shown to cross the blood-brain barrier and bind to MC4R in the hypothalamus, resulting in appetite suppression in mice [62]. In our patient cohort, no significant difference in circulating LCN-2 levels was observed between PDAC patients with

normal food intake and PDAC patients with less food intake. Of note, we could not study MC4R activity in those patients. Correlation analysis of patients' circulating LCN-2 levels and food intake, as well as investigation of MC4R activity in human hypothalamic cells after exposure to LCN-2 *in vitro* might help understanding the role of LCN-2 in regulating appetite.

Intramyocellular lipid species alterations and distribution in the process of cachexia were identified by using LC-MS/MS-based lipidomics and MALDI-MSI. Several intramyocellular lipid species belonging to the glycerolipids, glycerophospholipids, and sphingolipids classes showed a significant difference between cachectic patients with inflammation and non-cachectic patients. Studying the biological functions of these altered lipid species should be a high priority in future studies. Another important question that should be addressed concerns the source of the altered intramyocellular lipid species in cachectic patients. Lipidomics analysis of plasma or serum from pancreatic cancer patients with or without cachexia should be encouraged in this context.

Within human myofibers, oxidative (type 1) and nonoxidative (type 2) types can be distinguished according to their myosin heavy chain isoform expression. Increased type 2 myofibers have been reported in cachectic patients [82]. Given that type 1 myofibers and type 2 myofibers differ with respect to their metabolic properties [83], and since increased intramyocellular lipid accumulation was observed in cachectic patients, application of novel MALDI-MSI with immunohistochemical stainings may be a promising approach to explore fiber type-specific lipids and lipid metabolism in cancer cachexia. In addition, reprogramming of lipid metabolism in cancer cells has been shown to support tumor progression [84], and lipid profiling of tumors from cachectic or non-cachectic patients could therefore be interesting for future studies.

In combination with patients' clinical records and data on cachexia-associated phenotypes, pancreatic tumor organoids are an excellent model for future studies of the mechanisms driving cancer-associated cachexia. For instance, the cross-talk between tumors and host organs like adipose tissue, liver, and gut could be studied by using a multi-organoid-on-a-chip system [85]. Likewise, co-culture systems of tumor organoids and immune cells will be of benefit to study tumor immunology and its link with cancer cachexia. In this context, our

ongoing studies show that tumor-derived factors from cachectic patients promote M1 macrophage polarization and enhance macrophage phagocytosis rates. Further studies of macrophage functionality such as mitochondrial respiration, lipid uptake, and reactive oxygen species generation are foreseen. Finally, application of patient-derived organoids as a model for cancer drug discovery and personalized therapy could be a promising approach to improve cancer treatment of cachectic and non-cachectic patients.

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Summary

Cancer cachexia is a multifactorial and devastating syndrome that is associated with poor survival of patients. The common features of cachexia include significant loss of body weight (both skeletal muscle mass and fat mass), elevated systemic inflammation, anorexia, nausea, and fatigue. Another aspect of cachexia that is strongly predictive of patient survival is myosteatosis, which refers to the accumulation of ectopic fat within muscle tissue. Cancer cachexia affects 50%-80% of cancer patients and directly causes 20% of cancer-associated deaths. Among cancer patients, cachexia is highly prevalent in those with pancreatic cancer (up to 80%), followed by patients with gastro-oesophageal cancer, head and neck cancers, and lung cancer. Despite significant advances in cancer treatment in the past decade, no practical guide for early diagnosis of pre-cachexia and no effective treatment for cancer cachexia has been developed and implemented in clinical practice. Studies in mouse models of pancreatic cancer cachexia have shown that several proinflammatory cytokines such as IL-6, TNF-α, and IL-α promote muscle wasting. However, the neutralization of a single cytokine is unlikely to be effective against cancer-associated cachexia in patients, and more insight into the role of the respective branches of the immune system in cachexia progression is required to develop an effective treatment. Furthermore, the mechanisms leading to the development of myosteatosis remain poorly characterized. To address these issues, complement system activation, the role of neutrophil-derived lipocalin, and myosteatosis were studied in the context of pancreatic cancer cachexia.

The complement system was discovered more than 100 years ago. It is an ancient key component of the innate immune system, playing a vital role in host defense against infection. Several studies have shown that aberrant complement system activation is also associated with inflammatory conditions such as rheumatoid arthritis, renal diseases, chronic neurodegenerative diseases, as well as cancer [1-4]. In the context of cancer cachexia, the complement system has received little attention. It is well described that systemic inflammation is a hallmark of cancer cachexia and the complement system has been shown to contribute to metabolic inflammation. In **Chapter 2**, we hypothesized that systemic inflammation in patients with cancer cachexia was associated with complement activation. The levels of circulating complement factors including C1q, mannose-binding lectin (MBL), C3a, and terminal complement complex (TCC) were determined in pancreatic cancer patients

without cachexia and in cachectic patients with or without systemic inflammation (as defined by a CRP levels >10 mg/mL). We observed that systemic C3a levels were higher in cachectic patients with inflammation as compared to patients without inflammation or without cachexia. Accordingly, TCC concentrations gradually increased in these patient groups. C3a and TCC concentrations were strongly correlated. Although concentrations of C1q and mannose-binding lectin did not differ between groups, C1q levels were correlated with both C3a and TCC concentrations. Altogether, in this study, we revealed that systemic inflammation in patients with cancer cachexia is associated with the activation of key effector complement factors. Moreover, the correlations between C1q and C3a/TCC suggested that the classical complement pathway could play a role in complement activation in patients with pancreatic cancer cachexia.

In **Chapter 3**, we explored the link between neutrophil activation and cachexia, focusing on neutrophil-released LCN-2, and we assessed whether LCN-2 levels were associated with appetite and nutritional status of patients with pancreatic cancer. A set of circulating neutrophil activation markers including calprotectin, myeloperoxidase (MPO), elastase, and bactericidal/permeability-increasing protein (BPI) was determined in PDAC patients in relation to cachexia and LCN-2 levels. Our results showed that cachectic patients with high systemic LCN-2 levels had higher concentrations of calprotectin, myeloperoxidase, and elastase than non-cachectic patients or cachectic patients with low LCN-2 levels. Systemic inflammation (defined by the CRP/albumin ratio) was also higher in cachectic patients with high LCN-2 levels as compared to non-cachectic patients or cachectic patients with low LCN-2 levels. Spearman correlation analysis revealed a significantly positive correlation between systemic LCN-2 levels and those of neutrophil activation markers calprotectin, myeloperoxidase, elastase, and BPI. Given that complement factors such as C3a and C5a have the ability to trigger and amplify neutrophil activation, and since we reported complement system activation in pancreatic cancer patients with cachexia (Chapter 1), we further extended the correlation analysis between complement factors and neutrophil activation markers in our study cohort. Importantly, we observed a positive correlation among these variables. This suggests that complement activation may underlie neutrophil activation in pancreatic cancer cachexia.

To investigate whether LCN-2 was associated with appetite, we compared systemic LCN-2 levels in pancreatic cancer patients with normal food intake and reduced food intake as assessed by validated questionnaires. No significant difference in systemic LCN-2 between the groups was observed. However, borderline significantly (p=0.00578) elevated LCN-2 levels were observed in severely malnourished PDAC patients. In conclusion, different from the recently reported effect of appetite suppression by LCN-2 in a mouse model of pancreatic cancer cachexia, the results of our study do not support that systemic LCN-2 contributes to cachexia by suppressing appetite in pancreatic cancer patients. In contrast, our data do support that LCN-2 is released by activated neutrophils in these patients.

Low muscle mass (sarcopenia) is one of the criteria for the diagnosis of cancer cachexia which has been well studied in the past decade. However, the mechanisms underlying poor muscle quality (myosteatosis) and its impact in the context of cachexia received comparatively less attention. In **Chapter 4**, we investigated whether intramyocellular lipid accumulation in pancreatic cancer patients is associated with inflammation and other defining aspects of cancer cachexia, and identified the types of intramyocellular lipids in patients with cancer cachexia and their distribution. Body composition was analyzed by using L3-CT scans. Rectus abdominis muscle biopsies were collected during surgery from PDAC patients for muscle morphology, lipidomics, and qPCR analyses. We observed that cachectic patients with inflammation had significantly lower muscle radiation attenuation as compared to those without inflammation or weight loss, reflecting increased lipid accumulation in the muscle of those former patients. In line with this, intramyocellular lipid content was lower in patients without cachexia as compared to those with cachexia with inflammation or without inflammation. Although the expression of muscle atrophy-related genes did not differ significantly among the studied groups, a notable leftward shift in the frequency of smaller muscle fibers was observed in cachectic patients with inflammation. Untargeted lipidomics analyses revealed alterations in intramyocellular lipid classes and species in pancreatic cancer patients without cachexia compared with cachectic patients with or without inflammation. In particular, a higher relative abundance of intramyocellular glycerophospholipids and a lower relative abundance of intramyocellular glycerolipids were found in cachectic patients with inflammation, as well as certain elevated ceramides species. In addition, genes coding for

enzymes involved in *de novo* ceramides synthesis such as SPT1/2, KDSR, Cers1-2, Cers4-6, and DEGS1 tended to show an increased expression in the skeletal muscle of cachectic patients with inflammation. We were not only able to determine the levels of intramyocellular lipid species but could also visualize the altered intramyocellular lipid species such as PC (34:1), PC(33:2), and TG (48:1) by using mass spectrometry imaging. Taken together, these findings indicate that patients with cachexia exhibit intramyocellular accumulation of specific lipid species that may be partly related to elevated ceramide synthesis.

Tumor-derived factors are known to play a key role in driving the progression of cancer cachexia. However, whether tumor-derived factors have direct actions promoting lipid accumulation in skeletal muscle during cancer cachexia remains uncertain. Therefore, in **Chapter 5**, we investigated the effect of the human pancreatic tumor organoid secretome on lipid accumulation in C2C12 myotubes. Pancreatic tumor organoids were established from six PDAC patients (three with cachexia, three without cachexia), and conditioned medium (CM) was collected from these tumor organoids. We exposed the differentiated C2C12 muscle cells to 50% CM plus fatty acids for 8 hours. Lipid accumulation in myotubes was assessed by Oil-red O staining and live cell imaging. LC-MS/MS-based lipidomics was performed to determine global lipid changes in myotubes after treatment. Lipid metabolism-related genes were analyzed by RNA sequencing. CM from pancreatic tumor organoids of cachectic patients caused significant lipid accumulation in differentiated C2C12 from 6 hours onward, which was not seen with CM from organoids of non-cachectic patients or control medium. We observed that the pancreatic tumor organoid secretome induced alterations in intramyocellular lipid species, mainly from the glycerolipids, glycerophospholipids, and sphingolipids classes. Furthermore, several genes related to lipid uptake were upregulated and genes related to fatty acid oxidation were suppressed in C2C12 myotubes after exposure to fatty acids plus CM from human pancreatic tumor organoids of cachectic patients. A trend toward decreases in mitochondrial membrane potential and key genes (PPARGC1A and NRF1) related to mitochondrial biogenesis was observed. Although the pancreatic tumor organoid secretome of cachectic patients tended to decrease myotube fiber diameter, muscle atrophy genes Foxo32 and Trim63 were not altered, and no apoptosis was observed. This result suggested that pancreatic tumor organoid secretomes do not induce muscle atrophy. GO/KEGG

enrichment pathway analyses revealed a significant pathway with biological relevance to cytokine-cytokine receptor interaction in myotubes after exposure to fatty acids plus human pancreatic tumor organoid factors. These results imply that the human pancreatic tumor organoid secretome induces lipid accumulation in C2C12 myotubes. This process may be caused by disruption of lipid metabolism pathways and mitochondria dysfunction. Our findings highlight the important role of factors directly released by pancreatic tumor cells in promoting lipid accumulation in skeletal muscle.

Finally, in **Chapter 6**, the main findings of this thesis are discussed and future perspectives are presented. Altogether, our studies highlighted the important role of inflammation and the factors released by cancer cells and immune cells during the development of cancer cachexia.

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Impact

Relevance

Cancer cachexia is a multifactorial and devastating syndrome characterized by significant body weight loss including loss of skeletal muscle mass and fat mass that cannot be fully reversed by conventional nutritional approaches. Cancer cachexia is associated with muscle weakness, increased systemic inflammation, loss of appetite, increased therapy toxicity, poor quality of life, and reduced survival. Cachexia affects 50-80% of patients and is directly responsible for 20% of cancer deaths. Given that most pancreatic cancer patients present with locally advanced or metastatic disease already at the time of diagnosis due to a lack of symptoms in the early stages, pancreatic cancer patients have the highest prevalence (up to 80%) of cachexia, and experience loss of more than 10% of body weight on average. Although the clinical management and treatment of cancer have been considerably improved in the past decade, no effective treatment for cancer-associated cachexia has been identified. In addition, the mechanisms behind cancer associated-cachexia remain incompletely understood.

However, catabolic mediators released by cancer cells or immune cells appear to play a key role in the development of cancer cachexia. For instance, pro-inflammatory factors IL-6, TNF-α, and IL-1β have been reported to induce lipolysis of adipose tissue and muscle atrophy both *in vitro* and *in vivo*. Although genetic deletion or pharmacologic inhibition of these factors ameliorates muscle wasting in mouse models of cancer cachexia, neutralization of single mediators has not been successful in overcoming cancer-associated cachexia in patients. Therefore, there is an urgent need for a better understanding of the underlying mechanisms of cancer cachexia to develop effective cachexia treatment.

Scientific impact

In this thesis, we first studied the association between systemic inflammation and the central complement factors as well as neutrophil activation markers in pancreatic cancer patients with or without cachexia. We revealed that systemic inflammation in patients with cancer cachexia was associated with the activation of key effector complement factors. Furthermore, a positive correlation between neutrophil activation markers and complement factors C3a and

TCC was observed in pancreatic cancer patients. Based on these observations, future studies should work out 1) the source of circulating complement proteins in cachectic patients; 2) systemic inflammation, complement activation and neutrophil activation, cause or consequence? 3) whether complement proteins are increased in skeletal muscle, adipose tissue as well tumor and what's the biological function of complement protein on these tissues. Secondly, no difference in circulating LCN-2 level was observed between cachectic PDAC patients versus non-cachectic PDAC patients, which differs from a previous study showing a significant higher circulating LCN-2 levels in cachectic mouse vs. non-cachectic mouse. Furthermore, LCN-2 levels of patients with normal versus reduced food intake were not different. In exploring the role of LCN-2 in regulation of appetite, these inter species differences deserve validation. Thirdly, nature and distribution of intramyocellular lipid species were assessed in pancreatic cancer patients with or without cachexia by combinding LC-MS/MS-based lipidomics and MALDI-MSI. This multimodal approach provid a new approach for intramyocellular lipid metabolism research in the cachexia field, whereby differences in special localization of lipids species can be detected. Furthermore, using patient-derived pancreatic tumor organoids, we also showed that the pancreatic tumor organoid secretome promotes lipid accumulation in mature myotubes. The use of patient-derived organoids in co-culture systems may pave the way for future research on tumor-host communication and tumor immunology in cachexia. For instant, to study the effect of tumor organoids secretome on macrophage polarization.

Target groups

In this thesis, we focused on pancreatic cancer cachexia and showed a close relationship between inflammation, complement activation, neutrophil activation, and myosteatosis. The results as described in here can potentially benefit all types of cancer in which cancer cachexia has been proven to play a role. This would include patients with gastro-oesophageal cancer, head neck cancer, lung cancer, colorectal cancer, haematological cancers, breast cancer, and prostate cancer. Myosteatosis has been associated with insulin resistance, aging, obesity, type 2 diabetes [1] as well as poor prognosis in cancer patients [2], and our findings on the possible contribution of pro-inflammatory cytokines on myosteatosis provides novel insights into the

pathophysiological mechanisms underlying myosteatosis which potentially also apply to these other fields. It could stimulate pharmaceutical companies to develop new drugs targeting tumor-derived pro-inflammatory cytokines for the treatment of myosteatosis.

Societal impact

Pancreatic cancer remains one of the most lethal malignancies, with a five-year survival rate of around 5%. The risk factors for developing pancreatic cancer include aging, diabetes, and chronic pancreatitis. Both the incidence and mortality rate of pancreatic cancer continue to increase due to population growth and aging. According to the data from the Global Cancer Observatory, approximately 844,000 new pancreatic cancer cases will be diagnosed in the world in 2040, and pancreatic cancer will lead to about 801,000 deaths worldwide. To date, surgical resection remains the only curative option for patients with pancreatic cancer. However, only 15-20% of pancreatic cancer patients are initially eligible for surgery, and the five-year survival rate for these patients is poor at around 20% following surgery (in the USA). A study in a larger cohort collected between 2001 to 2010 has revealed that total healthcare costs for patients with pancreatic cancer (n=5,262) were higher than for controls (n=15,786) (person/month, $15,480 vs. $1001) [3]. In the same study, the healthcare costs were significantly higher during treatment of the metastatic stage compared to the initial treatment phase of non-metastatic disease ($21,637 vs. $10,358, $p < 0.001$) [3]. In general, cachectic patients had a longer hospitalization stay compared to non-cachectic patients (6 vs. 3 days), which also leads to a higher cost per stay ($4641.30 higher) [4]. Therefore, a better understanding of the mechanisms underlying cancer cachexia as provided in this thesis could help pharmaceutical companies to develop new drugs against cancer-associated cachexia and benefit cachectic patients as well as reduce health care cost.

Anorexia (loss of appetite) is frequently associated with cancer, resulting in progressive weight loss (a key feature of cancer cachexia). A study in both drosophila and mouse tumor models revealed that anorexia could occur earlier than cachexia [5], suggesting that anti-anorexia treatment could be effective against the development of cancer cachexia. Several signals such as GDF-15-GFRAL, Dilp8/IINSL3 (insulin-like 3)-Lgr3/8 (leucine-rich repeat-containing G

protein-coupled receptor 3/8), as well as LCN-2-MC4R have been shown to suppress appetite in patients or experimental models of cancer cachexia. Therefore, the neutralization of these signaling mediators could be a potential therapeutic direction for treating the anorexia-cachexia syndrome. To date, several GDF-15 antibodies including CTL-002, NGM120, and PF-06946860 are tested in clinical trials with cancer patients against the anorexia-cachexia syndrome [6], but there are no approved available treatments for cancer-associated anorexia yet. Our study showed that circulating LCN-2 was not different between PDAC patients with normal and PDAC patients with reduced food intake, which should be noted before pharmaceutical companies develop antibodies to neutralize the LCN-2-MC4R signaling pathway against cachexia-associated anorexia. It is also worth mentioning that a trend toward increasing circulating LCN-2 was observed in malnourished patients. Given that LCN-2 is involved in intestinal and metabolic inflammation, and since gut barrier dysfunction and intestinal inflammation are associated with cachexia progression, it is tempting to speculate that circulating LCN-2 may contribute to development of cachexia by impairing nutritional uptake in the intestine.

One of the underestimated aspects of cancer cachexia is myosteatosis (also known as fat infiltration in skeletal muscle), which is associated with decreased muscle quality and poor prognosis in cancer patients. Although previous studies have shown that intracellular lipid droplets increase with the development of cancer cachexia, the mechanism behind cancer-associated myosteatosis remains poorly understood. In cancer patients, myosteatosis can be assessed by using a CT image at the L3 vertebral level without extra financial burden because CT is commonly used in the clinic for identifying the tumor location in these patients. Therefore, CT-scan-based body composition analysis should be recommended to all cancer patients in the clinic. Patients with myosteatosis have also been associated with increased risk of hospitalizations [7, 8]. A better understanding of the effect of tumor-derived factors as provided in this thesis could help to identify new therapeutic targets for myosteatosis in cancer cachexia.

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List of publications

List of publications

Min Deng, Merel R. Aberle, Annemarie A.J.H.M. van Bijnen, Gregory van der Kroft, Kaatje Lenaerts, Ulf P. Neumann, Georg Wiltberger, Frank G. Schaap, Steven W.M. Olde Damink, Sander S. Rensen. Lipocalin-2 and neutrophil activation in pancreatic cancer cachexia. *Frontiers in Immunology.* 2023, 14: 1286

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Min Deng, Merel R. Aberle, Jianhua Cao, Rianne D.W Vaes, Ron M. A. Heeren, Steven W.M. Olde Damink, Sander S. Rensen. The secretome of pancreatic tumor organoids derived from cachectic patients promotes lipid accumulation in skeletal muscle cells.

Min Deng, Jianhua Cao, Gregory van der Kroft, Merel R. Aberle, Andrej Grgic, David P.J. van Dijk, Ulf P. Neumann, Georg Wiltberger, Benjamin Balluff, Frank G. Schaap, Ron M. A. Heeren, Steven W.M. Olde Damink, Sander S. Rensen. Identification of intramyocellular lipid alterations in human pancreatic cancer cachexia by mass-spectrometry imaging.

Valerie d'Antonio, **Min Deng**, Rianne Vaes, Mo Hadfoune, Steven Olde Damink, Sander Rensen. Tumor organoid-derived factors from cachectic pancreatic cancer patients induce a pro-inflammatory macrophage phenotype – role of macrophage migration inhibitory factor.

Yan Sun[*], **Min Deng**[*], Olivier Gevaert, Shaimaa Hesham Bakr, Merel Aberle, Steven W.M. Olde Damink, Sander S. Rensen. Tumor metabolic activity is associated with myosteatosis and reduced survival in patients with non-small cell lung cancer. ([*]Authors share co-first authorship)

Conference presentations

Min Deng, Jianhua Cao, Gregory van der Kroft, Merel R. Aberle, Andrej Grgic, Ulf P. Neumann, Georg Wiltberger, Benjamin Balluff , Ron M. A. Heeren, Frank G. Schaap, Steven W.M. Olde Damink, Sander S. Rensen. Intramuscular lipid alterations in human pancreatic cancer cachexia. *19th International Medical Ph.D. Conference (2022)* (Oral presentation)

Min Deng, Jianhua Cao, Gregory van der Kroft, Merel R. Aberle, Andrej Grgic, Ulf P. Neumann, Georg Wiltberger, Benjamin Balluff , Ron M. A. Heeren, Frank G. Schaap, Steven W.M. Olde Damink, Sander S. Rensen. Intramuscular lipid alterations in human pancreatic cancer cachexia. *the 15th International Conference on Cachexia, Sarcopenia and Muscle Wasting*, Portugal, 2022 (Oral presentation)

Min Deng, Merel R. Aberle, Annemarie A.J.H.M. van Bijnen, Gregory van der Kroft, Kaatje Lenaerts, Ulf P. Neumann, Georg Wiltberger, Frank G. Schaap, Steven W.M. Olde Damink, Sander S. Rensen. Elevated systemic lipocalin-2 levels in cachectic patients are associated with

neutrophil activation. *the 15th International Conference on Cachexia, Sarcopenia and Muscle Wasting*, Portugal, 2022 (Poster presentation)

Min Deng, Rianne D.W Vaes, Annemarie A.J.H.M. van Bijnen, Steven W.M. Olde Damink, Sander S. Rensen. Activation of the complement system in patients with cancer cachexia. *Symposium Host defense against infection: Complement and beyond*, the Netherlands, 2022 (Poster presentation)

Min Deng, Jianhua Cao, Gregory van der Kroft, Merel R. Aberle, Andrej Grgic, Ulf P. Neumann, Georg Wiltberger, Benjamin Balluff , Ron M. A. Heeren, Frank G. Schaap, Steven W.M. Olde Damink, Sander S. Rensen. Intramuscular lipid alterations in human pancreatic cancer cachexia. the Dutch Translational Metabolism Conference, the Netherlands, 2022 (Poster presentation)

Min Deng, Rianne Vaes, Steven W.M. Olde Damink, Sander S. Rensen. The human pancreatic tumor organoid secretome suppresses macrophage mitochondrial respiration without affecting macrophage function. *the 13th International Conference on Cachexia, Sarcopenia and Muscle Wasting*, online, 2020 (Poster presentation)

Curriculum vitae

Min Deng was born on December 20th, 1993 in Jiangxi province, China. After graduating from high school, he studied bioscience at Nanchang University and obtained his bachelor's degree in June 2015. During his bachelor's study, he learned transcriptional data analysis. In September 2015, he started Botany at Nanchang University under the supervision of prof. Liping Luo. During his master's study, he developed a method to detect residues of multiple pesticides simultaneously, quickly, and efficiently in honey using a modified neutral desorption-extractive elective ionization mass spectrometry (ND-EESI-MS) without any sample pretreatment. He owned 2 patents and his master's thesis "Detection of pesticide residues in honey based on ND-EESI-MS and identification of syrup adulteration by MPT-MS" was awarded an Excellent Master's Thesis of Jiangxi Province in 2019. After 3 years of study, he received his Master's degree in June 2018 and he was awarded the title of outstanding graduate when he graduated. In the same year, he was awarded a national grant from China Scholarship Council and started his PhD training at the Department of Surgery of Maastricht University under the supervision of prof. Steven Olde Damink and Dr. Sander Rensen. During his PhD trajectory, he focused on studying the innate immune system activation in pancreatic cancer patients with cachexia and the effects of the pancreatic tumor organoid secretome on macrophage polarization and skeletal muscle lipid accumulation. The results of his PhD works are presented in this thesis. After his PhD defense, he will continue his work in the field of cancer cachexia as a postdoctoral researcher at the Department of Molecular Cell Biology, Oslo University Hospital, and at the Department of Pediatric Research, Oslo University Hospital. His research will focus on elucidating multi-organ interactions in cancer cachexia by using human organoid models with -omics and bioinformatical approaches.