The selfish tumour

Cachexia-associated changes in skeletal muscle

Miranda van der Ende
Propositions

1. Finding an appropriate control group to study early development of cachexia is one of the greatest challenges when investigating this progressive condition in humans. (this thesis)

2. Cytokines released by tumours only play a marginal role in the direct induction of muscle wasting. (this thesis)

3. Competition kills early data sharing.

4. Despite many initiatives, publishing negative outcomes or failed experiments is still hardly valued in the scientific world.

5. Being a dyslectic scientist presents challenges, but also offers advantages.

6. Better science education will reduce fear for new developments like SARS-CoV-2 vaccine.

Propositions belonging to the thesis, entitled

"The Selfish Tumour: Cachexia associated changes in skeletal muscle."

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The selfish tumour

Cachexia-associated changes in skeletal muscle

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Chapter 1

General Introduction
Cancer cachexia – definition, prevalence and consequences

Cachexia is a syndrome that is characterised by disease-induced loss of muscle mass with or without loss of fat mass [1, 2]. It can occur in patients with chronic inflammatory diseases, for example kidney disease, heart failure, Chronic Obstructive Pulmonary Disease (COPD) and cancer [3]. Anorexia, inflammation, insulin resistance and increased muscle protein breakdown are frequently associated with cachexia. As a result, cachexia is strongly linked with increased morbidity and mortality [1, 2]. In its aetiology and development, it is distinct from muscle loss due to starvation, ageing, malabsorption, and hyperthyroidism. Although between diseases symptoms of cachexia show a high degree of similarity, underlying mechanisms and outcomes may differ. In line with this, various forms of cancer and anti-cancer treatments differ in the extent to which they induce muscle wasting [4, 5].

In the Western world about 30% of all individuals with cachexia are cancer cachexia patients [6]. Moreover, up to 80% of the patients with advanced stage cancer suffer from cachexia. Of the European population about half of the colorectal cancer patients develop cachexia, while 80-90% of the lung and pancreas cancer patients are at risk to develop the syndrome [7]. Cachectic cancer patients suffer from fatigue, depression, anxiety, reduced appetite and declining mobility resulting in reduced physical activity, impaired ability to perform daily activities and, consequently, a decreased quality of life [8, 9, 10, 11, 12]. This wide array of symptoms are often variable and largely experienced subjectively and therefore there is a need for useful diagnostic criteria.

In recent years the diagnosis of cachexia has been debated extensively. For cancer, the best accepted definition of cachexia comprises a set of criteria as follows: “a loss of lean tissue mass, involving a weight loss greater than 5% of body weight in 12 months or less in the presence of chronic illness, or as a body mass index (BMI) lower than 20 kg/m²” [2]. Using these criteria patients will be diagnosed when they show weight loss of >5% over the past 6 months OR when their BMI is lower than 20 with >2% weight loss OR when their appendicular skeletal muscle index is low [2]. Some refinement has been made by combination of these diagnostic criteria with information on either the presence of anorexia and inflammation [13], or the presence of five different physiological factors to come to a refined cachexia score (CASCO) [14, 15]. The latter criteria are useful to characterize patients at risk for cancer-induced cachexia, who do not classify as cachectic based on BMI or percentage of weight loss alone.

However, in all these criteria loss of body weight is the leading indicator, while it has become apparent that specifically the loss of skeletal muscle mass and function contributes to the increased morbidity and mortality. Therefore, the definition does not cover the whole spectrum of cancer patients with muscle mass and function loss. Specifically, the population of obese patients
with decreased muscle mass will not be detected with these criteria. A high body fat mass is often seen in certain forms of cancer, in particular during the initial stages. A high BMI is for example associated with a higher incidence of colon cancer [16, 17]. These patient groups should be diagnosed for muscle mass and function loss. Similarly, severe depletion of skeletal muscle, measured using CT scan data, is a significant predictor of negative treatment outcomes in metastatic breast cancer patients with a high BMI [18, 15]. This effect is described to be even more pronounced in obese patients [18, 19, 20], underlining the importance to look beyond BMI in cancer patients, for example by using CT scan data [21]. In conclusion, cancer cachexia is a syndrome with a large impact, of which the diagnosis is complicated as it is a complex syndrome in which loss of muscle mass and function is underdiagnosed.

Molecular mechanism of cancer cachexia and treatment

In cancer cachexia the crosstalk between different organs plays an important role [22, 23, 24]. Next to the tumour (Figure 1.1), brown adipose tissue has been reported in animal models to contribute to fat burning and therefore weight-loss [25]. Breakdown of white adipose tissue was shown to contribute to insulin resistance and therefore to muscle breakdown [26]. Next to that, the heart is a muscle itself which in a late stage of cancer cachexia has been reported to manifest with cancer-induced muscle mass loss [27]. A dysfunctional heart contributes to hypoxia, which on its turn contributes to muscle mass loss. For the gut, findings from animal models indicate that tumour-induced intestinal permeability can contribute to inflammation, which further amplifies cachexia [28]. Moreover, tumour-induced inflammation can activate the liver to produce acute phase proteins, which reduces the amount of amino acids available for the muscle [29]. The tumour-induced changes in the different organs change many cellular, inflammatory and endocrine processes, which, in turn, induce changes in skeletal muscle (Figure 1.1). In this thesis, the focus will be on the mechanisms taking place in the cachectic skeletal muscle and its response to the presence of a tumour. Different processes in skeletal muscle which play a role during cachexia include: 1) an imbalance between protein synthesis and breakdown; 2) structural remodelling of the myofibre; 3) hampering of muscle degeneration and regeneration; and 4) alteration of mitochondrial functioning [30].

First, the imbalance between protein synthesis and breakdown is in itself also caused by different processes. For example, the PI3K/Akt/mTORC1 pathway, which contributes to muscle protein synthesis, has been reported to be inhibited in cancer cachexia [31]. On the other hand, the suppression of mTORC1 which removes the inhibition of autophagy could prevent the loss of muscle mass [31, 32]. Additionally, there are three pathways that contribute to increased muscle protein breakdown: the lysosomal system, cytosolic proteases and the ubiquitin (Ub)-proteasome pathway [33]. Second,
Figure 1.1: Graphical representation of the organs and processes that may contribute to skeletal muscle wasting in cachexia. Next to the tumour (which is in the top centre) brown adipose tissue, white adipose tissue, brain, heart, gut and liver all play a role in the crosstalk. Loss of skeletal muscle mass and function can be considered the result of changes in the following processes: 1) Impairment of the net protein balance; 2) Structural remodelling of the myofibre; 3) Degeneration and regeneration; 4) Mitochondrial dysfunction.

Structural remodelling of the muscle tissue regarding fibre type ratio is also a feature of cancer cachexia. What exactly happens is still under debate as some research shows a switch from type I (slow) to type II (fast) muscle fibres [34, 35, 36]. Others show a predominant wasting of the type II fibres [37, 38], but also no selective loss of a specific muscle fibre type has been found [39, 40]. Third, skeletal muscle degeneration and regeneration is affected by cancer cachexia [41]. Degeneration was found to be increased in a mouse model of cancer-induced cachexia [42] while regeneration, also known as myogenesis, is inhibited during cachexia [43, 44].

The last of the four processes mentioned above is the altered muscle mitochondrial functioning during cachexia [30]. A primary function of
mitochondria is the generation of cellular energy in the form of adenosine-tri-phosphate (ATP) from energy substrates, especially carbohydrates, fatty acids and amino acids. Carbohydrates are principally converted to pyruvate in glycolysis, which generates ATP and is a high-capacity low efficiency process to generate ATP. Pyruvate then enters mitochondria and is converted into acetyl-CoA by pyruvate dehydrogenase [45]. Acetyl-CoA is converted in the tri-carboxylic acid cycle (TCA cycle, also known as citric acid cycle or Krebs cycle), where the energetic substrates NADH and FADH2 are produced [46]. These energetic substrates can also be generated from fatty acids, via beta oxidation, or amino acids. NADH and FADH2 feed oxidative phosphorylation (OXPHOS), which generated a proton gradient over the impermeable mitochondrial inner membrane, establishing an electrochemical gradient that drives the production of ATP from ADP and inorganic phosphate (Figure 1.2). The OXPHOS machinery consists of several complexes; NADH:ubiquinone reductase (complex I), ubiquinone or coenzymeQ (CoQ), quinol-cytochrome c reductase (complex III), cytochrome C (CytC) and cytochrome c oxidase (complex IV), which transfer electrons and establish the proton gradient which is used by F0F1 ATP-synthase (Complex V) [47]. The proton gradient is used to synthesize ATP. The TCA cycle protein succinate-Q oxidoreductase (complex II) and the beta-oxidation protein Electron-transferring-flavoprotein dehydrogenase (ETF-DH) can also feed OXPHOS by transfer of electrons via CoQ.

![Figure 1.2: Different complexes of the oxidative phosphorylation (OXPHOS). I = NADH:ubiquinone reductase (complex I). II = succinate-Q oxidoreductase (complex II). CoQ =ubiquinone or coenzymeQ. III= quinol-cytochrome c reductase (complex III). CytC = cytochrome C. IV = cytochrome c oxidase (complex IV). ATP synthase = F0F1 ATP-synthase (Complex V).](image)

Several processes are involved to ensure mitochondrial functioning. To understand the role of mitochondria in cachexia it is important to realise that mitochondria, although often depicted as bean-like structures, form in fact a complex and dynamic network throughout the cell. If part of the mitochondrial
system is damaged, it can be separated from the system (fission) and be removed by mitophagy to form an overall better functioning system [48]. Fission of mitochondria is also needed for their transport within a cell to locations where energy is needed, where they fuse again. Enhanced fusion of mitochondria may help to boost mitochondrial capacity. Fusion and fission are also important in the regulation of the mitochondrial cellular mass, where mitochondria are newly formed by biogenesis and removed by mitophagy. In this way, a dynamic and healthy mitochondrial content is maintained. Reported higher expression of genes involved in elongation and maturation of the phagophore supports an activation of mitophagy and might indicate an increased recognition and removal of damaged mitochondria [49, 50, 51, 52, 53, 54]. Moreover, literature suggest that gene expression of both genes controlling fission and genes controlling fusion-related processes are downregulated [55, 56, 50, 57]. These data might be interpreted as a decrease in the dynamic character of the mitochondrial system. Taken together, these changes in mitophagy, fission and fusion will result in a reduction of mitochondrial function in cachectic muscle. This is further supported by findings showing a decreased mitochondrial density and mitochondrial oxygen consumption per amount of mitochondrial mass in skeletal muscle during cancer-induced cachexia [58, 59, 60]. While a general picture of mitochondrial dysfunction in cancer cachectic muscle is becoming clearer, more in-depth research is necessary to evaluate the extent of the effects.

The molecular mechanisms mentioned above in the skeletal muscle seem to be crucially involved in cancer cachexia. However, the question remains how these mechanisms are activated. A possible mechanism is inflammation, a process that is generally seen in cancer cachexia [61]. The tumour is thought to secrete pro-inflammatory mediators including cytokines, but also other factors like microRNAs [62, 63, 64]. Many pro-inflammatory mediators are thought to be present in the tumour secretome. Known to be directly secreted by tumour cells are Leukemia Inhibitory Factor (LIF), C-X-C motif chemokine ligand 10 (CXCL10), C-C motif chemokine ligand 2 (CCL2 or MCP1), C-X-C motif chemokine ligand 1 (CXCL1) and Oncostatin M (OSM) [65, 66]. However, this is not a complete overview as a large screening has not been done. Additionally, not only tumour-secreted factors influence the cachectic body, but also molecules that are secreted by immune cells in the direct environment of the tumour. Moreover, the combination of the factors secreted by the tumour and adjacent immune cells activate more distant organs and immune cells [67]. Tumour necrosis factor alfa (TNFα), Interleukin 6 (IL6), zinc-α2-glycoprotein (ZAG, or otherwise known as lipid mobilizing factor), proteolysis-inducing factor (PIF), myostatin, activin but also free fatty acids are suggested to be involved in this tumour-immune induced activation [68].

While TNFα and IL6 are measured in patients suffering from cancer cachexia, it is not known which of these immune signalling molecules or combination of molecules are potential treatment targets [69, 70]. TNFα
inhibitors, for example, are clinically available, however, blocking TNFα is not effective in the treatment of cancer cachexia [71]. In addition, many anti-cytokine treatment studies are performed, but their results do not allow to draw any conclusions or even indications for further research [72]. Next to the difficulties in treating cancer cachexia, it also often adversely affects cancer treatment efficacy, as weight loss and muscle loss are associated with a lower chemotherapy response rate and more post-operative complications [73, 74, 75, 70, 76]. Moreover, surgically removing the tumour does not always mean that the patients regain muscle mass [77]. Cancer cachexia is often associated with fatigue, which might be an indication to intervene using extensively monitored exercise treatment. However, normal exercise routines can be quite challenging for cachectic patients due to the fatigue. Therefore, low-intensity endurance exercise might be more effective in preventing cancer cachexia–induced muscle atrophy [78]. For COPD, there is already an evidence-based treatment combination of exercise and dietary adjustments [79, 80, 81]. For cancer patients, however, the available evidence supporting such an approach is still limited. At the same time, there is increasing consensus between experts that cachexia should be treated with a multimodal approach addressing dietary composition and intake, attenuating systemic inflammation, and stimulating physical activity by using a combination of drug(s), nutrition and therapeutic exercise [82, 12, 83, 84, 85]. Additionally, studies have shown that treatment of cancer induced cachexia is more effective when initiated at an early stage [86, 87, 88]. Unfortunately, the existing evidence underlying multimodal strategies is still considered to be too little and the current ASCO (American Society of Clinical Oncology) guideline recommends dietary counselling only, given the lack of evidence for pharmacological interventions [89]. More research is necessary to find out which molecules are present in the tumour secretome and what the effects of these molecules are. This apparent knowledge gap is partly caused by the fact that a lot of the data on molecular mechanisms and treatment options are obtained from models using only a specific type of cancer. Animal models can be useful, for example in an exercise study, which is not possible to perform blinded in humans. Other motives for in vitro or animal studies are the need to study multiple organs or testing a hypothesis in an isolated model. Therefore, the use of different models play an important role in the search for the mechanisms behind cancer cachexia.

Models of cancer cachexia

More research is necessary to unravel cachexia mechanisms and to develop treatment strategies. Studies in humans are preferred to study cancer cachexia, but these need to be designed properly involving the appropriate controls. In cancer studies often weight stable patients are used as controls [90, 91, 92, 93]. However, such patients may be weight stable, but this does not preclude that the processes that will lead to cachexia have already started,
as animal studies indicate extensive transcriptomic alterations concurrent to muscle wasting [94]. Therefore, cancer free controls should be used in human cachexia studies. When human participants are used not all organs or tissues can be analysed and in some studies, using for example exercise or nutrition interventions, it is impossible to blind the study for the participants. Next to this, isolated mechanisms cannot studied or a knockdown of a specific gene cannot be introduced in human participants. Therefore, different models for cancer cachexia research are in use: \textit{in vivo} animal models and \textit{in vitro} cell models. These models have several advantages: blinding can be applied much easier, all organs can be studied and isolated processes can be explored. Therefore, next to a patient study, a mouse model and an \textit{in vitro} model are used in this thesis to better understand cancer cachexia.

In many mouse models cancer cells are injected either in the flank of the animal or at the location at which the tumour is normally formed. Subsequently, the tumour will grow and the animal will start to develop cachexia. Cells that are often used to create a cachectic model are the colon 26 carcinoma cells (C26). The C26 mouse model will have progressive loss of skeletal muscle mass [95, 96]. Lewis lung carcinoma derived cells (LLC) also induce cachexia in a mouse model, but unlike C26, these cells are derived from a lung tumour instead of a colon tumour [96]. Lastly, KPC and 4662 are both murine pancreatic ductal adenocarcinoma-derived cell lines, they were relatively recently developed and can also be used in a mouse model [97, 98]. These animal models are established in cachexia research, and a lot of data is already available. Other types of models consist of, for example, orthotopically-placed tumour cells derived from a human tumour [99, 100]. A disadvantage of this model is that mice with a reduced immune capacity are needed as hosts, because otherwise the injected cells would be immediately rejected. Apart from the models where tumour cells are implanted in animals there are also genetic models (Apc\textsuperscript{Min/}+ mice) [101]. Next to mouse models, rats are used to study cancer cachexia [100]. Although many animal models are at hand, their use is not always to be preferred because of the animal burden, limited sample size and labour intensiveness.

An option to study cachexia mechanistically and reduce the use of animal models is by using \textit{in vitro} models. These models have the advantage that they can be used to study the direct effects of tumour cells on muscle cells. One model that is often used for \textit{in vitro} muscle tissue are the murine C2C12 myoblasts/myotubes (C2C12). Differentiation of C2C12 myoblasts into myotubes can be studied [102], which reflects muscle growth and regeneration. Morphology, enzyme activity, gene or protein expression can be analysed. Among the most important readout parameters to analyse C2C12 differentiation are: the number of myotube nuclei, myotube length, expression of key myogenesis markers or creatine kinase (CK) activity. Creatine kinase is an enzyme that plays a role in the regulation of the concentration of ATP within a cell. Cells that have high ATP requirements have higher levels of CK [103]. Furthermore, global gene expression analysis of the C2C12 will result in information about differentially regulated pathways after exposure to different
culture conditions. Using either a co-culture or by exposing C2C12 myoblasts to a conditioned cell culture medium of the cachexia-inducing cancer cell lines, cachexia can be mimicked in vitro [104, 105]. These models are suitable for screening, as experiments do take relatively little time and can be performed in parallel. The drawback is that there is a discrepancy between these in vitro models and the in vivo situation and therefore an ex vivo model could bridge the gap. However, more research will be necessary to establish such a model, and particularly methods to confirm if cachexia is successfully induced.

Thesis outline – Knowledge gaps and research questions

Understanding the molecular mechanism of cancer cachexia is important, because there are many remaining knowledge gaps. To this end the focus of this thesis, which is named: ‘The selfish tumour: Cachexia-associated changes in skeletal muscle’ was on the following questions:

1. To what extent is skeletal muscle in the C26 cachexia mouse model responsive to whole-body vibration training?
   (a) What are the effects of whole-body vibration training on muscle functional parameters in the C26 cachexia model?
   (b) How are these effects manifested at the level of muscle gene expression?

2. What are the effects of conditioned media (CM) of different cachexia-inducing cancer cell lines on differentiating skeletal muscle cells and what is the role of individual mediators of the CM?
   (a) Can cachexia-inducing cancer cell CM hamper C2C12 myotube differentiation in vitro?
   (b) Which specific immune signalling molecules are present in CM of different cachexia-inducing cancer cell lines?
   (c) Is it possible to create a mixture of immune signalling molecules that mimic the effects of the CM on C2C12 myoblast/myotubes?

3. How is mitochondrial functioning in skeletal muscle changed during cancer cachexia?
   (a) How do published results compare on the level of gene (genome wide and single genes) and protein expression on the topic of muscle mitochondria in cancer cachexia?
   (b) What are the effects of CM of different cachexia-inducing cancer cell lines on mitochondrial gene expression in differentiated C2C12?
(c) How do the gene expression data of the in vitro model and publicly available in vivo data compare on cancer cachexia-induced changes in mitochondrial gene expression?

4. Can the skeletal muscle gene expression results be confirmed in cancer patients?

(a) Can we set up a protocol to study gene expression in skeletal muscle tissue of cancer patients?

(b) Is it possible to explore preliminary data of included cancer patients?

Research questions 1 a and b were dealt with in chapter 2, in which the potential of low-intensity exercise in a cachectic C26 mouse model was explored. Whole-body vibration training was used as low-intensity exercise. Body parameters and ex vivo muscle force were measured as well as gene expression in skeletal and heart muscle. To investigate research questions 2 a, b and c, a study was designed in which C2C12 myoblast/myotubes were incubated with conditioned media of cachexia-inducing cancer cell lines with a different origin. Molecular analysis of the C2C12 cells was done and the results will be discussed in chapter 3. Additionally, the concentration of many immune signalling molecules was measured and the effects of a mixture containing the measured molecules on the C2C12 cells were analysed. To answer research question 3 a, the literature about the known role of skeletal muscle mitochondria during cancer cachexia and published genome-wide expression data was explored in chapter 4. This data was bundled and resulted in information on mitochondrial biogenesis, fission and fusion, mitophagy and function. Next, for research questions 3 b and c the role of the mitochondrial functioning in skeletal muscle during cancer cachexia was explored in more depth in chapter 5, using the gene expression data from the study described in chapter 3 and using published genome-wide expression mouse cachexic muscle data. The genes that are involved in mitochondrial processes were studied using the MitoCarta 3.0 gene set. To answer research question 4 a human observational study has been designed, this design can be found in chapter 6. In this study, called COMUNEX, a biopsy of the skeletal muscle of colon cancer patients will be collected and compared to control biopsies on gene expression level. Research question 4 b will be answered in chapter 7, in which the preliminary data of the COMUNEX study are presented. Last, in chapter 8 the general discussion summarises the results of this thesis and explores future possibilities for research into mechanisms in muscle cancer cachexia.
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Chapter 2

Effects of whole-body vibration training in a cachectic C26 mouse model

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Abstract

BACKGROUND Targeted exercise combined with nutritional and pharmacological strategies is commonly considered to be the most optimal strategy to reduce the development and progression of cachexia. For COPD patients, this multi-targeted treatment has shown beneficial effects. However, in many, physical activity is seriously hampered by frailty and fatigue. In the present study, effects of whole-body-vibration-training (WBV) were investigated, as potential alternative to active exercise, on body mass, muscle mass and function in tumour bearing mice. METHODS Twenty-four male CD2F1-mice (6-8 weeks, 21.5±0.2g) were stratified into four groups: control, control + WBV, C26 tumour-bearing, and C26 tumour-bearing + WBV. From day 1, whole-body-vibration was daily performed for 19 days (15min, 45Hz, 1.0g acceleration). General outcome measures included body mass and composition, daily activity, blood analysis, assessments of muscle histology, function, and whole genome gene expression in m. soleus (SOL), m. extensor digitorum longus (EDL), and heart. RESULTS Body mass, lean and fat mass and EDL mass were all lower in tumour bearing mice compared to controls. Except from improved contractility in SOL, no effects of vibration training were found on cachexia related general outcomes in control or tumour groups, as PCA analysis did not result in a distinction between corresponding groups. However, analysis of transcriptome data clearly revealed a distinction between tumour and trained tumour groups. WBV reduced the tumour-related effects on muscle gene expression in EDL, SOL and heart. Gene Set Enrichment Analysis showed that these effects were associated with attenuation of the upregulation of the proteasome pathway in SOL. CONCLUSION These data suggest that WBV had minor effects on cachexia related general outcomes in the present experimental set-up, while muscle transcriptome showed changes associated with positive effects. This calls for follow-up studies applying longer treatment periods of WBV as component of a multiple-target intervention.
List of abbreviations

AUC  Area under the curve
BMD  Bone mineral density
COPD Chronic obstructive pulmonary disease
CSA  Cross-sectional area
CT   Contraction time
DEXA Dual energy X-ray absorptiometry
dF/dt Rate of change of force
dNGS Diluted normal goat serum
EDL  M. extensor digitorum longus
ELISA Enzyme-linked immunosorbent assay
FDR  False discovery rate
FF   Force Frequency
Fmax Maximal force
GEO  Gene Expression Omnibus
GSEA Gene Set Enrichment Analysis
HBSS Hanks’ Balanced Salt Solution
KEGG Kyoto Encyclopedia of Genes and Genomes
MHC  Myosin Heavy Chain
PBS  Phosphate Buffered Saline
PC   Principal Component
PCA  Principal Component Analysis
RMA Robust Multi-array Analysis
RT   Relaxation Time
SOL  M. Soleus
sPLS-DA sparse Partial Least Squares Discriminant
WBV  Whole body vibration
WGA  Wheat germ agglutinin
Introduction

The cachexia syndrome, among others, characterised by disease-induced loss of muscle mass, can occur in patients with chronic kidney disease, heart failure, Chronic Obstructive Pulmonary Disease (COPD) and cancer [1, 2]. It is associated with reduced treatment efficacy and a reduced quality of life [3, 4, 5, 6]. Although the symptoms of this syndrome show a high degree of similarity for different diseases, the causes are multiple and their contribution to the development of cachexia may differ per disease and treatment trajectory [1, 7]. Moreover, it has been shown that crosstalk between different organs plays an important role, which makes cachexia a complex syndrome that is difficult to treat [8, 9, 10]. There is increasing consensus between experts that cachexia should be treated with a multimodal approach addressing dietary composition and intake, attenuating systemic inflammation, and stimulating physical activity [6, 4]. For COPD, there is already an evidence-based treatment combination of exercise and dietary treatment [11, 12, 13]. For cancer patients, however, less data is available supporting such a treatment.

A systematic review of the effects of physical exercise on muscle mass and strength in cancer patients provided evidence that resistance training, aerobic training, or a combination of these can improve muscle strength [14]. However, coexisting fatigue and frailty are often limiting factors in these patients [15]. Therefore, there is a need for training methods that are easily accessible for frail patients. Vibration training might meet this demand, since it provides an easily accessible and low intensity type of exercise. Patients stand on a vibrating surface, which transmits the vibration from the plate to the patient [16]. In older individuals, positive effects of whole-body-vibration training (WBV) on VO$_2$max and muscle strength have been observed [17]. Moreover, vibration training increased exercise capacity in patients treated for respiratory cancer [18]. Additionally, it was found that resistive vibration exercise could prevent the decrease in mitochondrial respiration during bed rest [19]. However, literature data are scarce, and studies are difficult to compare due to differences in set-up. A meta-analysis reported a beneficial effect on leg muscle strength in elderly [20], whereas a systematic review concluded that there would be only weak proof of efficacy of WBV in elderly [21]. Due to the overall inconsistent results of WBV, it remains unclear whether WBV could be a beneficial intervention to improve muscle mass and function in elderly. However, with a recent Delphi consensus study a more consistent form of reporting WBV studies is at hand [22]. Moreover, WBV has never been thoroughly investigated during disease-driven net catabolic conditions. Therefore, in this study, the effects of WBV alone using a murine cancer cachexia model were investigated.

Effects of WBV in rodents found so far seem promising but have also provided contradicting results. For example, WBV showed to be beneficial in chronic pain, it reduced mechanical and thermal sensitivity in male Wistar rats [23]. In healthy mice, one study has shown that low-intensity WBV can
partially improve muscle contractility, in particular strength and relaxation rates [24]. Although that WBV is having a positive effect in young mice, this effect could not be found in aged mice [25]. Another study demonstrated effectiveness of WBV in suppressing muscle atrophy pathways both in vivo and in vitro. Apart from effects on muscle, WBV is also able to improve bone health in mice and rats [26, 27]. Investigating the effect of vibration training in a mouse model for Duchenne muscular dystrophy showed no effect on bone or muscle improvement [28]. However, no studies were found in literature investigating a condition of cancer-induced atrophy, additionally no Delphi study is performed for animal WBV studies yet. So, the question whether WBV might be able to attenuate muscle wasting during this disease remains unknown.

To this end, the effects of WBV were investigated in a colon-derived-tumour-induced C26 cachexia mouse model, studying cachexia outcomes at different levels, including the muscle transcriptome, markers for bone and muscle function, muscle mitochondrial biogenesis, body composition and muscle performance. It is hypothesized that WBV might be able to attenuate muscle wasting in colon-derived-tumour-induced C26 cachexia mouse model.

Materials and Methods

Tumour model

Twenty-four male CD2F1 mice weighing 20g (6-8 weeks, BALB/c x DBA/2, Charles River, The Netherlands) were individually housed in macrolon type 3 cages with sawdust and tissues as nesting material, in a climate-controlled room (21 °C ± 1 °C) with a 12:12 hour dark-light cycle. Sample size was small because of the explorative nature of this study. Mice had ad libitum access to chow and water. Upon arrival, mice were stratified on body mass and divided into groups of 6 animals (Control [C], Control + whole body vibration training [C+WBV], Tumour bearing [T] and Tumour bearing + whole body vibration training [T+WBV]). Subsequently, mice were allowed to acclimatize for 1 week prior to the start of the experiment. Murine C26 adenocarcinoma cells, kindly obtained from the lab of Donna McCarthy (Ohio State University, USA), were cultured and suspended as described previously [29]. Tumour cells (1×10⁶ cells in 0.2 ml of Hanks’ balanced salt solution (HBSS)) were inoculated subcutaneously into the right inguinal flank of the mice under general anaesthesia (isoflurane/N2O/O2). HBSS was used as sham injection (0.2 ml). This study was conducted in accordance with institutional guidelines for the care and use of laboratory animals established by the Animal Ethics Committee of the University of Wageningen, and all animal procedures related to the purpose of the research were approved under the Ethical license of the national competent authority (registration number 2014075.e), securing full compliance the European Directive 2010/63/EU for the use of animals for
scientific purposes and the ARRIVE guidelines.

**Experimental Design**

From day -5 (D-5) onwards body mass, activity and grip strength were measured daily. On day 0 (D0), an injection with tumour cells or HBSS was given and blood was collected using a tail-vein cut. On D0, D7, D14 and D19, body composition, i.e. lean mass and fat mass, was measured using an EchoMRI Whole Body Composition Analyzer (EchoMRI, Houston, TX, USA). Starting on D1, mice were subjected to a whole-body vibration training (WBV) protocol, similar to previous rodent studies [24, 26, 28, 30], with 15 min of vibration training for 7 days/week, with a frequency of 45 Hz and 1.0 g acceleration.

The vibration platform used was an adjusted commercially available VG Professional (VibroGym, Badhoevedorp, The Netherlands) power plate. Four Plexiglas cages were mounted onto the power plate to enable WBV in 4 mice simultaneously. The power plate was calibrated using accelerometers on all four corners to ensure the frequency and acceleration specifics. On D19, at least 24h after the last training session and after anaesthesia, blood was collected by cardiac puncture and animals were killed. Subsequently, organs and hindlimb muscles were weighted and snap-frozen in liquid nitrogen. Carcass mass was determined as body mass excluding tumour weight. M. soleus (SOL) and m. extensor digitorum longus (EDL) were divided in two parts: one for histology and one for RNA isolation. The EDL and SOL of the other leg were used for *ex vivo* muscle function. Front legs were frozen for bone mineral density (BMD) measurement.

To assure compliance of mice with the WBV, a small pilot was executed prior to the experiment. Three additional control mice were used and apart from the vibration training no other treatment was applied. Mice did not show any signs of aberrant behaviour when exposed to the vibration training. Video recordings of mice subjected to WBV showing the behavioural response to the training are provided in supplemental video 1.

**Grip strength**

Forelimb grip strength was measured daily as previously described [31], using a calibrated grip strength apparatus from Panlab (Cornella, Spain), following the protocol delivered with the equipment. Each day, a set of five maximum effort repetitions was performed. From these five measurements, the average of the middle three measurements was determined. To eliminate measurement variation, grip strength was expressed per mouse as mean grip strength on D15-18 as a percentage of the mean on D-3 to D0.
Daily activity

Physical activity was monitored throughout the acclimatisation and study period starting at D-7, using activity sensors (dual technology detector DUO 240, Visonic; adapted by R Visser, NIN, Amsterdam, The Netherlands) according to an adapted protocol previously described [32]. Sensors translated individual disruption of the infrared beams caused by movements of the animals into arbitrary activity counts. Sensors were mounted above the home cages and connected through input ports and interface to a computer equipped with MED-PC IV software for data collection (MED associates, St Albans, VT, USA). Activity was expressed in counts per half hour. The activities of each 12hr dark cycle were summed to dampen the hour-to-hour variability. To eliminate measurement variation, daily activity was expressed per mouse as mean daily activity on D16-19 as a percentage of the mean on D-3 to D0.

Bone Mineral Density and DEXA

Frozen front legs were used for determination of BMD measured by dual energy X-ray absorptiometry (DEXA) scan, using a PIXImus imager (GE Lunar, Madison, WI, USA).

Blood plasma cytokines and PTHrP

The following cytokines were measured using a mouse cytokine Milliplex bead immunoassay (MCYTOMAG-70K-09, Merck chemicals [33], Amsterdam, The Netherlands): IFN$\gamma$, IL1-$\alpha$, IL4, IL6, IL10, IL15, MCP1, TNF$\alpha$ and VEGF. PTHrP was measured using a quantitative PTHrP enzyme-linked immunosorbent (ELISA) assay kit (SEA819Mu, Cloud-Clone Corp., Uscn Life Science Inc., Wuhan, Hubei, PRC).

Ex vivo muscle function

At the end of the experiment (D19), ex vivo muscle function of right SOL and EDL was measured according to an adapted protocol previously described [34]. Muscles stabilized in the organ bath for 30 minutes. Subsequently, optimal stimulation strength was determined. Force frequency characteristics (10-167 Hz, 250 ms (EDL) and 500 ms (SOL)) were determined after refreshing the organ buffer and 5 minutes of rest. Next, an exercise protocol was performed of 100 contractions (83 Hz, 250 ms every 1000 ms for EDL and 83 Hz, 500 ms every 2000 ms for SOL). At the used frequencies, complete tetanic contraction of the muscle was reached. Force signals of the force frequency curve and the exercise protocol were analysed for maximal force (F$_{\text{max}}$), contraction and relaxation time (CT and RT respectively) and rate of change of force (dF/dt). Area under the curve (AUC) for F$_{\text{max}}$ was determined for both force frequency and exercise protocols. CT, RT and dF/dt were analysed between 83-167 Hz.
**Immunofluorescent histology**

**Staining MHC type I in m. soleus**

Immunofluorescent staining was performed as previously described [35]. SOL sections (10 µm) were cut using a cryostat at -20°C, air dried and stored at -20°C until use. Based on MHC isoform, fibre-type abundancy was determined and classified as MHC type I and II using monoclonal antibody against mouse MHC type I (BAD-5, 1 µg/mL, as developed by Schiaffino; the Developmental Studies Hybridoma Bank, The University of Iowa, IA, USA) and immunofluorescent secondary anti-mouse immunoglobulin G2b antibody (IgG2b Alexa F488, 2 µg/mL; Fisher Scientific, Landsmeer, The Netherlands). Normal goat serum was diluted 1:10 (dNGS) in phosphate buffered saline (PBS). Sections were first air-dried for 15 minutes and then blocked with dNGS for 60 minutes at 37°C. Primary antibody dilution was prepared in dNGS, added to the sections and incubated for 60 minutes at 37°C. Sections were gently washed three times with PBS. Secondary antibody dilution was prepared in dNGS and added to the slides to incubate for 60 minutes at 37°C in the dark. After incubation, sections were gently washed again three times with PBS. Wheat germ agglutinin dilution (WGA, 20 µg/mL) was prepared in PBS. WGA was added to the sections and incubated for 20 min at 37°C in the dark. Sections were gently washed three times PBS before enclosure with Vectashield-hard set (with DAPI; Fisher Scientific).

**Fibre Area Analysis**

Gray-scale images of the sections were taken under a Leica DMIL LED microscope with a 10x objective (Leica Microsystems, Amsterdam, The Netherlands). Images were taken at 10x magnification. The microscope was equipped with Red (Excitation: BP 546/11 nm; Emission BP 605/75 nm), Green (Excitation: BP 470/40 nm; Emission BP 525/50 nm), and Blue (Excitation: BP 360/40 nm; Emission LP425 nm) filters, a Leica DFC450C camera (resolution of 52294 DPI), and LAS X 2.0 software (Leica). Images were combined using ImageJ 1.15f for Windows (National Institutes of Health, Bethesda). Total area of a muscle section was analysed using ImageJ. Muscle fibre properties were analysed using SMASH muscle image analysis application for MATLAB r2015b (Mathworks, Natick) [36]. Abundance of type II muscle fibres was measured as MHC type I negative fibres as percentage of total fibre count. Muscle fibre cross-sectional area (CSA) was determined by measuring minimal Feret’s diameter of fibres, a reliable measure for CSA [37]. The ratio of type II/type I fibre CSA and the relative abundance of type II MHC (total type II CSA as % of total muscle CSA) were also determined.

**qPCR gene expression**

RNA was isolated from gastrocnemius skeletal muscle tissue. Frozen tissue was added to ice-cold Trizol Reagent (Invitrogen Cat. No. 155596-026) and
homogenised for 60 seconds using the IKA Ultra-Turrax T25 homogenizer. After centrifugation chloroform was added. Samples were centrifuged again, and 2-propanol was added to the aqueous phase. The supernatant was removed after centrifuging, and the remaining pellet was washed once in 75% ethanol by vortexing, followed by centrifuging. Finally, the pellet was air-dried and resuspended in nuclease-free water by an incubation at 55 °C in a heating block. The RNA concentration and purity were assessed with the Nanodrop spectrophotometer (Nanodrop ND1000, Nanodrop technologies Wilmington, DE, USA). cDNA was synthesized using the Promega cDNA synthesis Kit (A3500, Promega Benelux B.V., Leiden, the Netherlands) following the manufacture’s instruction in a Hybaid PCR machine. qPCR was carried out using Sensi-Mix SYBR-green. A 3-fold dilution series consisting of six dilutions were prepared for the standard curve. Primer sets for Ampk (AMP-activated protein kinase), Pgc1α (Ppargc1a, Peroxisome proliferator-activated receptor-γ coactivator 1 alpha), Sirt1 (silent mating type information regulation 2 homolog 1), Akt1, Atrogin1 (Fbxo32), Murf1 (Trim63), β-actin and Tbp (TATA-sequence binding protein) were used (table S2.1). β-actin and Tbp were used as reference genes in order to normalize the data for the target genes. Relative fold change was calculated with the 2-∆∆Ct method as described previously [38] and in the User Bulletin #2 (ABI PRISM 7700 Sequence Detection System) of Applied Biosystems.

**Protein expression**

Total protein was extracted from frozen *gastrocnemius* skeletal muscle tissue by homogenising the tissue in RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1mM EDTA, 1% NP-40, 0.25% Na-deoxycholate, 10x phosphatase inhibitor (Roche Diagnostics) and 25x Protease inhibitor (EDTA complete)) using an Ultra-Turrax homogeniser. The protein concentration was quantified with the BCA protein assay (Pierce) according to the manufacturer’s directions. Equal amounts of each protein sample (20 µg) were loaded on an 8-16% SDS gel (Biorad) and separated by SDS-PAGE. The separated proteins were transferred on a PVDF membrane followed by a 1h blocking step in 5% (w/v) milk. Membranes were incubated with different primary antibodies AKT1 (1:1000, Cell Signaling, #9272S), p-AktSer473 (1:2000, Cell Signaling, #9271S), AMPKa (1:1000, Cell Signaling, #2532S), p-AMPKaThr172 (1:1000, Cell Signaling, #2535S), Ppargc1a (1:1000, Invitrogen, PA5-72948) and p-hPpargc1a Ser571 (1:500, R&D Systems, AF6650), SIRT1 (1:1000, Cell Signaling, #2493S) overnight at 4°C. The membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody (1:5000) as secondary antibody for 1h at room temperature. For every washing step the membranes were washed for three times for 3 minutes followed by three times for 10 minutes with 1x TBST. Protein bands were detected with chemiluminescence and the Bio-Rad Imager. Protein bands were normalized to total protein after staining the membranes with Coomassie Brilliant Blue- R-250 and de-staining with a
mixture containing 10% Acetic-acid and 40% Methanol. Band intensities were quantified with the Bio-Rad's Image Lab software. Images of the blots of AMPK, AKT1 and PGC1α can be found in figures S2.1, S2.2 and S2.3 respectively.

**Statistics**

**General**

All data are expressed as means ± SEM. Statistical analyses were performed using Graphpad Prism 5 (Graphpad Software Inc., La Jolla, California, USA). Differences in daily body mass, body composition and ex vivo measures (Fmax, CT, RT and dF/dt) were tested using a repeated measures two-way ANOVA with group and time as factors. Differences at a specific point were all tested using a two-way ANOVA with tumour and training as factors. All post hoc testing was done with a Bonferroni multiple comparison correction. Differences were considered significant at p<0.05.

**PCA + Random forest**

Principal component analysis (PCA) and random forest analysis were performed using R (R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/). Variables with 2 or fewer missing values were included, missing values were replaced with the mean of the variable. There were 63 variables used for the analyses (table S2.2). The package moments was used to calculate the Shapiro Wilk p-value, skewness and kurtosis value (table S2.2). Random forest analysis was done using the randomforest package [39]. For PCA analysis data was auto scaled. Plots were made using the R libraries ggplot2 [40] and ggpubr. Used R scripts are available via https://github.com/mirandavdende/vibrogym-study.

**Microarray gene expression**

**Microarray**

RNA from EDL, SOL and heart was isolated (RNaseasy Micro kit, Qiagen, Venlo, the Netherlands). Subsequently, RNA was quantified by Nanodrop analysis and integrity was checked by an Agilent 2100 Bioanalyser with RNA 6000 microchips (Agilent Technologies, South Queensferry, UK). Total RNA was labelled with the GeneChip WT plus Reagent Kit and hybridized to GeneChip Mouse Gene 1.1 ST Array (Affymetrix, Inc. Santa Clara, Ca, USA). Sample labelling, hybridization to chips and image scanning were performed according to the manufacturers' instructions.
Microarray data analysis

Microarray quality control was performed in MADMAX, a pipeline for statistical analysis of microarray data [41]. Data were normalized using the robust multi-array analysis (RMA) algorithm [42] as implemented in the Bioconductor package AffyPLM. Probe sets were identified with genome information according to Dai et al. [43] based on annotations provided by the Entrez Gene database, which resulted in the profiling of 22,135 unique genes (custom CDF v22). Differential expression of probe sets (genes) was determined using linear models (package limma) and an intensity-based moderated t-statistic [44, 45]. T and T+WBV groups were compared to C group for EDL, SOL and Heart samples. P-values were adjusted for multiple testing by Benjamini-Hochberg false discovery rate (FDR) procedure [46]. Probe sets with an adjusted p-value of \( p < 0.01 \) were considered regulated. Venn diagrams and scatter plots were made using the R libraries ggplot2 [40] and VennDiagram [47]. To find genes most regulated by either tumour or training, a sparse Partial Least Squares Discriminant (sPLS-DA) analysis was performed using the mixOmics package [48]. Changes in individual genes were related to changes in pathways by gene set enrichment analysis (GSEA) [49] and the subset of metabolic and signalling pathways retrieved from the expert-curated Kyoto Encyclopedia of Genes and Genomes (KEGG) database [50]. Only gene sets consisting of more than 10 and fewer than 500 genes were considered, which resulted in the inclusion of 226 gene sets. For each comparison, genes were ranked on their t-value that was calculated by the empirical Bayes method. Statistical significance of GSEA results was determined using 10,000 permutations. GSEA and visualization was performed using the Bioconductor package clusterProfiler [51]. Microarray data has been submitted to the Gene Expression Omnibus (GEO) database under accession number GSE121972. Used R scripts are available via https://github.com/mirandavdende/vibrogym-study.

Results

No effect of training in tumour bearing mice on general cachectic parameters

Nineteen days after tumour inoculation, body mass, fat mass and lean mass started to significantly decrease in tumour bearing mice (T and T+WBV) (figure 2.1 a, b and c), with fat mass reduction preceding the other characteristics on day fourteen. Upon sacrifice (day 19), the carcass mass (figure 2.1 d) was also significantly lower in the T and T+WBV groups. Almost all organs, muscles (except for SOL) and fat compartments showed decreased mass due to a tumour effect (table S2.3). However, the mass of the spleen and intestine were both larger in tumour bearing mice, which could be explained by tumour growth and inflammatory reaction. Plasma INF-\( \gamma \) and
IL-6 showed an increase due to the tumour while the other tested cytokines were not affected (figure S2.4). Mean grip strength and daily activity at the end of the experiment were both lower in the tumour bearing mice (T and T+WBV) compared to the control mice (C and C+WBV) (figures 2.1 e and f). Front leg BMD was significantly lower, but plasma PTHrP was significantly higher in tumour bearing mice (T and T+WBV) compared to control mice (C and C+WBV) (figures 2.2 a and b). The gastrocnemius muscle of the T and T+WBV groups showed a significant increase in gene expression of Murf1 and Atrogin1, which are both markers for muscle mass breakdown (table S2.3). In all these parameters no effect of WBV was seen in both the C+WBV group and the T+WBV group, with one exception: EDL muscle mass was increased in C+WBV compared to control. Next to that, the C+WBV group showed a trend of a lower mean daily activity, although not significant.

**Figure 2.1:** Relative body mass [a], fat mass [b] and lean mass[c] over time, carcass mass at section [d], mean daily activity [e] and grip strength [f] at the end of the experiment. Data represent mean ± SEM. Asterisks indicate significant differences between C and T and dots indicate significant differences between C+WBV and T+WBV. */•, **/••, ***/••• and ****/•••• indicate p<0.05, p<0.01, p<0.005 and p<0.001 respectively.

**Training increases contraction and relaxation speed of SOL, but it has no effect on muscle fibre type**

EDL only showed minor differences in ex vivo muscle function (figures 2.3 a, b, c and f). Contraction time was slightly longer in the muscle of the tumour mice (T and T+WBV) (figure 2.3 d), however, this was not significant. Compared to the C and C+WBV groups respectively, the relaxation time was significantly
shorter in the T and T+WBV groups at 83 Hz (figure 2.3 e). In all cases, no significant changes due to the WBV training were observed.

In the SOL greater differences were seen between the tumour mice (T and T+WBV) and the control mice (C and C+WBV). A significantly lower F−max could be observed in both T and T+WBV groups at 167 Hz (figure 2.4 a). AUC for both the force-frequency and the exercise protocol was significantly lower in the T and T+WBV groups (figures 2.4 b and c). In the rate of force development, no differences were found (figure 2.4 f). In the SOL the clearest training effect was in the contraction and relaxation time. The contraction time of the T+WBV group was similar to that of the C and C+WBV groups while that of the T group seemed slower than in the other groups (figure 2.4 d). Also, only the T group showed significantly longer relaxation time compared to the C group, while the C+WBV and T+WBV groups did not significantly differ from each other (figure 2.4 e).

To further investigate the difference in ex vivo outcomes in the SOL, an immunofluorescent staining to measure fibre properties was performed. No differences between groups were found in total fibre cross sectional area (CSA), type I fibre CSA or type II fibre CSA (figures S2.5 a, b and c). A tumour effect in fibre CSA ratio (type II/type I CSA) was found, which was decreased in tumour bearing mice (T and T+WBV) compared to control mice (C and C+WBV) indicating a heavier wasting in type II fibres (figures S2.5 d and f).

Training in tumour bearing mice lowers AKT1 total protein expression

To assess mitochondrial biogenesis, gene and protein expression levels of Sirt1, Ampk, Akt1, and Pgc1α (Ppargc1α) were measured in m. gastrocnemius (table 2.1). SIRT1, AMPK, and AKT1 are regulators of PGC1α, which is considered the key protein in mitochondrial biogenesis. Sirt1 mRNA expression was upregulated in the T and T+WBV groups. Ampk mRNA expression did not change, but total protein expression was significantly lower in the T and T+WBV groups. AKT1, a potentially negative regulator of PGC1α and indirect promoter of protein synthesis, was upregulated at transcript level in the T and T+WBV groups. The mRNA expression level of Pgc1α was increased in the tumour bearing mice (T and T+WBV). However, protein expression levels of PGC1α were not significantly changed. Interestingly, total protein expression of AKT1 was significantly decreased by WBV training in the tumour mice (T+WBV), bringing it down to the level of the control group (C).

No training effect observed when using advanced analysis methods

Next, a random forest and PCA analysis was performed on a combined dataset of all results described above which consists of 63 variables (table S2.2). The random forest test is an ensemble learning method for classification, regression
**Figure 2.2:** Front leg BMD [a] and plasma PTHrP [b] at section. Data represent mean ± sem. ** and **** indicate p<0.01 and p<0.001 respectively.

**Figure 2.3:** EDL ex vivo muscle characteristics. Maximal tetanic force, at different stimulation frequencies [a] and area under the curve of the force-frequency (FF) relationship [b] and the exercise protocol [c]. Contraction time [d], relaxation time [e] and rate of force development [f]. Data represent mean ± SEM. Asterisks indicate significant differences between C and T and dots indicate significant differences between C+WBV and T+WBV. */ • and ****/ **** indicate p<0.05 and p<0.001 respectively.
**Figure 2.4:** SOL ex vivo muscle characteristics. Maximal tetanic force, at different stimulation frequencies [a] and area under the curve of the force-frequency relationship [b] and the exercise protocol [c]. Contraction time [d], relaxation time [e] and rate of force development [f]. Data represent mean ± sem. Asterisks indicate significant differences between C and T and dots indicate significant differences between C+WBV and T+WBV. */•, **/••and •••/••• indicate p<0.05, p<0.01 and p<0.005 respectively.

and other tasks that operates by constructing a multitude of decision trees. With this approach it is aimed to predict to which group the mice belong (C, C+WBV, T, or T+WBV). The Out of Bag (OOB) estimate of error rate is 73.91%, this means that 73.91% was not correctly classified by the random forest approach. The control mice (C and C+WBV) were not uniquely and correctly classified but rather dispersed in each other’s category which happened for the tumour mice (T and T+WBV) as well. This is also seen in the MDS plot (figure 2.5) in which these two distinct groups can be seen: tumour bearing mice versus the control mice, without any effect of WBV training.

Next, the scree plot of the PCA analysis (figure S2.6) showed that principal component (PC) 1 explains 41% of the variance in this model. When PC1, PC2, PC3, and PC4 were all plotted against each other (figure 2.6) it is clear that only in the plots in which PC1 is involved there is a distinction between tumour bearing mice (T and T+WBV) and control mice (C and C+WBV) (figures 2.6 a, b and c), again without effect of WBV training. When investigating the components in PC1 it was found that carcass weight, body mass, muscle mass, and body composition expressed as lean and fat mass, are the top 5 components. PC2, PC3 and PC4 are not able to make this separation between the groups (figures 2.6 d, e and f).
Table 2.1: Relative gene and protein expression in m. gastrocnemius (mean ± SEM). Two-way ANOVA with factors Tumour, Training and Tumour-Training interaction and Bonferroni Post-Hoc analysis. Significant effects are represented with * p<0.05, ** p<0.01, *** p<0.005, and **** p<0.001.

<table>
<thead>
<tr>
<th>Expression</th>
<th>C</th>
<th>C+WBV</th>
<th>T</th>
<th>T+WBV</th>
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<tr>
<td>Pgc1a mRNA</td>
<td>1.00±0.33</td>
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<td>3.34±0.99</td>
<td>2.22±0.25</td>
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<td>0.83±0.27</td>
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<tr>
<td>P-PGC1a/PGC1α Protein</td>
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<td>2.05±1.07</td>
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<td>0.85±0.22</td>
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<tr>
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<td>3.50±0.58</td>
<td>**** Tumour</td>
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<tr>
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<tr>
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<td>**** Tumour</td>
</tr>
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</table>

Training attenuates tumour-induced effects on gene expression level in muscle tissue

The physiological experiments described above showed mainly a tumour effect (T and T+WBV). The effects of the whole-body vibration training were mild and only seen in soleus muscle to increase the contraction and relaxation speed and in gastrocnemius muscle to decrease the AKT1 total protein expression. These findings were not enough to make a distinction between the WBV trained and the non-trained mice using random forest analysis or PCA analysis. However, these minor changes contrast with what was observed in the muscle gene expression profiles of these mice and this can provide an indication of how these cells are counteracting the induced effects.

In both EDL, SOL and heart samples, WBV training reduced the impact of the tumour on the muscle transcriptome. Importantly, fewer genes were significantly changed in the T+WBV group in all muscle tissues than in the T group when comparing each of these groups to the C group (figures 2.7 a, c and e). However, the direction of the change in gene expression was very similar in tumour mice (T and T+WBV) with clear correlations between the log-ratio of all genes (Pearson r of 0.94 [EDL], 0.89 [SOL] and 0.85 [Heart]) (figures 2.7 b, d and f). Moreover, the magnitude of change in gene expression was similar in all tissues where the slope of a linear regression fit was between 0.72 and 0.77 in all tissues. This means that the genes that significantly changed in both the T+WBV and T groups do this along the same
Figure 2.5: MDS plot (1 – Random Forest Proximities) using 63 variables. Green colour are the control groups and blue colour are the tumour groups, in both the darker colour represents the group with whole body vibration training (WBV).

line and to a similar degree.

Subsequently, it was tested if groups could be separated based on gene expression using a sPLS-DA with two components and a maximum of 50 genes per component (figure 2.8). Here it was noticed that the tumour effect (T and T+WBV) was very distinctly explaining 21 – 26% of variance (figures 2.8 a, b and c) with some common genes in the first component (figure 2.8 d). The second component separated groups based on WBV training. For all tissues, tumour bearing mice (T and T+WBV) were separated in trained and untrained where for the control mice (C and C+WBV) only in EDL the groups were separated. This indicates that there was a WBV training effect in the T and T+WBV groups, whereas there was little to no training effect in the C and C+WBV groups on the level of gene expression. The clearest separation based on WBV training was visible in the cardiac tissue with 9% explained variance. No commonalities were found in the genes driving this training effect in the three different muscle tissues (figure 2.8 e).

GSEA shows that the proteasome and RNA transport pathways are the most abundantly upregulated pathways for skeletal muscle with no significant regulation in heart muscle in the T and T+WBV groups (figure S2.7). The complement and coagulation cascades are the most abundantly upregulated pathway in all three muscle tissues, with it being upregulated in the T+WBV
Figure 2.6: PCA analysis with 63 variables plotting the first four principle components against each other. Green colour are the control groups and blue colour are the tumour groups, in both the darker colour represents the group with whole body vibration training (WBV). PC1 vs PC2 [A], PC1 vs PC3 [B], PC1 vs PC4 [C], PC2 vs PC3 [D], PC2 vs PC4 [E] and PC3 vs PC4 [F].

Group in EDL and heart, while for soleus it was upregulated in both groups (T and T+WBV). While thermogenesis and oxidative phosphorylation pathways are the most abundant pathways being down regulated in EDL, SOL and heart tissue in the T and T+WBV groups. The WBV prevented the upregulation of the proteasome pathway in the SOL of the T+WBV group but not in the EDL.
Figure 2.7: Microarray results showing the comparison of C with T and T+WBV group for three different tissues: EDL [a, b], SOL [c, d], and heart [e, f]. Venn diagrams [a, c, e] display number of genes with q<0.01 in T (green) and WBV (blue) mice. Scatter plots [b, d, f] show a correlation and linear regression of log-ratio of all genes from T+WBV (y-axis) and T (x-axis) compared to C mice with colors indicating significance.

Discussion

As it was hypothesized, WBV might be able to attenuate muscle wasting colon-derived-tumour-induced C26 cachexia mouse model. The current study primarily aimed to explore possible modulating effects on cancer-induced loss of muscle mass and function of whole-body vibration training (WBV) in the C26 cachexia mouse model. The cachectic effects found in this model for are in line with those reported in literature; a clear reduction in body, muscle and fat mass of a similar order of magnitude as reported in comparable studies was found [29, 52, 53]. Moreover, reductions in daily activity [29], grip strength [54], BMD [55], and the increase in muscle Murf1 and Atrogin1 expression
Figure 2.8: Microarray results showing a partial least squares discriminant analysis of all four groups with two components and 50 genes per component. sPLS-DA is performed per tissue, EDL [a], SOL [b] and heart [HRT][c]. Venn diagrams display genes per tissue in component 1 [d] and 2 [e].

[56] are similar to other studies. On cytokine levels a clear IL-6 induction and a small reduction of IFN\(\gamma\) were found, where others also found effects on TNF-\(\alpha\) and IL-4 [53]. When looking at muscle specific effects similar tumour-induced effects compared to other studies with decreased maximal force and increased muscle contraction and relaxation times were seen [29, 57]. Although no significant effect in the EDL was observed, this may result from a lack of statistical power since the data (figures 2.3 and 2.4) clearly shows a trend in the tumour effect reflected by a decreased maximum force and increasing contraction and relaxation times. For fibre type-specific effects, these results are also in line with other studies concerning the heavier tumour burden on type II fibres compared to that on type I fibres [57]. Taken together,
this tumour model largely supports previous findings which led us to conclude that it was suitable to explore effects of WBV on cancer-induced cachexia.

The most interesting finding was that WBV reduced the effects of the tumour on muscle gene expression in EDL, SOL and heart. The WBV prevented the upregulation of the proteasome pathway in the SOL but not in the EDL. Upregulation of the proteasome pathway and the complement and coagulation cascades are also found in another study with the C26 mouse model [58]. In isolated SOL, contractility was improved with contraction and relaxation time of the trained tumour group shifting to, and not being significantly different from, the trained control group. Training decreased AKT1 protein expression in the tumour group (T+WBV). Additionally, WBV effects in the control mice were found (C+WBV). Training increased EDL muscle mass in the control group and, surprisingly, there was a trend for WBV to reduce daily activity in these animals. Taken together, the overall effects of WBV training are small and mainly visible in gene expression, which might represent an adaptive response. The lack of specific effects are likely to be caused by the limited number of animals used, the short period of training or the severity of the cachexia in this study.

Literature on effects of WBV in rodents shows some contradicting results. In the ex vivo set-up of this study an improvement in contractility and a trend of improved strength were found. This is in line with other findings where WBV in healthy mice was shown to partially improve muscle contractility, specifically strength and relaxation rates [24]. Possibly, the reason for the absent effect on muscle morphology, like fibre type and cross-sectional area, could be due to the short period of training in combination with the severity of the cachexia in this study. This is supported by two other studies that also found no significant effect of WBV on myosin heavy chain (MHC) isoforms in the soleus muscle of mice [24, 59]. In contrast, a 6-week WBV training in healthy mice did show to increase total cross-sectional area of the soleus muscle as well as its type I and II muscle fibres [26]. A study into the long-term effects of WBV on the gastrocnemius muscle in rats found a decrease in type I content in favour of type II [60]. This could be beneficial for tumour bearing mice as type II fibres tend to be more affected by cachexia [57]. However, this should be further investigated in future studies. In a recent metabolomics study with aged mice it was found that WBV might postpone senility by attenuating lipid deposition and reducing chronic inflammation and the insulin resistance of skeletal muscle [61]. A study comparing aerobic training (treadmill exercise) with resistance training (ladder climbing) in C26 mice indicated possible beneficial effects of aerobic training and not of resistance training [54]. However, aerobic training was only able to mildly preserve muscle size, sensory motor function and relative m. gastrocnemius mass but failed to prevent weight loss [54]. This underlines the challenges in the treatment of cachexia with exercise. Another study in ApcMin/+ mice showed that moderate treadmill exercise is able to attenuate body and muscle mass loss in IL-6-dependent cachexia, moreover, mitochondrial oxidative capacity was improved [62]. The importance of the mitochondria can also be seen in the GSEA results of the
current study, where oxidative phosphorylation in muscle is one of the top enriched and downregulated pathways in the tumour bearing mice (T and T+WBV). The relevance of mitochondrial dysfunction is emergent from literature [63]. Nevertheless, these data support the view that in muscle metabolic adaptations take place induced by the tumour suggesting a switch from oxidative to glycolytic muscle metabolism. Moreover, proper muscle mitochondrial function relies on proper mRNA handling of nuclear encoded mitochondrial genes. Souza et al. showed the importance of mRNAs encoding essential mitochondrial regulators, as their stability is a function of muscle oxidative capacity [64]. Knowing this, one could hypothesize that the increase expression of the RNA transport pathway might be a compensation mechanism to counteract the downregulated oxidative phosphorylation pathway in tumour bearing mice. However, the upregulation in Pgc1α gene expression did not result in an higher PGC1α protein expression in the current study.

Unfortunately, the four groups could not fully be separated with PCA or random forest analysis. This could be attributed to the fact that the differences were limited or, alternatively, that the parameters to separate the training mice were not measured as for instance the gene expression profiles were not included in this analysis. This is a legit possibility because clear differences were seen in these profiles and the tumour bearing groups could be separated in trained versus untrained using a sPLS-DA. Taken together, these results and those from previous studies seem to indicate that different forms of WBV training have mostly small but distinct effects. This might indicate that WBV could have additive effect in cachexia treatment, however, it is not powerful enough on its own. One of the reasons might be the absence of a preventive effect of WBV on the downregulation of gene-expression of the oxidative phosphorylation pathway. Therefore, WBV in combination with either nutrition or exercise therapy or a combination of both is worthwhile to investigate. In Lewis lung carcinoma bearing mice, endurance training combined with supplementation of the poly unsaturated fatty acid eicosapentaenoic acid proved to partially rescue muscle strength and mass [65]. Moreover, a specific nutritional combination high in fish oil and leucine was able to reduce cachectic symptoms and improve functional performance and immune function [29, 53]. Therefore, it would be tempting to investigate whether combining whole body vibration training with endurance training and targeted nutrition might give synergistic effects. As stated before, cancer cachexia patients can struggle with exercise training [15]. WBV is easy to use and, although the small positive effect of WBV, it could be of added value to the therapy. It could be specifically helpful in a tailor made exercise program in patients with cancer-induced fatigue [66]. It might be worthwhile, to initiate follow-up studies that investigate a multiple-targeted treatment, including exercise and nutrition, or to study whole-body vibration training in a model more closely representing the situation in humans.
Strengths and limitations

As described in the discussion, the muscle wasting colon-derived-tumour-induced C26 cachexia mouse model used in the current study was similar to cachexia models in literature [29, 52, 53, 54, 55, 56]. Therefore, one strength of this study is the use of a solid cachectic model. An additional strength is the WBV protocol used, as it has yielded beneficial results in previous rodent studies [24, 26, 28, 30]. A limitation of this study is that the WBV protocol was not as effective as expected in this severe cancer cachexia model. A different WBV protocol could have yielded different results, as frequency and recovery time have a dose-dependent effect [67]. A Delphi consensus study for animals could help to have more consistent form of reporting WBV study protocols [22]. Despite the limitations, the current study has brought cachexia researchers a step closer to possible treatments.

Conclusion

In conclusion, this data suggest that with the present setup, WBV had only little effect on systemic endpoints but it triggered distinct subtle effects on muscle specific cachexia related pathways in tumour bearing mice. No effects were confirmed on body mass, body composition, inflammation, or bone mineral density, but this could be related to the limited number of animals that were part of this study, the short period of WBV training or the severity of cachexia. Although small, the muscle specific findings are very consistent, and no adverse effects were found. Specifically, the gene-expression data are promising, since far fewer genes significantly change in EDL, SOL and heart muscle of the tumour mice when they had undergone WBV training, showing a gene expression profile that shifts towards control mice. However, the C26 model might have been too acute to study long term effects of a low impact training such as WBV. It is important to publish these minor effects that WBV has in a severe state of cachexia. Additionally, this is the first study looking at the potential beneficial effects of WBV in cachexia. Since the possible effects on different outcomes were examined, both in vivo and ex vivo, it is possible to pinpoint the level at which vibration training might have an effect, namely an anticipating or adapting gene expression profile.

Declarations

This study was conducted in accordance with institutional guidelines for the care and use of laboratory animals established by the Animal Ethics Committee of the University of Wageningen, and all animal procedures related to the purpose of the research were approved under the Ethical license of the national competent authority (registration number 2014075.e), securing full compliance the European Directive 2010/63/EU for the use of animals for
scientific purposes. The manuscript does not contain clinical studies or patient
data. This research was funded by Wageningen University and VLAG
Graduate School. Microarray data has been submitted to the Gene
Expression Omnibus (GEO) database under accession number GSE121972.
Used R scripts are available via
https://github.com/mirandavadende/vibrogym-study. M van der Ende,
RLC Plas, M van Dijk, JT Dwarkasing, F van Gemerden, A Sarokhani, HJM
Swarts, EM van Schothorst, S Grefte, RF Witkamp and K van Norren give
their consent for publication and declare that they have no conflict of interest.

Authors’ contributions. Conceiving and design of the study was done by
RLC Plas, RF Witkamp and K van Norren. Collection of experimental data
was done by RLC Plas, M van der Ende, JT Dwarkasing, M van Dijk, F van
Gemerden, A Sarokhani, HJM Swarts and EM van Schothorst. Data analysis
was done by M van der Ende and RLC Plas. Writing of the paper was done
by M van der Ende, RLC Plas, S Grefte and K van Norren. All authors read,
commented, and approved the final manuscript. M van der Ende and RLC Plas
contributed equally to this manuscript and are therefore shared first author.

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Not applicable.
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[56] Lei Yuan, Jun Han, Qingyang Meng, Qiulei Xi, Qiuin Zhuang, Yi Jiang, Yusong Han, Bo Zhang, Qingfang, and Guohao Wu. “Muscle-specific E3 ubiquitin ligases are involved in muscle atrophy of cancer cachexia: an in vitro and in vivo study”. In: *Oncology reports* 33.5 (2015), pp. 2261–2268.


**Supplemental material**

*Figure S2.1:* Western blot images of AMPK protein [A] plus coomassie staining of the same blot [B] and phopho-AMPK protein [C] plus coomassie staining of the same blot [D]. Marker is indicated on the left side of the blot.

*Table S2.1:* Primer sequences.

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Figure S2.2: Western blot images of AKT protein [A] plus coomassie staining of the same blot [B] and phopho-AKT protein [C] plus coomassie staining of the same blot [D]. Marker is indicated on the left side of the blot.
Figure S2.3: Western blot images of PGC1α protein [A] plus coomassie staining of the same blot [B] and phospho-PGC1α protein [C] plus coomassie staining of the same blot [D]. Marker is indicated on the left side of the blot.
Figure S2.4: Plasma cytokine levels. Data represent mean ± sem. * and **** indicate p<0.05 and p<0.001 respectively.
Figure S2.5: SOL muscle fibre characteristics. Fibre cross sectional area of all fibres [a], type I dominant fibres [b] and type II dominant fibres [c]. Relative measures for fibre CSA ratio [d], type II dominant fibre abundance (in % of total fibres) [e] and type II dominant relative abundance (sum of total type II CSA as % of sum all fibre CSA) [f]. Data represent mean ± sem. * indicates p<0.05.
Figure S2.6: Scree plot plotting the principle components of the analysis with 63 variables.
Figure S2.7: Microarray results showing enrichment of KEGG pathways of tumour groups (with and without WBV) compared to control group without WBV.
Table S2.2: Shapiro Wilk p-value, skewness and kurtosis value of the 63 variables used to build the random forest and PCA models.

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Table S2.3: Organ masses (mg) and relative gene expression at section (mean ±SEM). Two-way ANOVA with factors Tumour, Training and Tumour-Training interaction and Bonferroni Post-Hoc analysis. Significant effects are represented with * p<0.05, ** p<0.01, *** p<0.005, and **** p<0.001.

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<td>2.5</td>
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<td>1.4</td>
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<td>M. Gastrocnemius</td>
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<td>7.5</td>
<td>125.6   ±</td>
<td>11.5</td>
<td>80.9       ±</td>
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<td>147.6   ±</td>
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<td>195.1   ±</td>
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<td>1118    ±</td>
<td>129</td>
<td>1039    ±</td>
<td>130</td>
<td>841        ±</td>
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<tr>
<td>Intestine</td>
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<td>103</td>
<td>1167    ±</td>
<td>82</td>
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<td>128</td>
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<td>Tumour</td>
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<td>n.a.</td>
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<td>264</td>
<td>1007       ±</td>
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<td>1.00 ± 0.23</td>
<td>0.76 ± 0.03</td>
<td>15.78 ± 3.18</td>
<td>13.84 ± 5.04</td>
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<tr>
<td>Atrogin Expression (m. gastrocnemius)</td>
<td>1.00 ± 0.27</td>
<td>0.79 ± 0.08</td>
<td>10.40 ± 1.65</td>
<td>10.69 ± 3.69</td>
<td>**** Tumour</td>
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</tbody>
</table>
Chapter 3

Prostaglandins can partly mimic the effects of cachexia-inducing cell line secretomes in differentiating muscle cells

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Manuscript in preparation
Abstract

BACKGROUND It is well known that tumours can exert direct negative effects on muscle tissue, which can contribute to the development of cancer cachexia. However, the underlying mechanisms and mediators involved in the communication between tumour and skeletal muscle are poorly understood. Cachexia-inducing cancer cell lines are used in both in vivo and in vitro research, they cause cachexia when used in an animal model. In the present study, we investigated the effects of conditioned media (CM) from cachexia-inducing cancer cell lines on differentiating skeletal muscle cells, and explored the role of individual mediators. METHODS Muscle-derived C2C12 cells were cultured (3-7 days) under differentiation conditions with CM from KPC, 4662, C26 and LLC cell lines. Creatine kinase (CK) activity and gene expression (qPCR) were measured as functional indicator and global gene expression was analysed by RNA sequencing. Conditioned media were screened for cytokines (111 in total) and levels of selected cytokines and prostaglandins were determined. Relevant mimic mixtures of the measured immune signalling molecules were composed and their effects on CK activity in differentiating C2C12 cells were evaluated. RESULTS The CM of the different cell lines significantly reduced the CK activity in C2C12 cells, corresponding to changes in CK gene expression. Global gene expression of the three and five days differentiated C2C12 cells was influenced by CM exposure, with immune pathways significantly upregulated. Many immune signalling molecules were present in the CM, but only mimic media containing prostaglandin E2 and prostaglandin F2A decreased CK activity, although quantitatively smaller compared to CM. CONCLUSION Creatine kinase activity in C2C12 cells was inhibited by CM derived from KPC, 4662, C26 and LLC cells, paralleled with a clear pro-inflammatory effect. The presence of prostaglandins in the CM appears to be relevant. Our results underline ongoing studies on the potential of modulating inflammatory processes to reduce cancer cachexia.
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BP</td>
<td>Biological Process</td>
</tr>
<tr>
<td>C26</td>
<td>Murine colon 26 carcinoma cell line</td>
</tr>
<tr>
<td>C2C12</td>
<td>Murine myoblast and myotube cell line</td>
</tr>
<tr>
<td>CCL2</td>
<td>C-C motif chemokine ligand 2 or monocyte chemoattractant protein 1 (MCP1)</td>
</tr>
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<td>CCL20</td>
<td>C-C motif chemokine ligand 20</td>
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<tr>
<td>CE</td>
<td>Collision Energies</td>
</tr>
<tr>
<td>CK</td>
<td>Creatine Kinase</td>
</tr>
<tr>
<td>CKm</td>
<td>Creatine Kinase muscle</td>
</tr>
<tr>
<td>CM</td>
<td>Conditioned Medium</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
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<tr>
<td>CRP</td>
<td>C-Reactive Protein</td>
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<tr>
<td>CX3CL1</td>
<td>C-X3-C motif chemokine ligand 1</td>
</tr>
<tr>
<td>CXCL1</td>
<td>C-X-C motif chemokine ligand 1 or keratinocytes derived chemokine (KC)</td>
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<tr>
<td>CXCL10</td>
<td>C-X-C motif chemokine ligand 10 or interferon gamma induced protein 10 (IP10)</td>
</tr>
<tr>
<td>CXCL12</td>
<td>C-X-C motif chemokine ligand 12</td>
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<tr>
<td>CXCL5</td>
<td>C-X-C motif chemokine ligand 5</td>
</tr>
<tr>
<td>DEGs</td>
<td>Differentially Expressed Genes</td>
</tr>
<tr>
<td>EM</td>
<td>Enzyme mixture</td>
</tr>
<tr>
<td>FDR</td>
<td>False Discovery Rate</td>
</tr>
<tr>
<td>FM</td>
<td>Fresh Medium</td>
</tr>
<tr>
<td>GEO</td>
<td>Gene Expression Omnibus</td>
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<tr>
<td>GO</td>
<td>Gene Ontology</td>
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<tr>
<td>GSEA</td>
<td>Gene Set Enrichment Analysis</td>
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<tr>
<td>IFNG</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>IGF1</td>
<td>Insulin-like growth factor 1</td>
</tr>
<tr>
<td>IGFBP3</td>
<td>Insulin-like growth factor-binding protein 3</td>
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<tr>
<td>IGFBP6</td>
<td>Insulin-like growth factor-binding protein 6</td>
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<tr>
<td>IKBK</td>
<td>I kappa B kinase</td>
</tr>
<tr>
<td>IL1α</td>
<td>Interleukin 1 alpha</td>
</tr>
<tr>
<td>IL6</td>
<td>Interleukin 6</td>
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<tr>
<td>IPA</td>
<td>Ingenuity Pathway Analysis</td>
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<td>KPC</td>
<td>Murine pancreatic ductal adenocarcinoma cell line</td>
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<tr>
<td>LIF</td>
<td>Leukemia Inhibitory Factor</td>
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<tr>
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<td>Murine Lewis lung carcinoma cell line</td>
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<tr>
<td>LOD</td>
<td>Limit of Detection</td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear factor-kappa B</td>
</tr>
<tr>
<td>NSAIDs</td>
<td>Non-steroidal anti-inflammatory drugs</td>
</tr>
<tr>
<td>OSM</td>
<td>Oncostatin M</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>---------------------------------------------------------------------------</td>
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<tr>
<td>PCA</td>
<td>Principal Component Analysis</td>
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<td>Proprotein convertase subtilisin/kexin type 9</td>
</tr>
<tr>
<td>PGE2</td>
<td>Prostaglandin E2</td>
</tr>
<tr>
<td>PGF2α</td>
<td>Prostaglandin F2 alpha</td>
</tr>
<tr>
<td>QC</td>
<td>Quality Control</td>
</tr>
<tr>
<td>RM</td>
<td>Reaction mixture</td>
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<tr>
<td>RPLP0</td>
<td>Ribosomal protein, large, P0</td>
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<tr>
<td>SPP1</td>
<td>Secreted Phosphoprotein 1 or Osteopontin (OPN)</td>
</tr>
<tr>
<td>TBP</td>
<td>TATA-sequence binding protein</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor Necrosis Factor alpha</td>
</tr>
<tr>
<td>TRIM63</td>
<td>Tripartite Motif Containing 63 or MURF1</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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Introduction

Up to 80% of the cancer patients suffer from skeletal muscle wasting, also called cachexia. In the European Union about half of the colorectal cancer patients develop cachexia [1, 2, 3], while 80-90% of the lung and pancreas cancer patients are at risk for the syndrome [4]. Unfortunately, effective treatment of cachexia is still lacking [5]. Cachexia is characterized by loss of skeletal muscle mass, with or without loss of fat mass [6, 7]. This muscle mass loss is caused by different processes in the muscle itself; 1) an imbalance between protein synthesis and breakdown; 2) structural remodelling of the myofibre (from type I to type II muscle fibres); 3) hampering of muscle degeneration and regeneration; and 4) alteration of mitochondrial functioning [8]. As a result, myogenesis, the formation of skeletal muscular tissue, is inhibited during cancer cachexia [9]. Recently, it became apparent that decreased skeletal muscle regeneration also plays a role in the development of cachexia [10]. However, still little is known about how cancer cells modulate skeletal muscle. A paper from the group of Guttridge et al. indicates a role for soluble factors excreted by the tumour [11]. To further investigate this concept, we aimed to identify tumour cell-secreted immune signalling molecules that induce cachexia using different cachexia-inducing cancer cell lines.

In mechanistic studies on cancer cachexia, both in vivo animal models and in vitro cells models are being used. In many of the in vivo models, cancer cells are injected either in the flank of the animal or at the location at which the tumour normally occurs. As a consequence of the growing tumour the animal will start to develop cachexia. The colon 26 carcinoma cell line (C26) is one of the cachexia-inducing cancer cell lines that is often used to study cancer-induced cachexia [12, 13]. Additionally, the Lewis lung carcinoma-derived cell line (LLC) [14] and murine pancreatic ductal adenocarcinoma derived cell lines (KPC and 4662) [15, 16] are used in cachexia models. Direct effects of cancer cells on muscle can be studied in vitro by exposing muscle cells to conditioned medium (CM) of cachexia-inducing cancer cell lines, which are also used in vivo. As a model for muscle tissue, murine C2C12 myoblasts/myotubes (C2C12) are frequently used. Differentiating C2C12 cells are a model for muscle growth and regeneration, and functional and molecular responses can be readout parameters after incubation with CM [17]. Among the most important readout parameters are, the number of myotube nuclei, myotube length, expression of key myogenesis markers or creatine kinase (CK) activity. Creatine kinase is an enzyme that plays a role in the regulation of the concentration of adenosine triphosphate (ATP) within a cell. Cells that have high ATP requirements have higher levels of CK [18]. The CK activity in C2C12 is, for example, significantly reduced by tumour necrosis factor alpha (TNFα) (5 to 10 ng/mL) [19, 20], which has been suggested to have a direct catabolic effect on skeletal muscle in cachexia [21].

Up till now, various tumour-secreted factors have been shown to affect
muscle. Insulin like growth factor binding protein 3 (IGFBP3), C-X-C motif chemokine ligand 1 (CXCL1), and C-C motif chemokine ligand 2 (CCL2) were able to lower the number of nuclei per myotube in C2C12 cells, all in the concentration of 80 ng/mL [22]. Additionally, it was found that pancreatic cancer cell-derived IGFBP3 (0.1-5 µg/mL) decreases myogenesis in C2C12 cells [23]. Prostaglandin E2 (PGE2), thought to be mainly involved in tumour growth or development [24], also upregulates expression of both interleukin 6 (IL6) and tripartite motif containing 63 (TRIM63) [25]. Other prostaglandins, including prostaglandin F2 alfa (PGF2α), are thought to stimulate growth of skeletal muscle cells [26]. Although these results indicate that there could be a direct role for tumour-secreted factors in muscle wasting, these studies did not perform a wide screening and did not investigate whether the concentration of secreted factors used corresponded to that secreted by tumour cells.

The primary goal of the present study is to identify unrecognized immune signalling molecules secreted by cancer cells that have a role in cachexia development. To this end, the effects of CM of four different cancer cachexia-inducing cancer cell lines (KPC, 4662, LLC and C26) on myogenesis were investigated using CK activity and C2C12 gene expression (by RNA sequencing) as readouts. Ingenuity pathway analysis (IPA) of whole genome gene expression was used to identify the most prominently regulated processes. Profiles of soluble immune modulators in CM from the cancer cell lines were established and based on the results, different mixtures in cell culture medium (‘mimics’) were composed. These mimics were used to explore the (relative) importance of the CM components by analysing whether the mimic can reproduce (part of) the effects of CM on the C2C12.

**Materials & Methods**

**Culture media and cells**

The following culture media were used; C2C12 differentiation medium (DM-C2C12; DMEM (GlutaMAX with pyruvate) + 2% horse serum heat inactivated (30 minutes 56°C) + 1% Penicillin/Streptavidin (P/S, Penicillin (10,000 IU) and Streptomycin (10,000 µg/mL))), growth medium (GM; DMEM + 10% fetal calf serum (FCS) + 1% P/S) and GM-low glucose medium (GM-LG; DMEM (low glucose GlutaMAX™ with pyruvate) + 10% FCS + 1% P/S). C26 (kindly obtained from Donna McCarthy, Ohio State University, USA), LLC (kindly obtained from Josep Argiles, University of Barcelona, Spain) and C2C12 (kindly obtained from Francina Dijk, Nutricia, the Netherlands) cells were maintained in GM. KPC and the genetic variation KPC-4662 (4662) (kindly obtained from Elizabeth Jaffee and Daniel Marks, Oregon Health and Science University, USA) were maintained in GM-LG.
Conditioned Medium & Immune factor Mimics

To make conditioned medium (CM), cells were taken from 90% confluent flasks and seeded 1:5 (C26 and LLC) and 1:3 (KPC and 4662) in a T75 flask. Cells were allowed to adhere to the flask for two hours in their maintenance medium (GM or GM-LG), thereafter cells were washed twice with phosphate buffered saline (PBS) and 12 mL of DM-C2C12 was added. All cells were incubated at 37°C and 5% CO2 for 96 hours to create CM. After 96 hours, CM was harvested and pooled per cell line, centrifuged (5 minutes at 500 g), aliquoted and stored at -20°C.

Mimic medium A, based on the cytokine profiler array (R&D Systems, see below) and luminex ELISA (R&D Systems, see below) consisted of CXCL1, vascular endothelial growth factor (VEGF), C-X-C motif chemokine ligand 16 (CXCL16), serpin E1, C-C motif chemokine ligand 20 (CCL20), proprotein convertase subtilisin/kexin type 9 (PCSK9), IGFBP3 and C-X-C motif chemokine ligand 5 (CXCL5). Mimic medium B, based on IPA upstream regulator analysis (Qiagen, see below) and ELISAs (R&D Systems, see below), consisted of secreted phosphoprotein 1 (SPP1), IL6, CCL2 and leukemia inhibitory factor (LIF). Mimic medium C contained two prostaglandins, PGE2 and PGF2α. The last mixture, mimic medium D, contained all components of all three mixtures. The concentrations of the inflammatory compounds are provided in Table 3.1.

Cytokine measurements

The proteome profiler array mouse XL cytokine array kit (R&D Systems, ARY028) was used to measure cytokine levels of the CM samples according to manufacturer’s instructions. Arrays were measured using a ChemiDoc imaging system (Biorad). Analysis of the intensity of the spots was done in Fiji using the DotBlot_Analysis.ijm macro (https://github.com/ahklemm/AnalyzeDotBlots). Cytokine concentrations were further quantified using the mouse magnetic luminex assay (R&D Systems, LXSAMSM-08) for CCL20, CXCL1, CXCL16, IGFBP-3, CXCL5, PCSK9, Serpin E1 and VEGF according to manufacturer’s instructions. CM samples were measured on the MAGPIX System (Luminex Corporation). DuoSet ELISA kits of IL6 (R&D systems, DY406), LIF (R&D systems, DY449), MCP-1/CCL2 (R&D systems, DY479) TNFα (R&D systems, DY410) and Quantikine SPP1 ELISA (R&D systems, MOST00) were performed following manufacturer’s instructions.

LC-MS measurement of PGE2 and PGF2α

PGE2 (≥98% purity), PGF2α (≥98% purity), PGE2-d4 [≥99% purity deuterated forms (d1-d4)] were purchased from Cayman Chemicals and supplied by Sanbio (Uden, The Netherlands). Butylated hydroxytoluene (BHT) (99%) and ethanol (EtOH; absolute for analysis) were obtained from
Table 3.1: Cytokines and prostaglandins used. * This is the final concentration on the cells, e.g. 33% of the concentration measured in the CM as the CM itself was also applied to the cell at a concentration of 33%.

<table>
<thead>
<tr>
<th>Other name(s)</th>
<th>Reference number</th>
<th>Final Conc. (ng/mL)*</th>
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<td>Preprotech, 250-27</td>
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<td>Prostaglandin F2A</td>
<td>Sanbio, 16010-5</td>
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</table>
Sigma-Aldrich (Zwijndrecht, The Netherlands). Acetonitrile \( \geq 99.9\% \) and methanol (MeOH) HiPerSolv CHROMANORM for LC-MS, suitable for UPLC/UHPLC instruments were obtained from VWR International B.V. (Amsterdam, The Netherlands). Formic acid (99\%, ULC/MS-CC/SFC) was purchased from Biosolve Chemicals (Valkenswaard, The Netherlands).

After thawing, 1 mL MeOH containing 1000 pg/mL of PGE2-d4, RvD2-d5 and DHEA-d4 was added to 200 \( \mu \)L CM. The samples were shortly vortexed and placed on ice for 30 minutes. Hereafter, samples were centrifuged for 5 minutes at 3000 g and 4\(^\circ\)C, after which the supernatant was collected in clean borosilicate glass tubes. Prior to HLB solid phase extraction, 4.8 mL ultrapure water containing 0.125\% FA was added to the collected supernatant. Solid phase extraction was performed on HLB solid phase extraction columns (Oasis; 60 mg, 3cc) from Waters Chromatography B.V (Etten-Leur, The Netherlands). The columns were activated by two-times 1 mL MeOH, equilibrated by two-times 1 mL ultrapure water containing 0.1\% FA, before loading the samples. After loading, the columns were washed with 2 mL 20\% MeOH in ultrapure water (MilliQ Integral 3 system from Millipore, Molsheim, France) containing 0.1\% FA, and allowed to dry for 15 minutes. Compounds were eluted by adding 2 mL MeOH. Samples were collected in borosilicate glass tubes containing 20 \( \mu \)L of 10\% glycerol and 500 \( \mu \)M BHT in EtOH, and dried in a Turbovap evaporator from Biotage (Uppsala, Sweden) at 35\(^\circ\)C under a gentle stream of nitrogen (2.7 L/min). Samples were reconstituted in 50 \( \mu \)L EtOH. Samples were stored at -80 \(^\circ\)C before running UPLC-MS/MS analysis. UPLC-MS/MS methodology was performed as previously described [28]. For each run, 3 \( \mu \)L of sample was injected on a Acquity C18 BEH UPLC column (2.1 x 100 mm, 1.7 \( \mu \)m) from Waters Chromatography B.V. (Etten-Leur, The Netherlands). A detailed overview of the applied MS settings is given in Table S3.1. Applied MRM transitions and collision energies (CE) were optimized for each compound individually (Table S3.2). Spiked quality control (QC) samples were used for accuracy determination.

Results were analysed using MassLynx 4.1 software from Waters Chromatography B.V. (Etten-Leur, The Netherlands). The limit of detection (LOD) was defined as the concentration corresponding to the smallest integrated peak area included in the calibration curve. Calibration of the samples was performed using \( 1/X^2 \) weighing of calibration samples, after which sample concentrations were calculated. QC samples were used for accuracy determination. For the animal experiment, absolute mean \( \pm \) SD was calculated per group, in which LOD values were used to replace non-detectable concentrations.

C2C12 culture and sample harvest

C2C12 were maintained sub-confluent in GM. For the differentiation experiments, cells were seeded in a 24 wells plate at 1.14x10\(^5\) cells/mL and 1 mL per well. After 24 hours, medium was changed to DM (fresh medium control (FM)), DM with 33\% CM or DM with a immune factor mixture
mimicking the CM (Table 3.1). At day 3 and 5 of the differentiation, medium of all conditions were refreshed. Additionally, on day 3, 5 and 7, samples were harvested for protein and/or RNA isolation (duplicate or triplicate per day). Experiments were repeated (N) 3 or 4 times. Cells were washed once with PBS before harvesting. For protein samples, CelLyticM (Sigma, C2978) with added cOmplete, EDTA-free Protease Inhibitor Cocktail (Sigma-Aldrich, 11873580001) was used. Cells were incubated 15 minutes on a shaker at RT and homogenized by scraping. Homogenate was centrifuged at 12000 g at 4°C and supernatant was transferred to a clean chilled tube. Protein lysates were stored at -80°C. For the RNA samples, cells were collected with TRIzol Reagent (Invitrogen, 15596018) or RLT buffer (Qiagen, 74004) + β-mercaptoethanol. Lysates were homogenized and stored at -20°C.

**Creatine Kinase assay**

Protein concentrations were measured using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, 23225) following the manufacturer’s instructions. Protein lysates were diluted to 0.025 mg/ml using CelLyticM (Sigma, C2978). The reaction mixture (RM, Table 3.2) and enzyme mixture (EM) were made fresh every experiment. The chemicals were dissolved in MilliQ water and the pH was set to 7.4 with acetic acid. The EM consisted of 42 µL glucose-6-phosphate dehydrogenase (140 units/mg protein, Sigma, 10127671001) and 34 µL hexokinase (450 units/mg protein, Sigma, 11426362001) dissolved in 1016.88 µL PBS.

To measure creatine kinase (CK), 20 µL sample or blank were pipetted per well in the 96 wells plate and 150 µL of RM was added. Plates were incubated at 37°C for 10 minutes and 10 µL EM was added. Absorption was measured at 340 nm with a kinetic program for 44 minutes with an interval of 24 seconds at 37°C. Between every measurement the plate was shaken for 1 second at medium speed, using the Spectramax M2 (molecular devices). Creatine kinase activity was calculated by using a lag time of 500 sec., and Vmax (absorbance in milli-units per minute) was calculated over 90 points. After subtraction of the background (blank) the following calculation was used: CK activity (µmol/mg⁻¹/min⁻¹) = (Vmax x reaction volume in µL) / (6220 (molar absorption coefficient of NADPH at 340 nm) x cm height (= 0.5625 cm for 180 µL volume) x mg protein in the well x 1000 (conversion from milli-OD to OD)).

**RNA isolation and sequencing**

TRIzol C2C12 samples were used for sequencing. Samples were thawed and 0.2 mL chloroform per 1 mL of TRIzol was added and incubated for 2–3 minutes. Samples were centrifuged for 15 minutes at 12000 g at 4°C and the aqueous phase was transferred to a new tube. From this aqueous phase the RNA was isolated using RNeasy Micro Kit (Qiagen, 74004) following the manufacturer’s instructions. RNA was eluted in 14 µL RNase-free water and RNA yield was determined by nanodrop. RNA quality was determined using the Bioanalyzer.
Table 3.2: Chemicals used in Creatine Kinase assay reaction mixture.

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Code</th>
<th>MW</th>
<th>Final conc.</th>
<th>Conc. in RM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imidazole</td>
<td>Merck / AM15</td>
<td>68.08</td>
<td>100 mM</td>
<td>120 mM</td>
</tr>
<tr>
<td>Glucose</td>
<td>Sigma / BB05</td>
<td>179.49</td>
<td>20 mM</td>
<td>24 mM</td>
</tr>
<tr>
<td>Mg-acetate</td>
<td>Merck / AF17</td>
<td>214.79</td>
<td>10 mM</td>
<td>12 mM</td>
</tr>
<tr>
<td>Adenosine diphosphate</td>
<td>Sigma A5285 / FV1</td>
<td>458.15</td>
<td>10 mM</td>
<td>12 mM</td>
</tr>
<tr>
<td>Adenosine monophosphate</td>
<td>Sigma A1752 / FV4</td>
<td>415.49</td>
<td>25 mM</td>
<td>30 mM</td>
</tr>
<tr>
<td>Nicotinamide adenine dinucleotide phosphate</td>
<td>Sigma N0505 / FV5</td>
<td>828.19</td>
<td>2 mM</td>
<td>2.4 mM</td>
</tr>
<tr>
<td>Phosphocreatine</td>
<td>Sigma P7936 / FV6</td>
<td>327.11</td>
<td>35 mM</td>
<td>42 mM</td>
</tr>
<tr>
<td>Acetyl-cysteine</td>
<td>Sigma A9165 / CK2.02</td>
<td>163.66</td>
<td>20 mM</td>
<td>24 mM</td>
</tr>
<tr>
<td>Di(adenosine-5')pentaphosphate</td>
<td>Sigma D4022 / stock 10 mg/mL in PBS</td>
<td>915.5</td>
<td>10 μM</td>
<td>12 μM</td>
</tr>
</tbody>
</table>

Technical triplicates were pooled, resulting in a total of three biological replicates per conditions. All samples were diluted to a concentration of 40 ng/μL and shipped in dry ice to BGI tech solutions (Hongkong). At BGI tech solutions, DNBseq eukaryotic transcriptome re-sequencing (strand specific) was done applying at least 20M clean reads per sample. Filtered sequencing reads were collected (6-9 GB data per sample).

Transcriptome analysis

The analysis of the data was done on the high performance computer infrastructure hosted by Wageningen University & Research. Quality control was done using FastQC [29], next, alignment was done using STAR [30]. Reads were counted using HTSeq [31] and files were exported to do further analysis using R [32] in Rstudio [33]. DESeq2 [34] was used to detect differentially expressed genes (DEGs), P-values were adjusted for multiple testing by the Benjamini-Hochberg false discovery rate (FDR) procedure [35]. An adjusted p-value (or FDR) of p < 0.05 was considered significantly regulated. Venn diagrams and scatter plots were made using the R libraries ggplot2 [36] and VennDiagram [37]. Changes in individual genes were related to changes in pathways by gene set enrichment analysis (GSEA) [38], analysis and visualization was performed using the package clusterProfiler.
Table 3.3: Primers used for qPCR.

<table>
<thead>
<tr>
<th></th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tbp</td>
<td>CAGTGCCCAGCATCACTATTTC</td>
<td>TGGAAAGCTGTGTTCTGGT</td>
</tr>
<tr>
<td>Rplp0</td>
<td>CAATAAGGTGCCAGCCTGTGCTCG</td>
<td>GAAGAAGGAGGTCTTCTCGGTCTCCT</td>
</tr>
<tr>
<td>CKm</td>
<td>GGCCTGCAGAAAGATTGAGGA</td>
<td>CTTGGGGTGCTTGGCTCAGGT</td>
</tr>
</tbody>
</table>

[39] and DOSE [40]. Analysis was done for Biological Process (BP) gene ontology (GO) terms and only gene sets consisting of more than 50 and fewer than 1000 genes were considered. For each comparison, genes were ranked on their GeneRatio (the number of genes of input list associated with the given GO term / the total number of input genes). Additionally, data were analysed through the use of Ingenuity Pathway Analysis (IPA) [41]. Upstream analysis was used to identify possible (cytokine) regulators, only cytokines were considered when they had a activation z-score > 2 and fold change > 0.9. Additionally all regulators that were flagged with ‘bias’ were removed from the analysis. Data has been submitted to the Gene Expression Omnibus (GEO) database under accession number GSE000000.

RNA isolations and qPCR

RLT samples were used for qPCR. RNA was isolated using the RNeasy Micro Kit (Qiagen, 74004) following the manufacturer’s instructions. Technical duplicates were pooled and cDNA was made using the reverse transcription system kit (Promega, A3500) using a Hybaid PCR machine. qPCR was carried out using Sensi-Mix SYBR-green. A 3-fold dilution series consisting of six dilutions were prepared for the standard curve. Primer sets for ribosomal protein, large, P0 (Rplp0), TATA-sequence binding protein (Tbp) and Creatin Kinase muscle (CKm) were used (Table 3.3). Rplp0 and Tbp were used as reference genes in order to normalize the data of the target genes. The average of the two reference genes and the standard curve was used to estimate DNA quantity and relative fold change was calculated.

Statistics

All data are expressed as means ± SEM. Statistical analyses were performed using Graphpad Prism 5 (Graphpad Software Inc., La Jolla, California, USA) or R and Rstudio [32, 33]. CK activity of FM was set to 100% to which CK activity of all other samples is related and ANOVA was performed using R, post hoc testing was done with the Tukey test. Residuals were tested with Shapiro-Wilk test for normality. For the qPCR results, differences were tested using a repeated measures two-way ANOVA with treatment and time as factors. Post hoc testing was done with a Bonferroni multiple comparison correction. Differences were considered significant at p<0.05.
Results

**Media of cachexia-inducing cancer cell lines decrease CK activity in C2C12 cells**

Differentiating C2C12 cells were exposed to CM to determine effects on CK activity. To correct for depleted nutrients in the CM, it was diluted to 33% using new cell culture medium (fresh medium; FM). This approach provided the C2C12 cells with adequate nutrient levels but also exposed the cells to the secretome in CM at relevant levels. A condition with only FM was used as control, CK activity of C2C12 cells is expressed as percentage of this control. Upon incubation with CM of KCP, 4662, LLC and C26 CK activity decreased significantly across day 3, 5 and 7 of the differentiation (Figure 3.1). No time (days of differentiation) or interaction effects were found, therefore the significance on specific days could not be tested. The KPC CM induced the most pronounced change in CK activity followed by LLC, 4662 and lastly C26. When comparing the KPC, 4662, LLC and C26 conditions among each other, no significant differences were found. To check whether nutrient depletion could be responsible for the effects, we compared nutrient depleted differentiation medium to FM, but no differences were observed (data not shown).

![Figure 3.1: CK activity % of FM control. N=3 (triplicate per N) Mean ± SEM ANOVA with post hoc Tukey. Significant effects compared to FM are applicable to the three different days within the condition and are represented with * p<0.05, ** p<0.01, *** p<0.005, and **** p<0.001. No time (days of differentiation) or interaction effects were found.](image)

**CK activity correlates with CKm expression**

To confirm the results seen in CK activity, gene expression of CKm was analysed using qPCR. Here, C2C12 incubated with KPC CM were compared to the control (FM). A significant downregulation of CKm is seen on day 5
Data of day 7 was not analysed as little differences were seen between day 5 and day 7 in CK activity, therefore from this point onward day 7 was left out the analysis. Additionally, there is a general significant effect of the KPC CM incubation (P = 0.0019) and of time (P = 0.0001). Furthermore, the levels of CK activity (% of control) and CKm gene expression correlate (Pearson r = 0.3554 and p-value = 0.0019, Figure 3.2 B), using the data of both day 3 and 5. CM exposure thus results in decreased CK protein and gene expression.

![Figure 3.2: (A) qPCR of CKm in three and five day differentiated C2C12 treated with KPC CM. N=7 (duplicate or triplicate per N) Mean ± SEM two-way ANOVA with post-hoc Bonferroni. (B) Pearson correlation of CK activity (% of control) and CKm gene expression. Pearson r = 0.3554 and p-value = 0.0019.](image)

**Gene expression profiles of C2C12 cells incubated with CM**

Next, gene expression responses were analysed more extensively for all CM incubations. RNA was isolated and sequenced of the C2C12 on differentiation day 3 and day 5. The normalized gene expression profiles of the samples were analysed using principal component analysis (PCA) (Figure 3.3 A). In PC1, explaining 55% of the variance, a clear distinction was observed between the samples harvested on day 3 and on day 5, confirming differences in response between these days. PC2 explains 18% of the variance, which is mainly due to the differences between the experiments (batch difference). The whole dataset consisted of 23169 different genes, the number of differentially expressed genes (DEGs) per cell line were calculated (Figure 3.3 B and C). The CM of all tested cachexia inducing cell lines provoked gene expression responses; the KPC and LLC CM exposures showed the most DEGs on both days, while C26 CM induced the smallest differential effect. For all cell lines, more DEGs were found on day 5 of C2C12 differentiation than on day 3.
Figure 3.3: PCA plot (A) of the variation of the normalized differences in RNA expression patterns of C2C12 on differentiation day three and differentiation day five, after incubation with different conditioned media of cancer cachexia-inducing cancer cell lines (KPC, 4662, LLC and C26). Number and percentage of DEGs of the total dataset on day 3 (B) and day 5 (C) of C2C12 differentiation with fresh differentiation medium (FM), N=3.

Immune-related processes in C2C12 are activated after CM incubation

To better understand how soluble mediators in CM affect muscle cells, the GO terms in Biological Process (BP) related to the gene expression profiles on day 5 were analysed (Figure 3.4). Of the significant top 20 upregulated and the top 20 downregulated GO terms, the 4662 CM incubation of C2C12 cells resulted in the highest significant regulated GO terms (total of 32 out of 40) and the C26 CM incubation in the lowest number (total of 5 out of 40). In general, among the upregulated GO terms, most were related to immune processes. Six GO terms are related to leukocyte migration, and other terms were related to responses to an external stimulus, bacterium and virus. The only GO term that was commonly, and highly significant, upregulated in all CM conditions was ‘cytokine-mediated signalling pathway’. The downregulated GO terms showed a different picture. In general, GO terms related to skeletal muscle and mitochondrial functioning (electron transport chain) were found to be significantly downregulated. The most significant downregulated GO term was related to the 4662 CM incubation and was ‘rRNA metabolic processes’. In general, the downregulated pathways seem mostly related to the wasting
process, while the upregulated pathways point to a role for cytokines or other immune parameters as possible inducers.

Figure 3.4: Highest significant top 20 upregulated (A) and downregulated (B) GO terms in Biological Process (BP) on day 5 after incubation of C2C12 with different CM. * = the name of this GO term was shortened, the full name is: Adaptive immune response based on somatic recombination of immune receptors build for immunoglobin superfamily domains.

Since cytokines have been implicated in muscle wasting [42, 22] and the upregulated GO terms suggested that inflammatory signalling could play a role, we looked further into the gene expression data for possible upstream regulators that could explain the upregulation in GO terms related to the immune system. The identified possible upstream regulatory immune signalling molecules are indicated in Table 3.4. The regulator TNFα was found in all four CM conditions. Additionally, the following cytokines were found in two conditions: SPP1 (4662 and LLC), interleukin 1 alpha (IL1α) (KPC and LLC), C-X-C motif chemokine ligand 12 (CXCL12) (KPC and 4662) and C-X3-C motif chemokine ligand 1 (CX3CL1) (KPC and C26).

Immune factor profiles in CM of different cachexia-inducing cancer cell lines

Next, we investigated which immune signalling molecules are present in the CM and could cause the immune response of the C2C12 cells upon CM incubation. A cytokine array was used to screen 111 different cytokines. Of
Table 3.4: Cytokine upstream regulators identified with IPA with an activation z-score > 2 and a fold change > 0.9.

<table>
<thead>
<tr>
<th>Upstream Regulator</th>
<th>Expr Fold Change</th>
<th>Molecule Type</th>
<th>Activation z-score</th>
<th>p-value of overlap</th>
<th>Predicted Activation State</th>
</tr>
</thead>
<tbody>
<tr>
<td>KPC IL2</td>
<td>1.956</td>
<td>cytokine</td>
<td>3.311</td>
<td>4.73E-06</td>
<td>Activated</td>
</tr>
<tr>
<td>KPC CXCL12</td>
<td>1.731</td>
<td>cytokine</td>
<td>2.274</td>
<td>5.46E-02</td>
<td>Activated</td>
</tr>
<tr>
<td>KPC CX3CL1</td>
<td>1.046</td>
<td>cytokine</td>
<td>2.878</td>
<td>1.76E-09</td>
<td>Activated</td>
</tr>
<tr>
<td>KPC IL1α</td>
<td>1.003</td>
<td>cytokine</td>
<td>2.211</td>
<td>5.45E-04</td>
<td>Activated</td>
</tr>
<tr>
<td>KPC TNFα</td>
<td>0.966</td>
<td>cytokine</td>
<td>2.498</td>
<td>6.72E-13</td>
<td>Activated</td>
</tr>
<tr>
<td>4662 TNFα</td>
<td>1.907</td>
<td>cytokine</td>
<td>3.079</td>
<td>2.22E-15</td>
<td>Activated</td>
</tr>
<tr>
<td>4662 CXCL12</td>
<td>1.438</td>
<td>cytokine</td>
<td>2.558</td>
<td>5.33E-03</td>
<td>Activated</td>
</tr>
<tr>
<td>4662 CCL2</td>
<td>1.221</td>
<td>cytokine</td>
<td>2.343</td>
<td>4.46E-10</td>
<td>Activated</td>
</tr>
<tr>
<td>4662 SPP1</td>
<td>1.198</td>
<td>cytokine</td>
<td>2.394</td>
<td>7.90E-04</td>
<td>Activated</td>
</tr>
<tr>
<td>LLC IL1α</td>
<td>1.829</td>
<td>cytokine</td>
<td>2.097</td>
<td>5.46E-07</td>
<td>Activated</td>
</tr>
<tr>
<td>LLC CSF2</td>
<td>1.53</td>
<td>cytokine</td>
<td>2.788</td>
<td>5.04E-09</td>
<td>Activated</td>
</tr>
<tr>
<td>LLC SPP1</td>
<td>0.944</td>
<td>cytokine</td>
<td>2.587</td>
<td>1.13E-01</td>
<td>Activated</td>
</tr>
<tr>
<td>LLC TNFα</td>
<td>0.917</td>
<td>cytokine</td>
<td>3.362</td>
<td>2.58E-27</td>
<td>Activated</td>
</tr>
<tr>
<td>C26 IFNG</td>
<td>1.202</td>
<td>cytokine</td>
<td>6.774</td>
<td>5.78E-38</td>
<td>Activated</td>
</tr>
<tr>
<td>C26 CX3CL1</td>
<td>0.906</td>
<td>cytokine</td>
<td>2.985</td>
<td>9.20E-05</td>
<td>Activated</td>
</tr>
<tr>
<td>C26 TNFα</td>
<td>1.963</td>
<td>cytokine</td>
<td>4.768</td>
<td>8.45E-22</td>
<td>Activated</td>
</tr>
</tbody>
</table>

The 111 cytokines tested, 75 were not present above the FM control levels. The levels of the 36 cytokines that were present above FM control are visualised in Figure S3.1. The relative levels of the cytokines varies greatly between the different CMs, except for cystatin C. The most abundant cytokines found are CCL20, MMP3, Insulin-like growth factor-binding protein 6 (IGFBP6) and osteoprotegerin.

To test whether the cytokines were indeed (partly) responsible for the CK and gene expression effects that were observed, several mimics were created consisting of immune factor mixtures in fresh cell culture medium at concentrations found in the CM. The immune signalling molecules and concentrations in KPC CM formed the basis to create this immune factor mimic. KPC CM was chosen because it evoked the strongest response in C2C12 cells based on DEGs and CK activity. In this CM, the relative levels of the cytokines CCL20, CXCL5, PCSK9, CXCL16, VEGF, Serpin E1, IGFBP3 and CXCL1 were highest in the cytokine array. To determine the exact level of immune signalling molecules for the mimic, their concentrations in all CM were measured. Additionally, based on the upstream regulator analysis and literature, the concentrations of SSP1, TNFα, IL6, CCL2, LIF and of the prostaglandins PGE2 and PGF2α were also measured. Quantification of this set of immune signalling molecules for all CMs can be found in Table 3.5. To our surprise the levels were not the highest for all immune signalling molecules in the KPC CM, the 4662 CM exceeded them partly. IL6, LIF and
Table 3.5: Concentrations in ng/mL measured in CM of KPC, 4662, LLC and C26 cells. ND = not detectable.

<table>
<thead>
<tr>
<th></th>
<th>KPC CM</th>
<th>4662 CM</th>
<th>LLC CM</th>
<th>C26 CM</th>
<th>FM</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCL1</td>
<td>2.088</td>
<td>0.049</td>
<td>0.772</td>
<td>0.112</td>
<td>0.027</td>
</tr>
<tr>
<td>VEGF</td>
<td>2.092</td>
<td>2.307</td>
<td>0.682</td>
<td>1.098</td>
<td>0.007</td>
</tr>
<tr>
<td>CXCL16</td>
<td>0.668</td>
<td>1.893</td>
<td>0.016</td>
<td>0.059</td>
<td>0.006</td>
</tr>
<tr>
<td>Serpin E1</td>
<td>16.358</td>
<td>6.335</td>
<td>0.958</td>
<td>6.073</td>
<td>0.012</td>
</tr>
<tr>
<td>CCL20</td>
<td>5.719</td>
<td>11.006</td>
<td>0.204</td>
<td>0.123</td>
<td>ND</td>
</tr>
<tr>
<td>PCSK9</td>
<td>62.893</td>
<td>68.996</td>
<td>0.538</td>
<td>0.513</td>
<td>0.406</td>
</tr>
<tr>
<td>IGFBP3</td>
<td>21.570</td>
<td>42.855</td>
<td>ND</td>
<td>0.346</td>
<td>ND</td>
</tr>
<tr>
<td>CXCL5</td>
<td>2.793</td>
<td>4.425</td>
<td>0.218</td>
<td>0.194</td>
<td>ND</td>
</tr>
<tr>
<td>CCL2</td>
<td>0.053</td>
<td>0.037</td>
<td>13.960</td>
<td>23.096</td>
<td>ND</td>
</tr>
<tr>
<td>IL6</td>
<td>0.003</td>
<td>ND</td>
<td>ND</td>
<td>0.026</td>
<td>ND</td>
</tr>
<tr>
<td>LIF</td>
<td>0.009</td>
<td>0.004</td>
<td>0.018</td>
<td>0.011</td>
<td>ND</td>
</tr>
<tr>
<td>TNFα</td>
<td>0.001</td>
<td>0.006</td>
<td>0.001</td>
<td>0.001</td>
<td>0.002</td>
</tr>
<tr>
<td>SPP1</td>
<td>367.642</td>
<td>1463.976</td>
<td>707.199</td>
<td>1958.777</td>
<td>ND</td>
</tr>
<tr>
<td>PGE2</td>
<td>3.915</td>
<td>0.277</td>
<td>2.040</td>
<td>3.773</td>
<td>0.019</td>
</tr>
<tr>
<td>PGF2α</td>
<td>2.718</td>
<td>16.128</td>
<td>0.150</td>
<td>0.545</td>
<td>0.012</td>
</tr>
</tbody>
</table>

TNFα were only present in very low amounts in all CMs.

A KPC immune factor mimic decreases CK activity in C2C12 cells

The four immune factor mimics that were composed to establish a possible causality consisted of, respectively, A) CCL20, CXCL5, PCSK9, CXCL16, VEGF, Serpin E1, IGFBP3 and CXCL1; B) PGE2 and PGF2α; C) SPP1, IL6, CCL2 and LIF; D) a combination of A, B and C. In the previous experiment, the CM was diluted in FM to 33% and therefore the immune signalling molecules in the mimic were also used at 33% of the concentrations as present in the KPC CM. C2C12 cells were exposed to all four mimics during differentiation and after three, five and seven days CK activity was measured and expressed as percentage of the control (Figure 3.5). The KPC CM significantly decreased CK activity of the C2C12 on all differentiation days similar to the previous experiment. Furthermore, immune factor mimics B and D were able to decrease the overall CK activity significantly, which both contain PGE2 and PGF2α. No time (days of differentiation) or interaction effects were found, therefore the significance on specific days could not be tested. The effects of the mimics were smaller than the effect of KPC CM on which they were based. In contrast to the CM, the effects of the immune factor mimics seemed to recover in time and on day 7 of the differentiation the levels were back at 100% of the FM control.
Discussion

This study identified immune signalling as most prominently induced pathway that was altered in differentiating C2C12 muscle cells exposed to CM of 4 different cachexia-inducing cancer cell lines; KPC, 4662, LLC and C26. This was accompanied by a decrease in myogenesis as indicated by a reduction of CK activity triggered by the CM. Immune-related factors secreted by tumour cells are also likely to be involved in these processes, as was shown by the significant decrease of CK activity when incubating the C2C12 with a mimic medium containing PGE2 and PGF2α. Together, this further substantiates a key role of inflammatory signalling, and especially of prostaglandins, in cancer cachexia. These findings also provide more clarity as to the question, already posed in 1996 [43], to what extent cytokines are the possible mediators of cancer cachexia.

Cancer cachexia-inducing CM was able to reduce myogenesis in C2C12 cells, which is in line with literature in which myogenesis was reduced in on average 4 day differentiated C2C12 cells incubated with C26 CM [44, 45, 46, 47, 48], LLC CM [48, 49] and KPC CM [50, 51], based on myotube diameter. In literature, an important role in impairing myogenesis and activating the muscle protein breakdown processes has been assigned to inflammatory cytokines [42, 52, 53]. The inflammatory cytokine TNFα (1 ng/mL or 5 ng/mL) can reduce CK activity [19, 20], while insulin-like growth factor 1 (IGF1, 30 ng/mL) increased CK activity [54]. This suggest that that TNFα has a direct catabolic effect on skeletal muscle, which causes loss of muscle mass, and therefore has an important role in cachectic inflammation [21]. However, these
Effects have not been validated by exposure studies using the measured endogenous concentrations in cachexia-inducing CM. This is also the case in the study of by Hogan et al., which makes it hard to interpret results of C2C12 incubations with cytokines [22]. In the current study the concentrations of TNFα in the secretome of the cachexia-inducing cancer cell lines was lower than 0.01 ng/mL, which is at least 100x lower compared to what was used in exposure studies in literature. This indicates that TNFα, in the present study, did not have a prominent role in the cachexia-inducing CM, but that other immune signalling molecules present were more important.

The literature about secreted immune factors by cachexia inducing cell lines is incomplete. The C26 cell line is by far the most studied cell line and it was found that C26 secretes LIF, C-X-C motif chemokine ligand 10 (CXCL10), CCL2, CXCL1 and Oncostatin M (OSM), but not TNFα, myostatin or Interferon gamma (IFNG) [44, 55]. We found that our C26 CM contained CXCL10, CCL2 and no TNFα, which is in line with literature. However, for LIF we found a concentration of 0.011 ng/mL, while in the study of Seto et al. a 40 fold higher concentration of 0.4 ng/mL was found [44]. For IL6 values of 0.007 ng/mL [44] and 0.028 ng/mL [55] have been reported, of which the latter was in line with the 0.026 ng/mL we found in the current study. However, from our research, C26 seems to behave differently from the KPC, 4662 and LLC cell lines, as C26 causes less DEGs and has a smaller effect on CK activity. This underscores the need to combine several cachexia-inducing cancer cell lines, as was done here, to prevent bias. Also, our data points towards cytokines that were previously not associated with the tumour secretome, like CCL20, CXCL5 and PCSK9, underscoring the need to use wide profiling tools in order to identify possible culprits. Additionally, we here found a role for prostaglandins in the secretome of cachexia-inducing cancer cell lines.

We found no effects of the identified cytokines in the CM on the differentiation of C2C12 cells. However, a possible causal role for prostaglandins was found, since only the mimics containing PGE2 and PGF2α had a significant effect reducing the CK activity in differentiating C2C12 cells. PGE2 is known to regulate protein turnover and exercise training adaptations in human muscle [56]. PGF2α was reported to stimulate PI3K/ERK/mTOR signalling which results in increased C2C12 myotube diameter [57], while cancer cachexia is known for its downregulation of mTOR expression in muscle [52]. Both PGE2 and PGF2α are produced via cyclooxygenases (COX) [58, 59]. Interestingly, our findings fit well with earlier observations on the potential beneficial role of non-steroidal anti-inflammatory drugs (NSAIDs) in cancer cachexia [60, 61]. These NSAIDs could be useful to block both the IKBK/NFκB pathway and COX [62, 63]. Additionally, transcription of most of the cytokines that we found in the secretome are regulated via nuclear factor-kappa B (NFκB) [64], including CCL20 [65, 66], CXCL5 [67], VEGF [68], Serpin E1 [69], CXCL1 [70], SPP1 [71], IL6 [72] and CCL2 [73]. A possible drawback of NSAID treatment is that the NFκB pathway is described as both pro- and anti-inflammatory [74] and research showed that apolipoprotein E could promote immune suppression in
pancreatic cancer through NFκB mediated production of CXCL1 [75]. Furthermore, NSAIDs have side-effects on the GI tract and, in case of COX-2 selective NSAIDS, the cardiovascular system [76, 77].

Although we found an effect of PGE2 and PGF2α, it did not decrease CK activity in the same extent as KPC CM. A large number of cytokines were assessed, but not all molecules secreted by the cachexia-inducing cancer cell lines were examined, and therefore it could be that we missed some important factors. This could possibly be studied by stimulating other pro-inflammatory processes or inhibiting specific pathways or molecules. Alternatively, the potential of cytokines modulators in the treatment of cancer cachexia could be further explored [78, 79] as it seems that general inflammatory effects contributes to cancer cachexia as seen in the gene expression pathways. Additionally, many cytokines, like LIF, IL6, VEGF and CCL2, have a role as myokines [80]. Also in contractile C2C12, an upregulation of CXCL1, IL6 and CXCL5 was found [74]. This makes it difficult to disentangle these physiological effects from those of tumour cytokines.

In conclusion, this study showed that inflammatory components of CM from cachexia-inducing cancer cell lines can inhibit myogenesis in C2C12 cells. Many immune pathways are upregulated in C2C12 cells exposed to these media. Additionally, we showed the importance of using different cell lines as a model for cachexia, as their secretion profile and CM effects differ. Importantly, we identified two prostaglandins as potential causal factors in cancer cachexia, a finding that needs further investigation and confirmation. This could ultimately be of high value towards the treatment of cachexia, either by specific drug or possibly by nutritional interventions that alter prostaglandin production.

Acknowledgments

The authors would like to thank Bart Lagerwaard, Jenny Jansen and Guido Hooiveld for their practical help and tips during this study. Additionally, we would like to thank Donna McCarthy (Ohio State University, USA) for the C26 cell line, Josep Argiles (University of Barcelona, Spain) for the LLC cell line, Francina Dijk (Nutricia, the Netherlands) for the C2C12 cell line and Elizabeth Jaffee and Daniel Marks (Oregon Health and Science University, USA) for the KPC and the genetic variation KPC-4662 cell lines. Also we would like to thank Francina Dijk, Veerle Ottenheim and Yukako Tokutake for their help with the protocol for the CK assay.
References


Shoya Inaba, Atsushi Hinohara, Masashi Tachibana, Kazutake Tsujikawa, and So-ichiro Fukada. “Muscle regeneration is disrupted by cancer cachexia without loss of muscle stem cell potential”. In: *PloS one* 13.10 (2018), e0205467.


[70] Gong Feng, Yoshihiro Ohmori, and Pi-Ling Chang. “Production of chemokine CXCL1/KC by okadaic acid through the nuclear factor-κB pathway”. In: Carcinogenesis 27.1 (2005), pp. 43–52.


Supplemental Material

**Table S3.1**: Detailed overview of the applied MS settings for analysis.

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</thead>
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</tr>
<tr>
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</tr>
<tr>
<td>Source offset (V)</td>
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</tr>
<tr>
<td>Desolvation temperature (°C)</td>
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</tr>
<tr>
<td>Gas flow – desolvation (L/hr)</td>
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</tr>
<tr>
<td>Gas flow – cone (L/hr)</td>
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</tr>
<tr>
<td>Nebuliser (Bar)</td>
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<tr>
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</tr>
<tr>
<td>HM resolution 1</td>
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</tr>
<tr>
<td>Ion energy 1</td>
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</tr>
<tr>
<td>LM resolution 2</td>
<td>3.0</td>
</tr>
<tr>
<td>HM resolution 2</td>
<td>15</td>
</tr>
<tr>
<td>Ion energy 2</td>
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</tr>
</tbody>
</table>

**Table S3.2**: Optimized MRM transitions and CE for UPLC-MS/MS quantification of selected compounds.

<table>
<thead>
<tr>
<th>Compound</th>
<th>MRM transition</th>
<th>CE (eV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ESI-Negative Mode</td>
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<td></td>
</tr>
<tr>
<td>PGD2</td>
<td>m/z = 351.1 &gt; m/z = 271.2</td>
<td>15</td>
</tr>
<tr>
<td>PGF2α</td>
<td>m/z = 353.2 &gt; m/z = 193.1</td>
<td>25</td>
</tr>
<tr>
<td>PGE2-d4</td>
<td>m/z = 355.2 &gt; m/z = 275.2</td>
<td>15</td>
</tr>
</tbody>
</table>
Figure S3.1: Relative expression of 36 cytokines in LLC, C26, 4662 and KPC CM and FM.
Chapter 4

Mitochondrial dynamics in cancer-induced cachexia

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ABSTRACT

BACKGROUND Cancer-induced cachexia has a negative impact on quality of life and adversely affects therapeutic outcomes and survival rates. It is characterized by, often severe, loss of muscle, with or without loss of fat mass. Insight in the pathophysiology of this complex metabolic syndrome and direct treatment options are still limited, which creates a research demand. Results from recent studies point towards a significant involvement of muscle mitochondrial networks. However, data are scattered and a comprehensive overview is lacking. This paper aims to fill existing knowledge gaps by integrating published data sets on muscle protein or gene expression from cancer-induced cachexia animal models. METHODS To this end, a database was compiled from 94 research papers, comprising 11 different rodent models. This was combined with four genome-wide transcriptome datasets of cancer-induced cachexia rodent models. RESULTS Analysis showed that the expression of genes involved in mitochondrial fusion, fission, ATP production and mitochondrial density is decreased, while that of genes involved ROS detoxification and mitophagy is increased. CONCLUSION Our results underline the relevance of including post-translational modifications of key proteins involved in mitochondrial functioning in future studies on cancer-induced cachexia.
Introduction

Up to 80% of the patients with advanced stage cancer suffer from cachexia, a wasting syndrome that substantially contributes to morbidity and mortality [1]. Cachexia is characterized by loss of muscle mass, with or without loss of fat mass [2]. Patients suffer from fatigue, reduced appetite and declining mobility, resulting in reduced physical activity, impaired ability to perform daily activities and consequently a decreased quality of life [3, 4, 5]. Additionally, cachexia often adversely affects treatment outcomes, as weight loss and muscle loss are associated with a lower chemotherapy response rate and more post-operative complications [6, 7]. Studies have shown that treatment of cancer-induced cachexia is more effective when initiated at an early stage [8, 9, 10]. This not only demands for more insight in the initial events, but also for markers that allow timely detection.

Diagnosis of cancer-induced cachexia is based on body mass index (BMI) and the degree of ongoing weight loss. Patients will be diagnosed when they show weight loss of > 5% over the past 6 months, when their BMI is lower than 20 with > 2% weight loss, or when their appendicular skeletal muscle index is low [11]. These diagnostic criteria can be combined with information on either the presence of anorexia and inflammation [12], or the presence of five different physiological factors to come to a refined cachexia score (CASCO) [13]. The latter criteria are useful to characterize patients at risk for cancer-induced cachexia, who do not classify as cachectic based on BMI or percentage of weight loss alone.

Development of effective treatment options is hampered by the diversity of the patient population, their different types of tumours, and stages of disease and treatment. Therefore, the prevailing viewpoint is that treatment should be personalised and multimodal [14], using combinations of drug(s), nutrition and therapeutic exercise [15, 16]. However, notwithstanding many years of intensive research, additional mechanistic insight is needed to identify therapeutic targets and develop improved treatment strategies.

Available data indicate that the immune system plays a dual role. On one hand, it has an important role in controlling tumour growth. On the other hand, the tumour also attracts immune cells, e.g. by secreting chemokine (CeC motif) ligand 2 (CCL2, also known as MCP1). Subsequently, the tumour promotes the secretion of specific cytokines by the attracted immune cells [17]. This generates a pro-inflammatory state. Examples of cytokines and factors involved include tumour necrosis factor alpha (TNFα), interleukin-6 (IL-6), interleukin-1 (IL-1), and interferon gamma (IFNγ) [18]. These pro-inflammatory factors have been identified as major contributors to both cancer-induced anorexia [19, 20, 21, 22, 23] and cancer-induced muscle protein breakdown [20, 24, 25]. These inflammation-driven processes include changes in energy metabolism and energy expenditure [25]. Different mechanisms contribute to increased energy expenditure, such as futile cycles in liver and muscle, browning of fat resulting in increased mitochondrial energy dissipation by activation of uncoupling proteins (UCPs), and a
decreased oxidative energy production resulting from abnormalities in skeletal muscle mitochondria [26, 27, 28, 29, 30]. On the other hand, anti-cancer treatments, such as surgery, chemotherapy and radiation are also contributing to muscle protein breakdown [31, 32, 33]. For example, Folfox (5-FU, leucovorin, oxaliplatin) and Folfiri (5-FU, leucovorin, irinotecan) upregulate extracellular signal-regulated kinases 1/2 (ERK1/2) and P38 mitogen-activated protein kinases (P38 MAPKs), leading to catabolism [34].

Different processes underlying the loss of muscle mass and function take place in muscle during cancer-induced cachexia. These include, but are probably not limited to; 1) changes in muscle protein synthesis and breakdown; 2) structural remodelling of the myofibres; 3) increased muscle degeneration and impaired regeneration; and 4) altered muscle mitochondrial functioning (Figure 4.1). An imbalance between protein synthesis and breakdown of muscle proteins is at the basis of the classical explanation of the decrease in muscle mass during cancer-induced cachexia [35, 36]. Indeed, impaired synthesis of new proteins [37] and increased protein degradation were found during cancer-induced cachexia. In the latter process, two important degradation pathways, the ubiquitin-proteasome and the autophagy-lysosome systems, are involved [38, 39]. Next to changes in protein synthesis and breakdown, recent research focuses on other processes activated by cancer-induced cachexia. For example, there are indications that structural remodelling of myofibres takes place in cachectic muscle. More specifically, a fibre type switch from type I to type II muscle fibres occurs [40, 41, 42]. However, this issue is still under debate, as other researchers found no evidence for a selective loss of one or the other fibre type [43]. Additionally, in two studies wasting of type II fibres was predominantly found during cancer-induced cachexia [44, 45], while another study found that muscle wasting is not fibre type dependent [46]. Furthermore, muscle degeneration and regeneration are also important processes during cancer-induced cachexia. Degeneration was found to be increased in a mouse model of cancer-induced cachexia [47], while regeneration seemed to be impaired at the final step of the differentiation, when myoblasts normally develop into myocytes, which are part of the myofibres [48]. However, the mechanisms underlying these findings have remained unclear thus far [49]. Yet another possible mechanism underlying cancer-induced cachexia that is currently evolving is based on altered muscle mitochondrial functioning, which is the focus of the present paper.

The last few years have witnessed a rapidly increasing interest in the role of muscle mitochondria during cancer-induced cachexia [50, 51, 52]. This is reflected by a number of recent papers indicating that several mitochondrial processes are not only changed in cancer cachectic animal models, but also contribute to cachexia development [53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67]. However, a comprehensive overview of the changes in muscle mitochondria-related pathways during cancer-induced cachexia is lacking so far. To meet this demand, the present paper aims to provide such an overview by critically evaluating and integrating available data on animal models from
**Figure 4.1:** Graphical representation of processes that may contribute to muscle wasting in cachexia. Loss of muscle mass and function can be considered the result of changes in the following processes: 1) Net protein balance i.e. an imbalance between protein synthesis and breakdown; 2) Structural remodelling of the myofibre: there is evidence for myofibre changes from type I to type II; 3) Degeneration and regeneration, when the myofibre is regenerating the nucleus becomes located centrally between the myofibrils; 4) Mitochondrial function, the muscle mitochondria functioning is altered during cachexia.

the literature and those stored in the public gene expression omnibus (GEO) database.

**Methods**

To provide a complete overview and examine the role of muscle mitochondria during cancer-induced cachexia in rodent models, we performed a structured search to identify all available and relevant literature, and complemented this with data from four relevant genome-wide transcriptome datasets. We focused on four mitochondrial pathways, which are: 1) mitochondrial biogenesis, 2) mitochondrial dynamics (fission/fusion), 3) mitophagy and 4) mitochondrial function.

**Structured literature search**

The following search criteria were used: the research paper is accessible via Pubmed; it includes data on a cachectic mouse or rat model; and it is published after 01/01/1990. Sixteen different search terms were used (Table 4.1) and all papers were manually screened. The criteria for inclusion were: the papers report data on gene or protein expression in skeletal muscle tissue, and; the research papers report statistical analysis between the tumour bearing group and the control group. A schematic representation of the structured literature search can be found in Figure 4.2.

In total 94 research papers were included. Some of the papers comprised two different animal models, which were included separately. Therefore, a total of 103 models were extracted. We relied on the statistical method of the original authors to compare the tumour bearing and control group. No differentiation between species/strain, type of cancer, muscle type or gender was made. In total 141 different genes and 170 proteins were found, however, only those genes or proteins that were reported in two or more models were
### Table 4.1: Search terms for the structured literature search

<table>
<thead>
<tr>
<th>Search terms</th>
<th>Date</th>
<th>Papers found</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer cachexia muscle mitochondria</td>
<td>11 Jan. 18</td>
<td>43 results</td>
</tr>
<tr>
<td>APC min cachexia</td>
<td>11 Jan. 18</td>
<td>26 results</td>
</tr>
<tr>
<td>Lewis Lung Carcinoma cachexia</td>
<td>11 Jan. 18</td>
<td>76 results</td>
</tr>
<tr>
<td>Colon-26 cachexia</td>
<td>11 Jan. 18</td>
<td>106 results</td>
</tr>
<tr>
<td>C26 cancer cachexia</td>
<td>15 Jan. 18</td>
<td>65 results</td>
</tr>
<tr>
<td>Yoshida ascites hepatoma 130 cachexia</td>
<td>15 Jan. 18</td>
<td>48 results</td>
</tr>
<tr>
<td>Yoshida sarcoma cachexia</td>
<td>15 Jan. 18</td>
<td>23 results</td>
</tr>
<tr>
<td>Walker 256 carcinosarcoma cachexia</td>
<td>15 Jan. 18</td>
<td>69 results</td>
</tr>
<tr>
<td>Bladder cancer cachexia</td>
<td>15 Jan. 18</td>
<td>17 results</td>
</tr>
<tr>
<td>MAC 16 adenocarcinoma cachexia</td>
<td>15 Jan. 18</td>
<td>2 results</td>
</tr>
<tr>
<td>LP07 cachexia</td>
<td>15 Jan. 18</td>
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</tr>
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<td>27 results</td>
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<td>MCG 101 cachexia</td>
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</tr>
<tr>
<td>Morris hepatoma 7777 cachexia</td>
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<tr>
<td>PROb BDIX cachexia</td>
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<td>1 result</td>
</tr>
<tr>
<td>Prostate Adenocarcinoma cachexia</td>
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<td>10 results</td>
</tr>
</tbody>
</table>

### Table 4.2: The number of genes and proteins found in the 94 papers of the structured literature search, depicted are the total number, the number found two or more times and the number that is used in figures 4.3, 4.4, 4.5 and 4.6

<table>
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<th></th>
<th>Total</th>
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<th>figures</th>
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<td>54</td>
<td>20</td>
</tr>
<tr>
<td>Protein</td>
<td>170</td>
<td>77</td>
<td>37</td>
</tr>
</tbody>
</table>
Search criteria:
- Paper available via pubmed
- Cachexic mouse or rat model
- Published after 01/01/1990

540 Research papers

Exclusion of 423 papers with no data on mRNA or protein on muscle

117 Research papers

Exclusion of 31 papers with no statistics between tumour baring and control animals

94 Research papers

Figure 4.2: Search strategy of the structured literature search. A total of 540 papers were found using the search terms of Table 4.1. A total of 423 papers were excluded because they did not contain data on mRNA or protein in muscle, and another 31 papers were excluded because no statistics were available between the tumour baring and control animals. This left 94 papers to be used in the current paper.
included in our analysis (Table 4.2). This resulted in a total of 54 genes and 77 proteins, of which 20 genes and 37 proteins were used, which are related to the mitochondrial pathways discussed (Figures 4.3, 4.4, 4.5 and 4.6). See Table A.1 (online) for details on the 94 used research papers.

The data of gene and protein expression from all the research papers are visualized in graphs. On the y-axis the different genes/proteins are represented, and on the x-axis the number of publications. This number represents the amount of times a specific gene or protein was found in our database. The protein data with the ‘p’-before the name of the protein indicates that phosphorylated protein was measured. Additionally, of some proteins the ratio between phosphorylated and non-phosphorylated protein was measured, which is also indicated in the graph.

Analysis of genome-wide transcriptome datasets

Four genome-wide transcriptome datasets of muscle tissue of cachectic mouse models (three C26 and one pancreatic cancer model) were obtained via the GEO database. It should be noted that the C26 tumour bearing mouse models may slightly differ with respect to cell batch and number used to generate the tumours. Next to this, different mouse strains could have been used, which affects reproducibility [68].

For specific information about the datasets see Table 4.3. Genome-wide transcriptome datasets were normalized, assigned to groups and analysed using MADMAX (https://madmax.bioinformatics.nl), which is a pipeline for statistical analysis of microarrays [69]. Fold change was calculated comparing the cachectic group with control animals, and statistical tests to obtain p-values were performed. The data were transferred to Excel and the ‘VLOOKUP’ function was used to search for the genes involved in the four mitochondrial pathways. An up- or down-regulation of a gene was considered true when it was significant (p-value <.05) in at least three of the four genome-wide transcriptome datasets. Based on this analysis, mitochondrial pathway figures were made using Adobe Illustrator. Upregulated, downregulated or unchanged genes were indicated by a yellow, blue, or grey colour, respectively.

Main text

Mitochondrial biogenesis

Mitochondrial biogenesis comprises both the growth of the mitochondrial network and the division of existing mitochondria in individual cells [74]. Although mitochondria have their own mitochondrial DNA (mtDNA), many of the mitochondrial proteins are encoded by the nuclear DNA (also known as NuGEMPs, nuclear genes encoding mitochondrial proteins) [75]. To ensure the coordination of mitochondrial biogenesis, a mechanism regulating the
Table 4.3: Details genome-wide transcriptome datasets

<table>
<thead>
<tr>
<th>Model</th>
<th>Muscle</th>
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<tbody>
<tr>
<td>GSE48363 [70]</td>
<td>Colon-26 (C-26) cancer cachexia</td>
</tr>
<tr>
<td>GSE51931 [71]</td>
<td>Pdx1-cre;LSL-KrasG12D;1NK4a/arfl/fl mice, pancreatic cancer</td>
</tr>
<tr>
<td>GSE56555 [72]</td>
<td>Colon-26 (C-26) cancer cachexia</td>
</tr>
<tr>
<td>GSE63032 [73]</td>
<td>Colon-26 (C-26) cancer cachexia</td>
</tr>
</tbody>
</table>

expression of these NuGEMPs and mitochondrial genes is operating. Peroxisome proliferator-activated receptor-γ coactivator 1 alpha (Ppargc1a, also known as Pgc1a) is a major regulator of mitochondrial biogenesis and is encoded by the nuclear DNA [76, 77]. It interacts with and activates different transcription factors such as nuclear respiratory factor 1 (Nrf1) and GA repeat binding protein, alpha (Gabpa [78], also known as Nrl2), peroxisome proliferator-activated receptors (Ppars) and estrogen-related receptors (Errs). Thereby, it stimulates the expression of NuGEMPs and regulates mitochondrial metabolism [79, 80, 81]. Together, Ppargc1a and Gabpa promote the expression of mitochondrial transcription factor A (Tfam) [82], which is an activator of mtDNA transcription and involved in mtDNA packaging [83]. In addition, the interaction between Ppargc1a and; 1) Ppara and Ppard regulates lipid metabolism and fatty acid oxidation [84]; 2) Pparg regulates insulin sensitivity and adiponectin expression [85, 86], and; 3) estrogen related receptor alpha (Esrra) is thought to regulate mitochondrial oxidative phosphorylation [87].

Mitochondrial biogenesis is influenced by several factors including exercise, caloric restriction, oxidative stress, muscle regeneration and inflammation [74]. In these conditions, Ppargc1a is regulated post-translationally by inactivation or activation by (de)phosphorylation and (de)acetylation [77]. AMP-activated protein kinase (AMPK) and Mitogen-Activated Protein Kinase 14 (Mapk14) phosphorylate and activate Ppargc1a, thereby stimulating mitochondrial biogenesis [88, 89]. Another regulator of Ppargc1a is silent mating type information regulation 2 homolog 1 (Sirt1), which activates Ppargc1a through deacetylation [90]. Therefore, inhibition of Sirt1 leads to a decreased expression of Ppargc1a target genes in muscle [91]. Next to these post-translational modifications, Ppargc1a expression is regulated at transcriptional level by a number of proteins. These include for example Mapk14, which is highly responsive to reactive oxygen species (ROS), activating myocyte enhancer factor 2C (Mef2c) and transcription factor 2 (Atf2) via phosphorylation [92], both leading to an increased Ppargc1a transcription [93]. Likewise, forkhead box O1 (Foxo1),
Foxy3 and cAMP response element-binding protein (Creb) have the potential to increase Ppargc1a transcription [58, 94, 95, 96].

Ppargc1a deficiency in muscle leads to a decreased muscle respiratory capacity [97] and a decrease in glucose homeostasis in mice [98], which illustrates its relevance in mitochondrial biogenesis for muscle health. However, Ppargc1b may also have a role in regulating mitochondrial biogenesis. In contrast to Ppargc1a, Ppargc1b is not upregulated in response to exercise [99], which suggests that Ppargc1a and Ppargc1b are stimulated independently. Ppargc1b plays an important role in the expression of genes controlling mitochondrial protein import, translational machinery and energy metabolism [100]. Similar to NRFs, Ppargc1b stimulates the expression of NuGEMPs and therefore mitochondrial biogenesis [99]. Ppargc1b was also found to induce angiogenesis in muscle [101], and to increase mitochondrial fusion [102]. A third member of the Ppargc family is peroxisome proliferator-activated receptor gamma coactivator-related protein 1 (Pprc1) [103]. Pprc1 is little investigated, but it is a cofactor of Gabpa and Creb, with a role in early cell proliferation [104].

**Mitochondrial biogenesis in cancer-induced cachexia**

Literature and genome-wide transcriptome datasets of cachectic animal models revealed several changes in the expression of genes and proteins involved in mitochondrial biogenesis (Figure 4.3) [70, 71, 72, 73]. However, some inconsistencies are found between the different types of data (genome wide transcriptome, mRNA and protein), and results also vary with regard to the types of data. This hampers interpretation to some extent.

Conflicting results are reported regarding the expression of Ppargc1a mRNA in cancer-induced cachexia animal models. Some researchers describe an upregulation during cachexia [105, 106], while others report a downregulation [107, 108, 109, 110]. In some cases, no change in Ppargc1a gene expression between the cachectic and control animals has been found [109, 111, 112], which is in line with our results from the analysis of different genome-wide transcriptome datasets [70, 71, 72, 73]. The literature on Ppargc1a protein expression is more consistent, as most papers report a reduction of total Ppargc1a protein levels during cancer-induced cachexia [107, 108, 109, 113, 114, 115]. These data suggest that Ppargc1a regulation takes place at the translational level. Further interpretation of the role of Ppargc1a can be obtained by examining gene inactivation or overexpression models. Interestingly, no indication for an effect of whole body gene inactivation of Ppargc1a on muscle phenotype was found. However, muscle specific gene inactivation was reported to induce a switch from type I to type II fibres [116]. Additionally, muscle specific overexpression of Ppargc1a did not prevent or reverse muscle wasting in a cachectic animal model [117], suggesting that Ppargc1a modulation alone is not enough to resolve cancer-induced cachexia. Ppargc1b, on the other hand, is found to be downregulated during cancer-induced cachexia in the genome-wide
transcriptome datasets, while in published articles no clear effect was reported. A decrease in the expression of several mitochondria-related genes was seen when endogenous Ppargc1b was inactivated by siRNA [118], illustrating its importance. Additional research is necessary to unravel the role of Ppargc1b in cancer-induced cachexia. The expression of the third member of the Ppargc family, Pprc1, is found to be upregulated during cancer-induced cachexia in the genome-wide transcriptome datasets.

Based on the downregulation of Ppargc1a protein during cancer-induced cachexia one would expect that co-activators of Ppargc1a, which regulate mitochondrial metabolism, are also downregulated. Surprisingly, the gene expression of Ppard and Nrf1 were found to be increased in cachectic models. In contrast, expression of Esrra and Tfam mRNA was downregulated. Despite the absence of statistical information, it seems however, that Tfam protein levels are not different between C26 tumour bearing mice and control mice [119].

The activity of Ppargc1a is post-translationally regulated by deacetylation and phosphorylation. Only one publication reported data on the ratio between acetylated Ppargc1a and total Ppargc1a. However, these authors found no difference between cachectic and control animals [120]. The deacetylation of Ppargc1a is performed by Sirt1, of which gene expression is reported to be upregulated while protein expression is downregulated [108, 110, 120, 121] during cancer-induced cachexia. It should be noted that Sirt1 is primarily regulated by altering its activity and the exact effects of this modulation on cancer cachexia have not been established. The phosphorylation of Ppargc1a is regulated by AMPK and Mapk14. However, available data on these genes are inconclusive and did not allow to deduce what happens to AMPK and Mapk14 during cachexia.

Genes that regulate the transcription of Ppargc1a have an altered expression during cachexia. The regulation via Mef2c is lower, because both mRNA and protein expression of Mef2c are downregulated in cachectic animals [120, 122, 123]. Mice with an inactivation of the Mef2c gene in muscle, show a reduced number of slow muscle fibres, they accumulate glycogen in muscle, and display poor muscle structure [124, 125]. The expression of the genes Foxo3a and Foxo1, which also regulate Ppargc1a transcription, is higher during cancer-induced cachexia [26, 109, 110, 123, 126]. It is difficult to precisely delineate what happens at the protein level with Foxo3a and Foxo1, as sometimes only the phosphorylated protein, total protein, or the ratio between these two are measured.

In summary, individual genes and proteins involved in mitochondrial biogenesis seem to be affected by cancer-induced cachexia in animal models. However, no clear conclusion can be drawn on the entire pathway. Combining the transcription and protein data, mitochondrial biogenesis seems mainly affected by a reduction in protein levels of Ppargc1a, while at transcriptional level current evidence is less clear.
Figure 4.3: Graphical integration of muscle mitochondrial biogenesis during cancer-induced cachexia in animal models, based on literature available in the public domain and data available in the public GEO database. Colour indicates when an upregulation (yellow, contiguous border), downregulation (blue, dotted border) or no significant difference (white, black thin border) was found. See Table A.2 (online) for full gene names. Data from literature: Graphs made based on literature of protein and mRNA data comparing muscle tissue of control animals and animals with cancer-induced cachexia. The number of publications that found no difference are spread over both sides of the graph. Phosphorylated proteins are depicted in a separate graph and indicated with a ‘p-’ in front of the protein name. When the ratio between two forms of the protein was investigated it is indicated with ‘name1:name2’. Genome-wide transcriptome data: Figure made based on four genome-wide transcriptome datasets of control animals and animals with cancer-induced cachexia. Upregulation or downregulation is indicated when a significant difference was found in three or more of the datasets. Red arrows with flat arrow head = inhibition. Black arrows = stimulation. Dotted arrows = positive effect on gene expression. Green ovals with P indicate phosphorylation and green ovals with DA and flat bottom indicate deacetylation.
Mitochondrial fission and fusion

Contrary to the familiar idea of mitochondria being distinct sausage-shaped organelles, current evidence indicates that cells harbour a continuous mitochondrial network [127]. This mitochondrial network is very dynamic, with mitochondria constantly splitting (fission) and merging (fusion). Fission and fusion are both necessary to maintain mitochondrial function. Fission is needed to remove less functional parts (e.g. damaged by radicals) and in muscle also occurs during immobilization [128]. Fusion is needed for mixing mitochondrial content (e.g. mtDNA, substrates), to enhance oxidative capacity, and for expansion of the network in times of e.g. muscle training. Next to that, fission and fusion are necessary to maintain stable mtDNA and muscle function [129].

Dynamin 1-like (Dnm1l, also known as dynamin-related protein (Drp1)) plays a key role in fission. Embedding of Dnm1l in the mitochondrial outer membrane promotes fission [130]. This process first requires dephosphorylation by calcineurin, inducing recruitment, after which Dnm1l can bind to the adaptor proteins fission mitochondrial 1 (Fis1) and/or mitochondrial fission factor (Mff) [131]. Subsequently, fission takes place in conjunction with the endoplasmic reticulum and actin filaments to constrict the mitochondria [132]. Interestingly, when Dnm1l binds to mitochondrial elongation factor 1 (Mief1) fission will be inhibited [130].

In the process of fusion other proteins are involved. The fusion of the outer mitochondrial membrane is accomplished by mitofusin 1 (Mfn1) and mitofusin 2 (Mfn2) [133], which are both important for healthy mitochondria [134]. Mfn1 transcription is stimulated by Ppargc1a and inhibited by mitofusin-binding protein [135, 136]. To fuse the inner mitochondrial membrane, optic atrophy 1 (Opa1) is cleaved by YME1 Like 1 ATPase (Yme1l1) to form L-Opa1, which stimulates fusion. However, when Opa1 is cleaved by OMA1 Zinc Metallopeptidase (Oma1) it will result in S-Opa1, which inhibits fusion and therefore promotes fission [137].

Mitochondrial fission and fusion in cancer-induced cachexia

Little was found in literature on mitochondrial fission and fusion during cancer-induced cachexia in animal models. Therefore, most of the conclusions of this section are based on the genome-wide transcriptome datasets from the GEO database. When possible, these datasets were linked to the literature (Figure 4.4).

Combined analysis of literature and the four genome-wide transcriptome datasets of cancer-induced cachexia mouse models led to an interesting finding; Dnm1 and Oma1, involved in promoting fission, and Mfn2 and Opa1, involved in promoting fusion, were consistently reported to be downregulated during cancer-induced cachexia. At the same time, most other genes involved in fission and fusion do not change [70, 71, 72, 73]. Mfn2 gene expression was found to be upregulated during cancer-induced cachexia in one
publication [105], whereas other publications reported downregulation of both
gene expression [108] and protein expression [113, 138]. With respect to the
fission pathway, it is intriguing that two regulators of Dnm1l, calcineurin and
protein kinase cAMP dependent catalytic subunit (Prkac), are downregulated
in the genome-wide transcriptome datasets. Together with lower protein
expression levels of Dnm1l [114, 138] this may suggest reduced mitochondrial
fission. However, the unchanged mRNA expression of Fis1 (both in literature
[108, 112] and genome-wide transcriptome datasets) between healthy and
cachectic animals does not correspond with the upregulated protein
expression [113, 114]. Nevertheless, these results provide support for the
hypothesis that mitochondrial fission and fusion is impaired during
cancer-induced cachexia. This will most likely result in reduced turnover of
mitochondrial fusion and fission and thereby negatively affect the quality
control of the mitochondria by altering mitochondrial morphology and the rate
in which the mitochondria change their shape [139]. Several studies have
used electron microscopy to study the morphology of mitochondria in more
detail. In cancer-induced cachexia models, both a reduced [113] and an
increased [105, 112, 140] size of the mitochondria have been reported. Also
swelling, vesicle-like structures and disrupted triads were seen during
cachexia [105, 112, 122]. It can therefore be assumed that the changes in
morphology are associated with the decreased expression of fission and
fusion genes during cachexia.

In summary, mitochondrial fission and fusion is affected in muscle
mitochondria of animal models of cancer-induced cachexia. Expression of
genes and proteins involved in fission and fusion is lower, and mitochondrial
morphology is altered.

Mitophagy

Mitophagy is the selective degradation of damaged mitochondria, which,
together with biogenesis, is essential to maintain a healthy population of
mitochondria [141]. Mitophagy can be divided into several phases. It starts
with the initiation phase, after which the phagophore is formed during the
phagophore nucleation phase. This phagophore, a cup-shaped double
membrane, elongates and recognizes damaged mitochondria during the next
phase. Crucial for the recognition of damaged mitochondria are
post-translational modified proteins located on the outer membrane of the
mitochondria [142]. The phagophore encloses the damaged mitochondria,
which is called maturation [143], and eventually fuses with lysosomes in which
the mitochondria are degraded [144].

Mitophagy starts with an initiation signal through activation of Unc-51 like
kinase 1 (ULK1). ULK1 is normally suppressed via the Akt/ mTORC1 pathway
and stimulated by AMPK [141, 145, 146]. For phagophore nucleation to occur,
Beclin1 (Becn1) is needed. Next, the phagophore will elongate, which will
lead to the maturation of the phagophore [147]. Elongation of the phagophore
requires autophagy related proteins (ATGs) and microtubule-associated
Figure 4.4: Graphical integration of muscle mitochondrial fission and fusion during cancer-induced cachexia in animal models, based on literature available in the public domain and data available in the public GEO database. Colour indicates when an upregulation (yellow, contiguous border), downregulation (blue, dotted border) or no significant difference (white, black thin border) was found. See Table A.2 (online) for full gene names. Data from literature: Graphs made based on literature of protein and mRNA data comparing muscle tissue of control animals and animals with cancer-induced cachexia. The number of publications that found no difference are spread over both sides of the graph. Phosphorylated proteins are depicted in a separate graph and indicated with a ‘p-’ in front of the protein name. When the ratio between two forms of the protein was investigated it is indicated with ‘name1:name2’. Genome-wide transcriptome data: Figure made based on four genome-wide transcriptome datasets of control animals and animals with cancer-induced cachexia. Upregulation or downregulation is indicated when a significant difference was found in three or more of the datasets. Black arrows = stimulation. Green ovals with P indicate phosphorylation. ER = endoplasmic reticulum.
protein 1 light chain 3 (Map1lc3, also known as LC3). Map1lc3-I is converted to Map1lc3-II and then built into the membrane to form a mature phagosome [142, 148]. This is regulated by Sirt1, because inactivation results in lower levels of Map1lc3 and therefore less mitophagy [149].

The elongating phagophore recognizes mitochondria that are marked as damaged, which occurs via two pathways. The first marking happens via inhibiting a continuous degradation of PTEN-induced putative kinase 1 (Pink1). Healthy mitochondria import and degrade Pink1 because of the negative membrane potential, which prevents mitophagy [150, 151]. When this does not occur Pink1 recruits parkin (Park2) to the mitochondrial outer membrane [152], initiating the ubiquination of mitochondrial substrates leading to mitophagy [153, 154]. The mitochondrial target substrates of ubiquitination include Mfn2, ras homolog family member T2 (Rhot2, also known as Miro), and voltage-dependent anion channel 1 (Vdac1) [155, 156, 157]. Sequestosome 1 (Sqstm1, also known as P62) promotes mitophagy via this pathway by stimulating mitochondrial clustering [158]. In the second pathway, specific proteins on the outer mitochondrial membrane of damaged mitochondria are expressed and recognized. These proteins include Bcl-2/adenovirus E1B 19kDa-interacting protein 3 (Bnip3), Bnip3-like (Bnip3l) [155] and FUN14 Domain Containing 1 (Fundc1) [159].

**Mitophagy in cancer-induced cachexia**

When reviewing the literature of cachectic animal models, we found publications on a couple of proteins involved in the mitophagy pathway in muscle tissue. As protein levels, derived from their corresponding genes, are very important for the regulation of mitophagy, the protein data from literature will form the backbone for this section, to which mRNA and genome-wide transcriptome data will be linked (Figure 4.5).

No consistent changes in protein or mRNA levels were found in cachectic muscle when looking at the regulation of nucleation and elongation (Becn1 [160, 161, 162, 163] and ATGs [162, 164]). However, the maturation phase might be affected. The ratio between Map1lc3-II and Map1lc3-I is found to be upregulated during cachexia in most publications [165, 166, 167]. The results on total protein amount of Map1lc3-II and Map1lc3-I are scattered, and therefore more information is needed to draw firm conclusions [112, 162, 164, 168]. No changes were found in the specific isoform Map1lc3b-II/Map1lc3b-I ratio [168, 169]. Expression of Sqstm1 and ubiquitin, both responsible for the tagging and thereby recognition of mitochondria by the phagophore, changes during cancer-induced cachexia. Sqstm1 is found to be upregulated at both protein and gene level (literature and genome-wide transcriptome datasets) in muscle tissue of cachectic animal models [161, 162, 163, 166, 169]. Along with this, ubiquitin gene and protein expression was reported to be increased in many publications [105, 170, 171, 172, 173, 174, 175], which is not only an indicator for mitophagy but also for muscle protein breakdown. Consistently, increased ubiquitin protein expression [115, 176], increased ubiquitinated...
proteins [165] and increased poly-ubiquitination of proteins [177] are reported during cachexia.

The second pathway of tagging and recognizing damaged mitochondria, via specific proteins, has been less investigated. Publications describe an upregulation of Bnip3 protein during cancer-induced cachexia [112, 114, 160, 167]. Moreover, gene expression was found to be upregulated both in literature and the genome-wide transcriptome dataset [126, 178, 179]. On the other hand, three other papers found no difference in gene expression of Bnip3 between cachectic and healthy animals. However, it must be said that protein and gene expression of Bnip3 are not necessarily linked. This was illustrated by the finding that Bnip3 protein levels increase with fasting and decrease after subsequent exercise, whereas mRNA levels remained elevated [180].

In the last step of mitophagy, the mitochondria-containing phagosomes fuse with the lysosomes and mitochondria are subsequently degraded. It is known that lysosome function is impaired during ageing, which becomes visible by the appearance of lipofuscin granules [181]. Similarly, during cancer-induced cachexia swelling of the mitochondria and vesicle-like structures is seen [105, 122], suggesting that lysosome function might be also impaired during cachexia.

Together these results show that proteins involved in elongation and maturation of the phagophore are upregulated during cancer-induced cachexia in animal models. The upregulation could be an indication of increased mitophagy. However, it could also be that higher expression of these genes is caused by a lower functionality lysosomes [163, 181]. Therefore, more research is needed to unravel what is happening exactly. Additionally, the recognition of damaged mitochondria is upregulated via both ubiquitination and Bnip3. Taken together, the available data indicate that mitophagy is affected and perhaps even increased in muscle during cancer-induced cachexia.

**Mitochondrial function**

Adequate mitochondrial performance is crucial for normal cell functioning. Examples are oxidative phosphorylation for energy production, ammonia detoxification, and both production and neutralization of ROS. One cause of having a diminished mitochondrial function is a decrease in the number of mitochondria (or mitochondrial density) in a cell. This is seen during ageing where mitochondrial density in muscle tissue drops causing lower muscle quality [182, 183]. Mitochondrial density often is investigated by measuring mtDNA or specific genes and proteins. Examples are citrate synthase, subunits of complex I and Vdac1 [184]. Besides mitochondrial density, which influences mitochondrial function, the activity of the mitochondrial proteins is variable. The variability in activity of these proteins is a result of post-translational regulation. Sirtuin 3 (Sirt3) is one of the important post translational regulators and is localized mainly in the mitochondria [185].
Figure 4.5: Graphical integration of muscle mitophagy during cancer-induced cachexia in animal models, based on literature available in the public domain and data available in the public GEO database. Colour indicates when an upregulation (yellow, contiguous border), downregulation (blue, dotted border) or no significant difference (white, black thin border) was found. See Table A.2 (online) for full gene names. Data from literature: Graphs made based on literature of protein and mRNA data comparing muscle tissue of control animals and animals with cancer-induced cachexia. The number of publications that found no difference are spread over both sides of the graph. Phosphorylated proteins are depicted in a separate graph and indicated with a ‘p-’ in front of the protein name. When the ratio between two forms of the protein was investigated it is indicated with ‘name1:name2’. Genome-wide transcriptome data: Figure made based on four genome-wide transcriptome datasets of control animals and animals with cancer-induced cachexia. Upregulation or downregulation is indicated when a significant difference was found in three or more of the datasets. Red arrows with flat arrow head = inhibition. Black arrows = stimulation. Green ovals with P indicate phosphorylation and green ovals with DA and flat bottom indicate deacetylation.
higher expression of Sirt3 is found in tissues with a high oxidative capacity [186].

Under fasting conditions, Sirt3 is upregulated. Additionally, mice lacking Sirt3 have higher levels of $\beta$-oxidation intermediates [187], which illustrates the importance of Sirt3 for mitochondrial functioning. Sirt3 regulates oxidative phosphorylation, by deacetylation of both succinate dehydrogenase (Sdh) and the NADH:ubiquinone oxidoreductase subunit A9 (Ndufa9) [188, 189]. Additionally, Sirt3 regulates the influx to the tricarboxylic acid (TCA) cycle by stimulating pyruvate dehydrogenase E1 (Pdha1), isocitrate dehydrogenase 2 (Idh2) and acyl-CoA synthetase short-chain family member 1 (Acss1), which generate acetyl-CoA [190, 191, 192]. Also $\beta$-oxidation is regulated by Sirt3, via the deacetylation of acyl-Coenzyme A dehydrogenase long-chain (Acadl, also known as Lcad), which is a key mitochondrial fatty acid oxidation enzyme [193]. Moreover, Sirt3 deacetylates and therefore activates acetyl-Coenzyme A carboxylase beta (Acacb) which leads to an increase in $\beta$-oxidation [194].

Next to the role of Sirt3 in oxidative phosphorylation, TCA cycle, and $\beta$-oxidation, it also plays a role in ammonia detoxification and reactive oxygen species (ROS) neutralization. Ornithine transcarbamylase (Otc), an enzyme of the urea cycle, is deacetylated by Sirt3 which thus promotes ammonia detoxification [195]. Moreover, Sirt3 increases the levels of catalase and superoxide dismutase 2 (Sod2) [196] resulting in increased capacity to detoxify ROS.

**Mitochondrial function in cancer-induced cachexia**

Reviewing literature of cachectic rodent models did not reveal many data on the pathways involved in mitochondrial functioning. At protein level, catalase, Sod2, cytochrome C (Cycs) and complex IV and at mRNA level Ucp2 and Pdk4 have been examined. From the results it is impossible to reconstruct what happens during cancer-induced cachexia, and this is why this section is largely based on the results found in the genome-wide transcriptome datasets (Figure 4.6). One interesting finding from the analysis of the genome-wide transcriptome datasets of cachectic mouse models is the downregulation of Sirt3. Expression of Sirt3 is regulated by Ppargc1a and Esrra [197]. A downregulation of both Ppargc1a (protein) and Esrra (mRNA) can be the cause for the lower Sirt3 expression. However, Sirt3 needs NAD+ (Nicotinamide Adenosine Dinucleotide) to be active, which is increased in conditions of low cellular energy such as caloric restriction [198]. Such a low energy status is likely to be present in the muscle mitochondria during cancer-induced cachexia, and therefore, despite lower gene expression, Sirt3 could still be highly active. Additional to the lower expression of Sirt3, the mitochondrial density markers citrate synthase, subunits of complex I and Vdac1 are downregulated in the genome-wide transcriptome datasets. Together with the reduction of mtDNA during cachexia [113, 199], this indicates a decrease in mitochondrial mass.

It is interesting to note that many of the nuclear DNA encoded subunits of
complex I, II, IV and V are found to be downregulated during cachexia by analysis of the genome-wide transcriptome datasets. Additionally, complex IV and Cycs protein expression are downregulated [28, 107, 108, 113, 115, 138, 166]. In literature, a decrease in muscle oxygen consumption, which is a measure for functional oxidative phosphorylation, is found during cachexia [29, 200, 201]. Likewise, genes involved in the TCA cycle are downregulated. This is confirmed by nuclear magnetic resonance (NMR) and gas chromatography/mass spectrometry (GC/MS) results, which indicate that the ATP synthesis rate and TCA cycle flux are reduced during cachexia [140]. Based on these results one would expect that lactate production increases due to the low mitochondrial energy production. However, lactate dehydrogenase RNA expression is found to be downregulated during cachexia in the genome-wide transcriptome datasets. Contrary, genes of the β-oxidation seem to be upregulated in cachectic mouse models.

It is proposed that increased ROS levels could drive the development of cancer-induced cachexia [61, 62, 202]. We found that there are indeed changes in the genes and proteins that facilitate ROS detoxification. Sod2 was found to be downregulated during cachexia in the genome-wide transcriptome datasets while no difference was found on protein level between healthy and cachectic animals [111, 114, 203, 204]. Additionally, catalase is upregulated during cachexia both in the genome-wide transcriptome datasets and in literature on protein level [177, 204, 205].

In summary, muscle mitochondrial functioning is downregulated at the level of oxidative phosphorylation genes, TCA cycle genes and mitochondria density markers in rodent models of cancer-induced cachexia. Additionally, there is a higher expression of some ROS detoxification genes and proteins.

**Summarizing discussion**

Our comprehensive analysis of literature data and its integration with publicly available data from genome-wide transcriptome datasets provides an up-to-date overview of changes in muscle mitochondrial pathways occurring in cancer-induced cachexia animal models. Figure 4.7 summarizes our general conclusions. Mitophagy is altered, there is a higher expression of genes involved in elongation and maturation of the phagophore and recognition of damaged mitochondria is upregulated. Together, this points towards a process of clearance of damaged parts of the muscle mitochondria. However, the upregulation of these processes might also suggest an underlying impaired mitochondrial clearance. Meanwhile gene expression of fission genes is lower, which is probably not beneficial for mitophagy. Additionally, also genes controlling fusion-related processes are downregulated, which could indicate involvement and decrease of biogenesis. Although most papers report a reduction of total Ppargc1a protein levels during cancer-induced cachexia, it is not clear what happens with biogenesis, as there is no apparent up- or downregulation of other genes or proteins involved. The changes in
Figure 4.6: Graphical integration of muscle mitochondrial function during cancer-induced cachexia in animal models, based on literature available in the public domain and data available in the public GEO database. Colour indicates when an upregulation (yellow, contiguous border), downregulation (blue, dotted border) or no significant difference (white, black thin border) was found. See Table A.2 (online) for full gene names. Data from literature: Graphs made based on literature of protein and mRNA data comparing muscle tissue of control animals and animals with cancer-induced cachexia. The number of publications that found no difference are spread over both sides of the graph. Phosphorylated proteins are depicted in a separate graph and indicated with a ‘p’ in front of the protein name. When the ratio between two forms of the protein was investigated it is indicated with ‘name1:name2’. Genome-wide transcriptome data: Figure made based on four genome-wide transcriptome datasets of control animals and animals with cancer-induced cachexia. Upregulation or downregulation is indicated when a significant difference was found in three or more of the datasets. Red arrows with flat arrow head = inhibition. Black arrows = stimulation. Green ovals with P indicate phosphorylation.
mitophagy, fission and fusion will result in a reduction of mitochondrial function. Moreover, there is less expression of genes involved in energy production, while there is a higher expression of genes involved in ROS detoxification. This increased expression of ROS detoxification genes could indicate that there is more ROS in the mitochondria, which will further contribute to a reduction of mitochondrial function. This conclusion of impaired mitochondrial functioning is further strengthened by reports of decreased mitochondrial density and mitochondrial oxygen consumption per unit of mitochondria during cancer-induced cachexia [29, 200, 201].

The lack of data on protein level and post-translational modifications of key proteins, and the lack of publicly available genome-wide transcriptome datasets from different animal models (3 out of the 4 datasets retrieved were from the C26 model) limits a thorough understanding of the mechanisms inducing mitochondrial dysfunction during cancer-induced cachexia. Differences between the levels of mRNA and protein in the cachectic animals might be caused by changes in the rate in which proteins are synthesized during cancer-induced cachexia [206, 207]. Post-translational modifications, like phosphorylation and deacetylation, are not often analysed in cachectic animal models. When looking at Ppargc1a for example, it is known that posttranslational modifications are key in the activity of Ppargc1a [77]. However, increasing mitochondrial biogenesis, by increased protein levels of Ppargc1a, did not prevent or reverse muscle wasting [117]. Similarly, Sirt1 is thought to be regulated mainly at the level of its activity by levels of its reaction co-substrate NAD+ and the balance between NAD+ and the deacetylation reaction co-product nicotinamide [208].

A next step would be to linking the data presented in this paper with data from patients with cancer-induced cachexia. However, this is likely to generate additional limitations and challenges. For example, in human patients there are many other factors, next to the tumour, which play a role in the mechanisms underlying muscle mass loss. Altered or decreased mitochondrial functioning is also known to play a role in other diseases, and thus co-morbidities might be contributing to this. For example, in mouse models, cancer-induced cachexia is provoked during an early life stage, while cancer patients who suffer from cachexia are mostly in a later stage of their life [209]. Older age is also associated with muscle loss (sarcopenia) and it is recognized for some time that mitochondria play a role in age-related sarcopenia as well [210, 211]. Additionally, altered morphology of mitochondria in general is seen in ageing [212]. Next to this, patients may change their lifestyle, for example by exercising less or even becoming physically inactive. Moreover, cancer patients are often anorectic which is likely to affect mitochondrial functioning [213, 214, 215]. Finally, mitochondrial dysfunction is also seen during fatigue, which is common during cancer [216]. The animal models included in our analysis also display some of these features. In the C26 model, the animals show reduced activity and even change their day-night rhythm, as also occurs in cancer patients [217]. In the Lewis Lung carcinoma model, food intake decreases when the tumour
develops, which is comparable to the anorexia development in cancer patients [218]. Last but not least, the treatment of the tumour can contribute to the wasting of the muscle, a process that can also been studied in animal models [138, 219].

In conclusion, the combined analysis of pooled data from available publications and publicly available genome-wide transcriptome datasets does not only provide novel insights in underlying pathways and potential target for intervention, but also shows that this relatively new in silico approach is promising and likely to be extendable to other diseases and clinical data. Our results also merit more emphasis on the role of mitochondrial dysfunction during cancer-induced cachexia. Since several key processes are regulated post translationally, analysis of protein modifications in cancer cachexia is warranted.

![Cancer Induced-Cachexia Diagram](image)

**Figure 4.7:** Interplay between the different mitochondrial processes during cancer induced-cachexia. A summary of the findings presented in this review. The changes in mitophagy and fission and fusion are hypothesized to contribute to a decrease in mitochondrial functioning resulting in a decreased ATP production in the presence of more ROS, for which an increased ROS detoxification is needed. The estimated average of the expression of many different genes that were found to be changed, is crudely indicated in the figure with small arrows and one question mark.

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Conflict of interest statement

Miranda van der Ende, Sander Grefte, Rogier Plas, Jocelijn Meijerink, Renger Witkamp, Jaap Keijer and Klaske van Norren declare that they have no conflict of interest.
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Chapter 5

Mitochondrial gene expression in cancer cachectic models

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Manuscript in preparation
Abstract

BACKGROUND To unravel the mechanisms of cancer cachexia, we need to understand how the muscle is affected. In this regard, new insights have been gained in recent years pointing to an important role of mitochondrial processes. However, precise effects of cachexia on expression of nuclear encoded muscle mitochondrial genes are not known. Therefore, the goal of this study was to explore if 1) effects of different cell lines known to induce cachexia when injected to mice (KPC, 4662, LLC and C26), are comparable to each other in an in vitro model, and 2) effects obtained with in vitro models for cachexia are comparable with those from in vivo cachectic mouse models.

METHODS Gene expression results of our in vitro models, murine C2C12 myoblasts/myotubes incubated with conditioned media (CM) of the KPC, 4662, LLC and C26 cell lines, were combined to create a general cachexia model (GCM). Next these results and data of publicly available datasets obtained from in vivo cachectic mouse models were studied in an untargeted manner using gene set enrichment analysis and the MitoCarta 3.0 dataset.

RESULTS About 70% of the significant differentially expressed MitoCarta genes were downregulated, while the whole genome expression had more upregulated genes. Conditioned media of the cachexia-inducing cancer cell lines KPC, 4662 and LLC were quite similar in causing changes in C2C12 muscle cells on MitoCarta gene expression level, while the effects of the C26 CM were small. Changes in MitoCarta genes most abundantly seen were related to energy metabolism processes (oxidative phosphorylation, TCA cycle or pyruvate metabolism). The in vivo data was quite comparable with the in vitro data, and eight MitoCarta genes were found to overlap between the GCM and the in vivo models. CONCLUSION In both in vitro and in vivo cachexia models comparable mitochondrial changes on global gene expression level were found in muscle cells using the MitoCarta 3.0 dataset. These MitoCarta genes were abundantly downregulated and oxidative energy metabolism processes was particularly affected. Dut, Ndufc1, Idh3a, Idi1, Cbr2, Coq7, Ndufa1 and Qdpr form a potential molecular signature of cancer cachexia.
Abbreviations

BP Biological processes (as search term)
C26 Murine colon 26 carcinoma cell line
C2C12 Murine myoblast and myotube cell line
CK Creatine kinase
CM Conditioned medium
DEGs Differentially expressed genes
DM-C2C12 C2C12 differentiation medium
DMEM Dulbecco’s Modified Eagle’s Medium
EM Enzyme mixture
FC Fold Change
FCS Fetal calf serum
FDR False discovery rate
GCM General Cachexia Model
GEO Gene Expression Omnibus
GM Growth medium
GM-LG Growth medium low glucose
GO Gene ontology
GSEA Gene set enrichment analysis
KPC Murine pancreatic ductal adenocarcinoma cell line
LLC Lewis Lung Carcinoma cell line
OD Optical Density
PBS Phosphate buffered saline
RM Reaction mixture
TCA cycle Tricarboxylic acid cycle
Introduction

Cancer patients suffer from fatigue, reduced appetite and declining mobility, resulting in decreased quality of life [1, 2, 3]. Advanced stage cancer patients often suffer from cachexia, a syndrome characterized by loss of muscle mass with or without loss of fat mass [4]. It substantially contributes to the morbidity and mortality of cancer patients [5] and impacts their quality of life [6]. Development of effective treatment options of cachexia is hampered by the diversity of the patient population, their different disease types, and stages and treatment of the disease itself [7, 8]. Therefore, the prevailing viewpoint is that treatment of cachexia should be personalised and multimodal [9], using combinations of drug(s), nutrition and therapeutic exercise [10, 11].

To develop effective preventive or curative intervention strategies, we need to better understand how the muscle is affected during cachexia, i.e. we need to know the molecular mechanisms associated with cancer cachexia. Literature indicates that mitochondrial function, next to protein balance, structural remodelling and impaired regeneration, is hampered in the cachectic muscle [12, 13, 14, 15, 16, 17, 18]. The expression of genes and proteins involved in mitochondrial fusion, fission, ATP production and mitochondrial density are decreased, while that of genes and proteins involving reactive oxygen species (ROS) detoxification and mitophagy is increased [12]. In addition, research papers indicate that several mitochondrial processes are not only changed during clinically manifest cancer cachexia, but also contribute to the early stages of cachexia development [19, 20, 21, 22, 23, 24, 25].

Several observations point to a role for mitochondria-related changes in cachectic muscle tissue. In a Lewis Lung Carcinoma (LLC) cachectic mouse model, the mitochondrial respiratory control ratio was about 50% lower in muscle after two weeks of tumour development compared to control animals [26]. Additionally, a study using the same model showed that after four weeks of tumour development the oxidative capacity and mitochondrial content decreased in the muscle of the cachectic animals [27]. Furthermore, body wasting could be partly counteracted by treatment with the mitochondria-targeted compound SS-31 [28]. In patients with gastrointestinal cancer cachexia, electron microscopic analysis revealed an increase in the intermyofibrillar mitochondrial area accompanied by increased gene expression of FISSION1 [29]. In an in vitro model employing the C2C12 murine myoblasts and myotubes significant alterations in skeletal muscle mitochondrial function and lipid accumulation under influence of breast cancer-derived factors were found [30]. In addition, our research has established inflammation as the major upregulated process after exposure of C2C12 cells to cancer cell line conditioned medium. In contrast, the downregulated processes extracted from whole genome gene expression data for the individual cell lines pointed to mitochondria-associated pathways, which were not further examined (chapter 3). Overall, it has become clear that mitochondria are strongly implicated in cancer cachexia, but little is
known about the associated molecular pathways.

The goal of this study was to fill this gap and to provide a better insight in how mitochondria, and associated mitochondrial processes, are changed during cancer cachexia. To this end, we used RNAseq gene expression data from differentiating C2C12 cells exposed to conditioned medium (CM) of four different cachexia inducing cell lines; KPC, 4662, LLC and C26 (chapter 3). Different from chapter 3, the datasets from the individual cell lines were pooled to provide a more general cachexia model (GCM) and the downregulated processes were analysed in detail. Changes specifically in mitochondrial gene expression were analysed in an untargeted manner, using the MitoCarta 3.0 dataset [31] and compared to publicly available gene expression datasets of cachectic mouse models. This resulted in improved insight in which mitochondrial processes are changed during cancer cachexia in cell and preclinical models.

Materials & Methods

Culture media and cells

The following culture media were used; C2C12 differentiation medium (DM-C2C12; DMEM (GlutaMAX with pyruvate) supplemented with 2% heat inactivated horse serum (30 minutes 56°C) and 1% Penicillin/Streptavidin (P/S, Penicillin (10,000 IU) and Streptomycin (10,000 µg/mL))), growth medium (GM; DMEM supplemented with 10% fetal calf serum (FCS) and 1% P/S) and GM-low glucose (GM-LG; DMEM (low glucose GlutaMAX with pyruvate) supplemented with 10% FCS and 1% P/S). Murine colon 26 carcinoma (C26, kindly obtained from Donna McCarthy, Ohio State University, USA), LLC (kindly obtained from Josep Argiles, University of Barcelona, Spain) and C2C12 (kindly obtained from Francina Dijk, Nutricia Research, the Netherlands) cells were maintained in GM. Murine pancreatic ductal adenocarcinoma cell line (KPC) and the genetic variation KPC-4662 (4662) (kindly obtained from Elizabeth Jaffee and Daniel Marks, Oregon Health and Science University, USA) were maintained in GM-LG.

Conditioned Medium

To harvest conditioned medium (CM), cells from 90% confluent flasks were taken and seeded 1:5 (C26 and LLC) and 1:3 (KPC and 4662) in a T75 flask. Cells were allowed to adhere to the flask for two hours in their maintenance medium (GM or GM-LG), thereafter cells were washed twice with phosphate buffered saline (PBS) and 12 ml of DM-C2C12 was added. All cells were incubated at 37°C and 5% CO2 for 96 hours to create CM. After 96 hours, CM was harvested and pooled per cell line, centrifuged (5 minutes at 500 g), aliquoted and stored at -20°C.
C2C12 culture and harvest

C2C12 were maintained sub-confluent in GM. For the differentiation experiments cells were seeded in 24 wells plates at 1.14x105 cells/ml and 1 ml per well. After 24 hours medium was changed to DM (control) or DM with 33% CM. At day 3 of the differentiation, medium of all conditions were refreshed. Additionally, on day 3 and 5 samples were harvested (triplicate per day) Experiments were repeated (N) 3 times. Cells were washed once with PBS before harvesting. For RNA samples cells were collected with TRIzol Reagent (Invitrogen, 15596018). Lysates were homogenized and stored at -20°C.

Creatine Kinase assay

Protein concentrations were measured using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, 23225) following the manufacturer’s instructions. Protein lysates were diluted to 0.025 mg/ml using CelLyticM (Sigma, C2978). The reaction mixture (RM, Table S5.1) and enzyme mixture (EM) were made fresh every experiment. The chemicals were dissolved in MilliQ water and the pH was set to 7.4 with acetic acid. The EM consisted of 42 µL glucose-6-phosphate dehydrogenase (140 units/mg protein, Sigma, 10127671001) and 34 µL hexokinase (450 units/mg protein, Sigma, 11426362001) dissolved in 1016.88 µL PBS. To measure creatine kinase (CK), 20 µL sample or blank were pipetted per well in the 96 wells plate and 150 µL of RM was added. Plates were incubated at 37°C for 10 minutes and 10 µL EM was added. Absorption was measured at 340 nm with a kinetic program for 44 minutes with an interval of 24 seconds at 37°C. Between every measurement the plate was shaken for 1 second at medium speed, using the Spectramax M2 (Molecular Devices). CK activity was calculated by using a lag time of 500 sec and Vmax (absorbance in milli-units per minute) was calculated over 90 points. After subtraction of the background (blank) the following calculation was used: CK activity (µmol/mg⁻¹/min⁻¹) = (Vmax x reaction volume in µL) / (6220 (molar absorption coefficient of NADPH at 340 nm) x cm height (= 0.5625 cm for 180 µL volume) x mg protein in the well x 1000 (conversion from milli-OD to OD)). Figures were made in Graphpad Prism 5 (Graphpad Software Inc., La Jolla, California, USA). CK activity of the control was set to 100% to which CK activity of all other samples is related and ANOVA was performed using R [32] in Rstudio [33], post hoc testing was done with the Tukey test. Residuals were tested with the Shapiro-Wilk test for normality.

RNA isolation and sequencing

TRIzol C2C12 samples were used for sequencing. Samples were thawed and 0.2 mL chloroform per 1 mL of TRIzol was added and incubated for 2–3 minutes. Samples were centrifuged for 15 minutes at 12000 × g at 4°C and the aqueous
phase was transferred to a new tube. From this aqueous phase the RNA was isolated using RNeasy Micro Kit (Qiagen, 74004) following the manufacturer’s instructions. RNA was eluted in 14 µL RNase-free water and RNA yield was determined by nanodrop. RNA quality was determined using the Bioanalyzer (Agilent) and the RNA 6000 Nano Kit (Agilent, 5067-1511). Technical triplicates were pooled, resulting in a total of three biological replicates per conditions. All samples were diluted to a concentration of 40 ng/µL and shipped in dry ice to BGI tech solutions (Hongkong). At BGI tech solutions, DNBseq eukaryotic transcriptome re-sequencing (strand specific) was done applying at least 20M clean reads per sample. Filtered sequencing reads were collected (6-9 GB data per sample).

Transcriptome analysis

The analysis of the data was done on the high performance computer infrastructure hosted by Wageningen University & Research. Quality control was done using FastQC [34], next, alignment was done using STAR [35]. Reads were counted using HTSeq [36] and files were exported to do further analysis using R [32] in Rstudio [33]. DESeq2 [37] was used to detect differentially expressed genes (DEGs), P-values were adjusted for multiple testing by the Benjamini-Hochberg false discovery rate (FDR) procedure [38]. An adjusted p-value (or FDR) of p < 0.05 was considered significantly regulated. Plots were made using the R libraries ggplot2 [39] and EnhancedVolcano [40]. Changes in individual genes were related to changes in pathways by gene set enrichment analysis (GSEA) [41], analysis and visualization was performed using the package clusterProfiler [42] and DOSE [43]. Analysis was done for Biological Process (BP) gene ontology (GO) terms and only gene sets consisting of more than 50 and fewer than 1000 genes were considered. For each comparison, genes were ranked on their GeneRatio (the number of genes of input list associated with the given GO term / the total number of input genes). Data has been submitted to the Gene Expression Omnibus (GEO) database under accession number GSE000000.

Analysis of GEO datasets

Data was downloaded from the GEO database (https://www.ncbi.nlm.nih.gov/geo/). RNA sequencing datasets of muscle from mouse models using the KPC, 4662, LLC or C26 cell lines were extracted from the GEO database (Table S5.2). DESeq2 [37] was used to detect DEGs, p-values were adjusted for multiple testing by the Benjamini-Hochberg FDR procedure [38]. Five datasets were extracted and analysed using this method: GSE114820, GSE123310, GSE132120, GSE152553 (spilt in male (M) and female (F) data), whereas GSE173250, GSE65936, GSE175570 and GSE133524 were excluded due to the format of the counts table provided in the GEO database, these are FPKM or VOOM values which cannot be used for DESeq2 analysis. Contact persons of these
datasets were kindly asked to provide us with original count tables, but unfortunately no response was received.

**MitoCarta 3.0 Analysis**

The Human MitoCarta 3.0 inventory was used to analyse genes encoding mitochondrial proteins [31]. The number of DEGs was calculated with the Padj cut-off value 0.05. To get a more detailed insight in which metabolic processes were affected, DEGS were divided into categories based on mitopathways, which was subsequently refined using NextProt and scientific literature. The DEGs were divided in: Oxidative Phosphorylation: CI, Oxidative Phosphorylation: CoQ, Oxidative Phosphorylation: CIII, Oxidative Phosphorylation: CIV, Oxidative Phosphorylation: CV, Oxidative Phosphorylation: Auxiliary, TCA cycle, Pyruvate metabolism, Fatty acid metabolism, Cholesterol/steroid metabolism, DNA/nucleotide metabolism, Translation, Amino acid metabolism, Protein import/processing/repair, Mitochondrial dynamics, B vitamin metabolism, Calcium transport, Iron metabolism, Small molecule transport, Antioxidant defence and oxidative signalling, Pro-apoptosis, Other, Unknown.

**Results**

**C2C12 myotubes exposed to conditioned media from cachexia-inducing cancer cell lines display predominantly upregulated DEGs**

Creatine kinase activity of differentiating C2C12 cells exposed, from the start of differentiation, to CM of KPC, 4662, LLC and C26 significantly decreased at day 3, 5 and 7 of differentiation, as published (chapter 3, Supplemental figure 5.1). To investigate general effects of cachexia, RNA sequencing results of differentiating C2C12 cells at 5 days of differentiation and exposure to CM of KPC, 4662, LLC and C26 CM were put together (n=12) to create a general cachexia model (GCM). 2090 significant DEGs of a total of 22541 genes were identified (padj < 0.05) when the GCM were compared to the control medium. A subset of 813 significant DEGs had log2 FC levels above 0.5 or below -0.5 (padj < 0.05), of which 489 DEGs (60%) were upregulated and 324 DEGs (40%) were downregulated. The higher number of DEGs with a positive log2 FC indicates a tendency for more pronounced upregulation compared to downregulation (Figure 5.1).
GSEA shows that mitochondrial pathways are downregulated in a cachectic model

Next, the GCM was compared to the separate incubations of C2C12 with the KPC, 4662, LLC and C26 CM using GSEA for the GO terms for biological processes (BP) (Figure 5.2). Not all of the GO terms found in the single incubations were present in the general cachectic model, which shows that the general cachectic model is not a copy of one of the cachexia-inducing cancer cell lines (KPC, 4662, LLC or C26). The CM of 4662 had the largest correspondence in both up and downregulated GO terms, while the C26 CM contribution was smaller and therefore fewer C26 GO terms end up in the overall top 20. The top 20 upregulated GO terms found after incubation with KPC CM and LLC CM were also found after incubation with 4662 CM. For the downregulated GO terms this is the case for all but one (sarcomere organization). A large number of the upregulated GO terms were related to the immune response in the GCM as well as in the separate CM, as was observed before (chapter 3). All downregulated GO terms were either related to mitochondrial functioning or to muscle functioning.

To better understand the behavior of mitochondria in cachexia, we focused on mitochondrial related GO terms in the GCM and found these to be related to mitochondrial translation, ATP synthesis coupled electron transport, mitochondrial gene expression and energy derivation by oxidation of organic compounds. These effects were also found for the different individual cachexia-inducing cancer cell lines, except for C2C12 exposed to C26 CM. A
detailed overview of the most changed GO pathways for the GCM showed that the highest regulated pathway is the mitochondrial respiratory chain complex assembly, which is suppressed (Supplemental figure S5.2). Of the top 25 downregulated genes in the GCM one is present in the MitoCarta 3.0 set, this is the sterol 26-hydroxylase Cyp27a1 (Table S5.3).

**Figure 5.2:** Highest significant top 20 upregulated (A) and downregulated (B) GO terms in Biological Process (BP) on day 5 after incubation of differentiating C2C12 with CM of 4 cachexia-inducing cancer cell lines (KPC, 4662, LLC and C26), for the general cachexic model (GCM) and the individual cell lines, padj < 0.05. * = the name of this GO term was shortened, the full name is: Adaptive immune response based on somatic recombination of immune receptors build for immunoglobin superfamily domains.

**About 70% of the MitoCarta DEGs are downregulated in an in vitro cachetic model**

To obtain a better insight specifically in alterations of mitochondrial processes, the MitoCarta 3.0 geneset is employed. First, the number of MitoCarta DEGs of the GCM were compared to the incubations with the CM of the individual cell lines (KPC, 4662, LLC and C26) at a p-value cut-off of 0.05 (Figure 5.3 A). The LLC incubation shows the most MitoCarta DEGs (199; compared to 123 for the GCM) and C26 incubation caused the least MitoCarta DEGs (50 DEGs). KPC (173 DEGs) and 4662 (150 DEGs) incubation were in between. The percentage of downregulated DEGs was 75%, 83%, 77% and 48%, for KPC, 4662, LLC and C26, respectively. Next, we analysed the GCM for the number of MitoCarta
DEGs using different P-values (Figure 5.3 B). As expected, the total number of MitoCarta DEGs is higher with a lower p-value cut-off of 0.5 (530 MitoCarta DEGs) compared to a p-value cut-off of 0.05 (123 MitoCarta DEGs). However, the percentage of downregulated MitoCarta DEGs at all four different p-values remains similar at 70% and is therefore highly consistent.

Of the 123 MitoCarta DEGs in the GCM at \( \text{padj} < 0.05 \), 21 were also present in all four CM exposures (KPC, 4662, LLC and C26), of which 10 were upregulated and 11 were downregulated. Also in the top 10 downregulated (Table 5.1) and the top 10 upregulated (Table S5.2) MitoCarta DEGs, a large overlap between the GCM and the four CM exposures can be found.

**Table 5.1: Top 10 downregulated MitoCarta DEGs at p-value (padj) < 0.05.**

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Log2FC</th>
<th>General process</th>
<th>KPC</th>
<th>4662</th>
<th>LLC</th>
<th>C26</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ckmt2</td>
<td>-1.15773</td>
<td>Oxidative Phosphorylation, Auxiliary</td>
<td></td>
<td></td>
<td>YES</td>
<td></td>
</tr>
<tr>
<td>Cyp27a1</td>
<td>-0.93603</td>
<td>Cholesterol and steroid metabolism</td>
<td>YES</td>
<td>YES</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td>Coq8a</td>
<td>-0.70923</td>
<td>Oxidative Phosphorylation, CoQ</td>
<td>YES</td>
<td>YES</td>
<td></td>
<td>YES</td>
</tr>
<tr>
<td>Cox6a2</td>
<td>-0.64521</td>
<td>Oxidative Phosphorylation, CIV</td>
<td>YES</td>
<td>YES</td>
<td></td>
<td>YES</td>
</tr>
<tr>
<td>Osgepl1</td>
<td>-0.62732</td>
<td>Translation</td>
<td>YES</td>
<td>YES</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td>Me2</td>
<td>-0.61571</td>
<td>TCA cycle</td>
<td>YES</td>
<td>YES</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td>Idi1</td>
<td>-0.54977</td>
<td>Cholesterol and steroid metabolism</td>
<td>YES</td>
<td>YES</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td>Pdha1</td>
<td>-0.48676</td>
<td>Pyruvate metabolism</td>
<td>YES</td>
<td>YES</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td>Ucp2</td>
<td>-0.48458</td>
<td>Small molecule transport</td>
<td>YES</td>
<td>YES</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td>Dbi</td>
<td>-0.47966</td>
<td>Fatty acid metabolism</td>
<td>YES</td>
<td>YES</td>
<td>YES</td>
<td>YES</td>
</tr>
</tbody>
</table>
**Figure 5.3:** Differentially expressed MitoCarta genes in 5 days differentiated C2C12 cells incubated with CM of cachexia-inducing cancer cell lines. (A) The number of down- and upregulated DEGs at p-value (padj) < 0.05 of GCm compared to the individual exposures (KPC, 4662, LLC and C26). (B) The ratio of down- and upregulated MitoCarta DEGs in the GCM is stable at different p-value (padj) cut-offs.

**Table 5.2:** Top 10 upregulated MitoCarta DEGs at p-value (padj) < 0.05.

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Log2FC</th>
<th>General process</th>
<th>KPC</th>
<th>4662</th>
<th>LLC</th>
<th>C26</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aldh1l1</td>
<td>1.417684</td>
<td>B vitamin metabolism</td>
<td>YES</td>
<td>YES</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td>Cyp11a1</td>
<td>1.198069</td>
<td>Cholesterol and steroid metabolism</td>
<td></td>
<td></td>
<td>YES</td>
<td></td>
</tr>
<tr>
<td>Mucub</td>
<td>1.062955</td>
<td>Calcium transport</td>
<td>YES</td>
<td>YES</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td>Sxsn5</td>
<td>0.939307</td>
<td>Small molecule transport</td>
<td></td>
<td>YES</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nt5dc2</td>
<td>0.924343</td>
<td>Other</td>
<td>YES</td>
<td>YES</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td>Prdx4</td>
<td>0.696773</td>
<td>Antioxidant defense, oxidative signalling</td>
<td>YES</td>
<td>YES</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td>Nipsnap1</td>
<td>0.618871</td>
<td>Other, neurotransmitter</td>
<td>YES</td>
<td>YES</td>
<td></td>
<td>YES</td>
</tr>
<tr>
<td>Pnpo</td>
<td>0.577955</td>
<td>B vitamin metabolism</td>
<td></td>
<td></td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td>Sardh</td>
<td>0.566066</td>
<td>Amino acid metabolism</td>
<td>YES</td>
<td>YES</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td>Cpt1a</td>
<td>0.537925</td>
<td>Fatty acid metabolism</td>
<td>YES</td>
<td>YES</td>
<td>YES</td>
<td>YES</td>
</tr>
</tbody>
</table>
Mitochondrial energy metabolism processes are affected in the GCM

To analyse the mitochondrial processes in more detail, the 123 MitoCarta DEGs that were significantly changed in the GCM, were examined in more detail. Of the 123 DEGs, 39 DEGs (32%) were upregulated and 84 DEGs (68%) were downregulated. These DEGs were assigned to processes, of which an overview can be found in Table S5.4. Here, we will describe them in a grouped manner. Oxidative phosphorylation was the most prominent process with 24 of 123 genes (20%) affected, which were all downregulated (Figure 5.4). These differential GCM OXPHOS genes belonged to Complex I, Coenzyme Q, Complex III, Complex IV and Complex V, indicating that the downregulation is not restricted to one specific complex of the oxidative phosphorylation. The suggestion of a general decrease in oxidative metabolism is enforced by the concomitant downregulation of TCA cycle and pyruvate metabolism, and the upregulation of amino acid catabolism (amino acid metabolism). Together these three processes represented 17 of 123 genes (14%). Fatty acid metabolism comprises various processes, including membrane lipid biosynthesis, regulation of homeostasis and fatty acid breakdown. Whether breakdown was up- or downregulated is difficult to interpret, since gene expression changes were insufficiently consistent.

A second striking feature was the downregulation of the expression of 6 genes related to apoptosis, of which 5 are pro-apoptotic. These are Bcl2l13 and Mtc2h2 and the key effectors Bax, Bid and Bbc3. BBC3 is better known as PUMA, the apoptosis effector of P53 surveillance [44]. Possibly related, all seven differentially expressed mitochondrial dynamics genes were downregulated. Among these seven genes are Dnm1l, Mtf and Marchf5 related to fission, Mfn2 related to fusion, and Opa1 related to fission-fusion homeostasis. This suggests that especially fission, but also fusion, seemed to be downregulated. Furthermore, Armcx3, a regulator of mitochondrial distribution was downregulated. In addition to mitochondrial dynamics, calcium influx is a major determinant of apoptosis. Three genes related to calcium import were regulated; the negative regulator of calcium uptake Mcub was upregulated, while the Micu1 and Smdt1 were downregulated. In addition, oxidative signalling seemed to be affected with downregulation of Sod2 and upregulation of Prdx3 and Prdx5. Together, the regulation of these processes may suggest upregulation of ‘mitochondrial survival’ and the changes in DNA and nucleotide metabolism may also be interpreted in this manner. For example, the protein encoded by Dut protects against deoxy-uridine incorporation into DNA.

In addition to the differential expression of genes related to oxidative metabolism and ‘mitochondrial survival’, several changes seem to be related to mitochondrial protein homeostasis. These are protein import (especially Dnlz, Timm23, Pmpca and Tomm70) and biosynthesis of iron-sulphur clusters (Abcd7, Iscu, Nfu1), which were all downregulated. Fkbp10 (protein folding) and Slc25A37 (iron import), as well as genes encoding proteins involved in
FMN/FAD synthesis and import (Pnpo, Slc25a32) were upregulated. Myg1, which controls mitochondrial translation to balance mitochondrial vs nuclear genes, was upregulated. Three t-RNA synthetases were downregulated. Although 2 mitochondrial ribosomal genes were downregulated, Mrpl1 and Mrpl42, there was no sign of massive changes in mitochondrial ribosomal gene expression.

Gene expression changes have to be interpreted with caution and especially their interrelation, however, the changes seem to be strongly consistent with a downregulation of oxidative metabolism, an increase in mitochondrial survival and alterations in mitochondrial protein homeostasis. In addition, to these categories, the expression of four genes related to neurotransmitter metabolism (classified under Other) were mostly upregulated, except from Qdpr which was downregulated. Two genes related to folate/pteridine metabolism (Aldh1l1 and Mthfd1l) were upregulated and four genes related to sterol/steroid metabolism were differentially regulated, of which the two genes encoding proteins involved in cholesterol metabolism (Idi1 and Cyp27a1) were downregulated.

Figure 5.4: Differentially expressed MitoCarta genes in the general cachexia model (GCM) analysed at p-value (padj) < 0.05.
Comparison of the muscle MitoCarta gene expression of the GCM to data of cachectic mouse models

To examine to which extent the in vitro observations are also reflected in vivo, gene expression data from skeletal muscle of published pre-clinical cachexia models were examined. GSE114820, GSE123310, GSE132120, GSE152553 (split in male (M) and female (F) data) and GSE173250 were analysed for their MitoCarta DEGs and compared to the GCM (Figure 5.5). Here, on average 78% of the DEGs were downregulated at p-value (padj) < 0.05, which is in line with the results found for the in vitro models. There is, however, a large variation in the number of DEGs between the different datasets. On average 343 MitoCarta DEGs were found in the in vivo datasets.

Figure 5.5: Differentially expressed MitoCarta genes of different cachectic mouse models analysed at p-value (padj) < 0.05. GCM = general cachexia model.

The various in vivo models contained 37 overlapping MitoCarta DEGs. These were sorted into the processes that were found in the GCM analysis (Figure 5.6). The process that is mostly found is oxidative phosphorylation (9 MitoCarta DEGs; 24%), which is downregulated as are the two genes related to TCA cycle and one of the two genes related to amino acid metabolism (amino acid catabolism), the latter category seems oppositely regulated of what was observed in vitro. The upregulation of Oxct1 (fatty acid metabolism) implies an upregulation of ketone body catabolism. No genes related to
pyruvate metabolism were seen. Six MitoCarta DEGs were related to ‘mitochondrial survival’ (pro-apoptosis, mitochondrial dynamics, calcium transport and antioxidant defence, oxidative signalling) and none MitoCarta DEGs to protein homeostasis.

![Figure 5.6](image)

**Figure 5.6:** Differentially expressed MitoCarta genes of different cachectic mouse models analysed at p-value (padj) < 0.05. GCM = general cachexia model.

Zooming in on individual genes, eight MitoCarta DEGs were found to be differentially expressed in both the *in vitro* GCM and in all *in vivo* models. These are *Dut* (Nucleotide metabolism), *Ndufc1*, *Ndutaf5*, *Coq7*, (Oxidative phosphorylation), *Idh3a* (TCA cycle), *Idi1* (Cholesterol and steroid metabolism), *Cbr2* (other, metabolism), and *Qdpr* (other, neurotransmitter) (Table 5.3). *Dut* was upregulated in the GCM, while all other genes were downregulated. The *in vivo* models corresponded to the GCM (and all four individual CM exposures), except for *Dut*, since *Ndufc1*, *Ndutaf5*, *Coq7*, *Idh3a*, *Idi1*, *Cbr2*, *Qdpr* and *Dut* were all downregulated.
Table 5.3: Log2FoldChange of the overlapping MitoCarta DEGs between the GCM and the in vivo models.

<table>
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<th>Symbol</th>
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<th>LLC - GSE132120</th>
<th>LLC - GSE152553_F</th>
<th>LLC - GSE152553_M</th>
<th>LLC - GSE173250</th>
<th>KPC - GSE123310</th>
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Discussion

Mitochondria in skeletal muscle cells are strongly implicated in cancer cachexia, but little is known in what manner they are affected. The goal of this study was to fill this gap and to provide a better insight in how mitochondria, and associated mitochondrial processes, are changed during cancer cachexia. To explore this, both in vitro and in vivo models were employed that make use of cell lines (KPC, 4662, LLC, C26) known to induce cachexia in mice. The effects of CM obtained from these cell lines on mitochondrial gene expression in differentiating C2C12 were studied, as well as by analysing publicly available data of cachectic mouse models. The CM of KPC, 4662 and LLC were found to be quite similar in inducing changes on MitoCarta gene expression level, while the effects of the C26 CM were only small. By integrating the datasets of C2C12 cells incubated with the KPC, 4662, LLC and C26 CM, a general cachexia model (GCM) was composed. About 70% of the significant MitoCarta DEGs present in GCM were downregulated, while the whole genome expression displayed more upregulated DEGs. The most evident regulated process was the downregulation of oxidative phosphorylation. This was also the most affected process in the in vivo models. Of the 37 overlapping individual MitoCarta genes that were found in all in vivo datasets, eight genes overlapped with the GCM (Dut, Ndufc1, Idh3a, Idi1, Cbr2, Coq7, Ndufaf5 and Qdpr). In all cases, except from Dut, the direction of expression of these genes was identical in vitro and in vivo.

One of the top downregulated processes in the overall gene expression data set is aerobic respiration, which consists of oxidative phosphorylation, TCA cycle and pyruvate metabolism. It was most affected in the MitoCarta gene sets of both the in vitro and in vivo cachexic models examined here. Except for complex II, all complexes of oxidative phosphorylation seem to be downregulated. Next to this, genes of the TCA cycle were also downregulated. In addition, four out of eight genes that overlap between the in vitro and in vivo models are related to oxidative phosphorylation (Ndufc1, Ndufaf5, Coq7) or TCA cycle (Idh3a). This downregulation agrees with the fatigue that is observed in cachexia patients, as the muscle is not able to generate energy
for skeletal muscle contraction [45]. While our data seem robust and agree with other observations [46, 29, 47, 48], it would be relevant to complement our analysis with functional data, for example by analysing mitochondrial oxygen consumption of a biopsy of cachexic muscle. In vitro, we also observed a strong downregulation of pyruvate metabolism, which was not seen in vivo. These differences may be due to the vastly different metabolic conditions, with abundant glucose being present in culture media in vitro. To further investigate this, it may be relevant to measure the response of C2C12 cells to CM using different media, for example, galactose rather than glucose to enforce a dependence on oxidative metabolism [49].

Remarkably, pro-apoptotic genes and genes involved in mitochondrial dynamics (mainly fission) were downregulated. We expected otherwise, since the muscle is wasting during cachexia. The downregulation that was seen in vitro seemed to be protective, which was enforced by the upregulation of Mcub and the downregulation of Micu1 and Smdt1, which together suggest that uptake of calcium into mitochondria is inhibited. The reason behind this 'mitochondrial survival phenotype' is unclear, but may be due to the rich culture conditions employed, also because it was not observed in the in vivo data. In vivo, no pro-apoptotic genes were identified among the 37 common differentially expressed genes. Similarly, protein homeostasis was clearly affected in vitro, while this was less evident in vivo, where protein import was only represented by Tomm40l, which was downregulated, similar to the downregulation of e.g. Tomm70a and Timm43 in vitro. Although highly speculative, this may be due to the fact that we studied differentiating muscle cell in vitro, which display growth and hence require substantial protein import, while in vivo most muscle cells will be differentiated myotubes/myofibres, which are likely less dependent on protein import. The difference in differentiation state may possibly also explain why the expression of Dut is different between in vitro (upregulated) and in vivo (downregulated). The protein encoded by Dut is an dUTPase, that removes PP from dUTP, to prevent incorporation of dUTP in DNA. Likely preventing toxic levels of dUTP is more important in active differentiating cells that may have higher levels of nucleotide biosynthesis compared to fully differentiated muscle cells [50]. Also here, it may be of interest to expose fully differentiated C2C12 myofibres to CM of the cachexia inducing cancer cell lines and analyse the gene expression response to come to a better understanding of the effect of differentiation state in the response to CM.

As indicated above, differences exist between the in vitro and the in vivo data. Part of this may be due to the different number of MitoCarta genes that are present in the GCM (123 DEGs) and emerge from the in vivo studies (37 DEGs), which will likely affect representation of especially ‘smaller’ processes. We also noted that growth conditions and differentiation status may play a role in the apparent discrepancy. Despite this, we were able to identify eight MitoCarta genes that were differentially expressed between in vitro and in vivo, representing a robust signature of cancer cachexia, also because all-but-one displayed the same direction of regulation in vitro compared to in...
vivo. Among these genes were Ndufc1, Ndufaf5 and Coq7 involved in oxidative phosphorylation, the TCA cycle gene Idh3a, and the dUTP detoxification gene Dut. The three other genes are Idi1, Cbr2 and Qdpr. The function of Cbr2 is not fully resolved. It was primarily investigated in pulmonary cells, where it has been linked to metabolism of endogenous carbonyl compounds [51], metabolism of prostaglandins [52] and to regulation of Wnt signalling [53]. Idi1 is involved in isoprenoid biosynthesis [54]. Isoprenoid biosynthesis is important for both cholesterol biosynthesis and steroid hormone biosynthesis. In the in vitro dataset several other genes related to cholesterol metabolism and steroid biosynthesis (Cyp11a1 and Fdxr upregulated, Cyp27a1 and Idi1 downregulated) were differentially regulated. Although this has to be confirmed at the metabolite level, the possible regulation of cholesterol/steroid hormone metabolism may be relevant in the context of muscle wasting, since anabolic androgenic steroids have been demonstrated to increase fat-free mass, muscle mass and strength [55, 56]. Of interest in this context is Mono-ADP ribosylhydrolase 1 (Macrodi; in Other), which was upregulated, was shown to enhance estrogen receptor 1-mediated transcription activity [57]. The protein encoded by Qdpr (downregulated in the GCM) is a key enzyme in the biosynthesis of tetrahydropteridin (BH4). In the larger in vitro dataset also three genes related to folate/pteridine metabolism were identified, including Mthld11, which is upregulated, this we have previously linked to BH4 biosynthesis in adipose tissue [58]. BH4 is an essential cofactor for phenylalanine, tyrosine, and tryptophan hydroxylases, and thus plays an important role in amino acid metabolism. It is known that amino acid and protein synthesis is hampered in cachectic muscle tissue [59] and amino acid uptake in the muscle is reduced [60]. Increased quantities of lysine, arginine, proline, and tyrosine and reduced quantities of glutamate and aspartate are seen in a mouse model for cachexia, which is related to breakdown of muscle proteins [61]. The observed upregulation of Qdpr may possibly be to restoration of amino acid homeostasis in wasting muscle. In addition, tyrosine hydroxylase is a key enzyme in the synthesis of melanin as well as in the neurotransmitters L-dopa, dopamine, nor-epinephrine and epinephrine. Cachectic patients are known to have increased plasma concentrations of nor-adrenaline, epinephrine, cortisol, and aldosterone [62]. Finally, tryptophan hydroxylase is essential for the biosynthesis of serotonin, which regulates skeletal muscle mass and metabolism [63, 64]. Upregulation of Qdpr may thus also serve the biosynthesis of neurotransmitters in cachetic skeletal muscle. Metabolite analysis in muscle tissue is needed to further explore this as a first step to establish the relevance in the context of cancer cachexia.

Notwithstanding the fact that there are some markers known to be affected by cancer cachexia that can be measured using ELISA, western blot or qPCR [65], suitable biomarkers, in terms of acceptable specificity, sensitivity and measurability, remain to be identified. Here, we have identified a gene set that was robustly associated with cancer cachexia in in vitro models and preclinical models. These genes were not identified in relation to cachexia before.
Together these markers constitute a cachexia signature. When confirmed in vivo in humans, these genes may be used, alone or, more likely, in combination, as a signature to follow progression of disease and efficacy of interventions. In addition, these genes constitute potential targets that may be exploited for beneficial intervention. It may be particularly interesting to examine the effect of folate supplementation on BH4 levels in cancer cachexia, as folate may have positive effects on skeletal muscle development [66]. It may also be interesting to examine CoQ supplementation in the context of the alterations seen in CoQ. It should be noted however, that longer time supplementation is needed to enhance CoQ10 levels in muscle.

In conclusion, we showed that the CM of cachexia-inducing cancer cell lines contains factors dysregulating mitochondrial functioning, similar to the in vivo models. The changes in mitochondrial processes can directly be related to fatigue [67], which is something cachectic cancer patients often experience [68]. Further research in humans is needed to validate our findings, which seems especially important for the cachexia signature genes. Additionally, our results underline the importance of using different models for cachexia research, as commonly used models show considerable differences in their effects.

Acknowledgments

The authors would like to thank Donna McCarthy (Ohio State University, USA) for the C26 cell line, Josep Argiles (University of Barcelona, Spain) for the LLC cell line, Francina Dijk (Nutricia, the Netherlands) for the C2C12 cell line and Elizabeth Jaffee and Daniel Marks (Oregon Health and Science University, USA) for the KPC and the genetic variation KPC-4662 cell lines. Also we would like to thank Francina Dijk, Veerle Ottenheim and Yukako Tokutake for their help with the protocol for the CK assay. Additionally, we would like to thank Bart Lagerwaard, Evert van Schothorst and Rogier Plas with their tips and help on the analysis of the data.
References


[57] WD Han, YL Zhao, YG Meng, L Zang, ZQ Wu, Q Li, YL Si, K Huang, JM Ba, H Morinaga, et al. “Estrogenically regulated LRP16 interacts with estrogen receptor α and enhances the receptor’s transcriptional activity”. In: Endocrine-related cancer 14.3 (2007), pp. 741–753.


Table S5.1: Chemicals used in Creatine Kinase assay reaction mixture.

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<th>Conc. in RM</th>
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Figure S5.1: CK activity % of the control. N=3 (triplicate per N) Mean ± SEM ANOVA with post hoc Tukey. Significant effects compared to the control are represented with * p<0.05, ** p<0.01, *** p<0.005, and **** p<0.001.
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Table S5.3: Top 25 of downregulated DEGs in the GCM.

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Figure S5.2: Highest significant activated and suppressed GO terms in Biological Process (BP) on day 5 after incubation of C2C12 for the general cachectic model (GCM), $p_{adj} < 0.05$.

Table S5.4: Log2 fold change and general process of the significant ($p_{adj} < 0.05$) MitoCarta DEGs of the GCM.

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Chapter 6

COMUNEX; The effects of colon cancer on muscle gene expression, body composition, muscle function, and muscle metabolism - Study protocol

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Submitted
Abstract

BACKGROUND Cancer cachexia is characterized by clinically relevant loss of muscle mass with or without loss of fat mass. When the latter is relatively minor and (or) adiposity existed prior to the disease, cachexia can manifest itself as sarcopenic obesity. More insight into the early development of cachexia and the underlying processes may offer new leads for effective treatment strategies. To this end, this study aims to investigate the differences in gene expression, body composition, muscle function and muscle metabolism in colon cancer patients compared to age-matched controls without colon cancer and without inflammation. METHODS In total 40 colon cancer patients undergoing a tumour resection (30 patients with a primary tumour and 10 with liver metastases) and 15 control patients undergoing an inguinal hernia repair (men, n=10) or an abdominal hysterectomy (women, n=5) will be included. The primary study parameter will be muscle gene expression. Secondary parameters will be pre-surgery body composition (determined using already available CT scans), muscle function parameters (grip strength and selected biopsy tissue markers), metabolic markers (measured in fat and muscle biopsies taken during surgery), biochemical markers (measured in blood/serum taken during surgery) and gene expression in fat biopsies. Comparisons will be made between the different groups and after one year following the surgery, by performing selected follow-up measurements (grip strength and blood/serum analyses).

CONCLUSION This study includes age-matched controls who have no cancer or inflammatory diseases of which we can collect a tissue sample from the rectus abdominis muscle. This allows the best possible comparisons between cancer and control patients. Our study aims to contribute to identifying possible biomarkers or treatment targets for cachexia. Additionally, by characterising different tissues and blood samples and relate the outcomes to body composition parameters, we aim to develop diagnostic profiles for cachectic or sarcopenic obese patients which could improve earlier discovery of these syndromes.

Keywords

Cachexia; Cancer; Skeletal Muscle; Observational Study; Gene Expression;
## Administrative information

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Nieuwe Kanaal 9A, 6709 PA Wageningen  
**Vlag Graduate School**  
Bornse Weilanden 118, 6708 WG Wageningen  
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| Role of sponsor {5c} | The protocol was discussed at general meetings to improve its feasibility and scientific value. |
Introduction

Background and rationale (6a)

Cachexia is a complex metabolic syndrome characterized by clinically relevant loss of muscle mass with or without loss of fat mass. Anorexia, inflammation, insulin resistance and increased muscle protein breakdown are frequently associated with cachexia. However, cachexia is distinct from starvation, age-related loss of muscle mass, primary depression, malabsorption, and hyperthyroidism, while being strongly associated with increased morbidity and mortality [1]. In many patients, cachexia is associated with reduced treatment efficacy and therefore, progression of this condition can severely impact therapy, quality of life and prognosis [2, 3, 4, 5, 6, 7, 8]. Formally, cachexia is defined as: “a loss of lean tissue mass, involving a weight loss greater than 5% of body weight in 12 months or less in the presence of chronic illness or as a body mass index (BMI) lower than 20 kg/m2” [9]. This definition, which has a focus on loss of muscle mass and function, would imply that a cachectic person not necessarily has to have a low BMI. Vickie Baracos described severe depletion of skeletal muscle (sarcopenia) to be a significant predictor of toxicity of the chemotherapy and time to tumour progression in metastatic breast cancer patients with high body weight [10, 11]. This effect is described to be even more pronounced in obese patients [10, 12, 13]. The increasing prevalence of obesity in the western world leads to a larger cancer patient population with sarcopenic obesity [14, 15].

Moreover, animal work has shown that not only skeletal muscles but also the heart is affected in cancer cachexia. In a tumour-induced cachexia model in mice, heart muscle function was diminished, which was associated with increased fibrosis, disrupted myocardial structure, and altered composition of contractile proteins of cardiac muscle [16].

Up to 80% of patients with advanced stage cancer suffer from cachexia [17] and in the western world about 30% of all individuals with cachexia are cancer cachexia patients [18]. Although the condition is frequently seen in various types of cancers, the highest incidences are observed in patients with lung cancer and tumours of the GI tract [19, 20]. Colon cancer (CC) is the third most common cancer worldwide [20, 21]. About 30% of CC patients exhibit weight and muscle loss at diagnosis [19, 20] and a hampered response to treatment (either surgical or chemotherapy). Moreover, surgically removing the cancer further adds to the muscle mass loss. Next to that it has been described that patient do not gain muscle mass after surgery [20].

Studies have shown that treatment of cancer-induced cachexia is more effective when initiated at an early stage [22, 23, 24]. This demands for more insight in the initial events, but also for markers that allow timely detection. Developing an effective treatment is hampered by the diversity of the patient population, their different types of tumours, and stages of disease and treatment. Therefore, the current opinion is that treatment should be multimodal [25] through a combination of drug, nutrition and therapeutic
exercise [26, 27]. To determine how treatment methods can be most effective, full mechanistic insight in changes in overall body composition and molecular processes underlying muscle metabolism and muscle function are of great importance.

Analysis of the muscle transcriptome would be most straightforward in elucidating the underlying mechanisms of cancer cachexia. However, not many human studies have been performed, among others because finding proper controls is a major challenge. While in animal studies non-diseased (sham) control animals are used, in patient studies weight stable cancer patients [28] are the easiest available. Additionally, patients are compared with themselves at different time points [29] or the gene expression is correlated to the degree of weight loss [30]. This makes it hard to study the molecular processes and compare the human studies to animal studies. In our study we include an age-matched control group of non-inflammatory surgery patients (inguinal hernia repair or abdominal hysterectomy). We include both primary CC patients and patients who have liver metastases due to CC, we expect that the patients with metastases have a higher degree of cachexia because the signals from the metastatic process can trigger and amplify the process of cachexia development [31]. In addition to assessing the transcriptome, these biopsies of patients and controls will be used for additional in-depth studies, including single fibre muscle force and muscle oxygen consumption. This will give us a good insight of the mechanistic changes during cancer cachexia.

Objectives {7}

The aim of the study is to investigate in detail the differences in muscle and adipose tissue gene expression, body composition, muscle function and muscle metabolism in CC patients compared to age-matched controls. We will also include patients with liver metastases due to CC to investigate our secondary aim, which is to investigate differences in outcome parameters between CC patients in different degrees of cachexia and how these factors are inter-related. Lastly, we will follow-up the participants of the study at one year after tumour removal to investigate the relation between the above-described factors and body composition, muscle function and blood biochemistry in time.

Trial design {8}

Observational study
Methods: Participants, interventions and outcomes

Study setting {9}
Patient data and biopsies will be collected in Gelderse Vallei Hospital in the Netherlands. Measurements in biopsies will be done at Wageningen University.

Eligibility criteria {10}

Inclusion criteria
To be eligible to participate in this study, a subject must meet each of the following inclusion criteria.

• Cancer patients:
  – Diagnosed with primary CC [31] or liver metastases due to CC [10]
  – Eligible for a primary tumour or liver metastases resection procedure
• Age-matched controls:
  – Eligible for an inguinal hernia repair (male, 10) or an abdominal hysterectomy (female, 5)

Exclusion criteria
A potential subject who meets any of the following criteria will be excluded from participation in this study.

• Cancer patients:
  – Having had chemotherapy or an operational procedure of the abdomen in the past 6 months
  – Suffering from malabsorption
• Age-matched controls:
  – Having had treatment for previous or current tumours

Who will take informed consent? {26a}
After diagnosis, patients will be operated within a period of 2-3 weeks. Patients will be informed about the study by the medical specialist and receives the information brochure for further information. If patients are willing to participate, they can contact the coordinating investigator for more information. Patients will subsequently sign the informed consent form.
Additional consent provisions for collection and use of participant data and biological specimens {26b}

NOT APPLICABLE

Interventions

Explanation for the choice of comparators {6b}

There will no intervention as we will compare cancer patients with control patients.

Intervention description {11a}

There will be no intervention as this is an observational study.

Criteria for discontinuing or modifying allocated interventions {11b}

Subjects may discontinue the study at any moment without the obligation to state the reason for discontinuation. Subjects may be withdrawn from the study by the principal investigator if they do not comply with the rules and regulations of the study. Subjects may be withdrawn from the study by the medical supervisor in case of reported serious adverse events or in case of other medical/social/psychological events as evaluated by the medical investigator and discussed with the principal investigator.

Strategies to improve adherence to interventions {11c}

We expect very little drop out since we only measure during regular visits so the burden for the patients is very low.

Relevant concomitant care permitted or prohibited during the trial {11d}

No concomitant care is prohibited during the study.

Provisions for post-trial care {30}

There will be no follow up since patients will continue to be monitored by the medical specialists at the hospital.
Outcomes {12}

Objectives

• **Primary Objectives:**
  Main aim of the study is to molecularly investigate differences in gene expression of muscle and adipose tissue, body composition, muscle function and muscle metabolism in CC patients compared to controls.

• **Secondary Objectives:**
  Secondary aims of the study are to investigate differences in the above-mentioned factors between CC patients in different degrees of cachexia (based on CT scan and tumour stage) and how these factors are inter-related.

• **Tertiary Objectives:**
  Third aim of the study is to investigate the relation between the above-mentioned factors on the day of the surgery and body composition, muscle function and blood biochemistry one year after tumour removal.

Study questions

1. Primary aim: Is there a difference between CC patients and controls in:
   a Primary parameter:
    • Transcriptomic profiles of muscle
   b Secondary parameters:
    • Body composition
    • Muscle function, i.e. handgrip, leg strength
    • Parameters of (protein) metabolism in muscle tissue and lipolysis in fat tissue
    • Transcriptomic profiles of adipose tissue
    • Biochemical parameters

2. Secondary aim:
   a Is there a difference in primary and secondary parameters between CC patients in different stages of cachexia or muscle mass loss? (Patients will be classified by cachexia criteria of Evans *et al.* [1] and/or Fearon *et al.* [9] or divided in tertiles/quartiles based on body composition)
   b What is the relation between primary and secondary parameters?

3. Tertiary aim:
   a What is the relation between primary, secondary parameters on the day of the surgery and body composition, muscle function and blood biochemistry one year after tumour removal?
Participant timeline {13}

Before the surgery (D-1) patients will come to the hospital, this visit will take place 1 to 3 weeks before the surgery. The following measurements are done: [1] questionnaire on physical and functional wellbeing and anorexia/cachexia (three subscales of the FAACT), [2] physical function test (chair rise test) and [3] one muscle function tests (hand grip strength).

On the day of the surgery (D0) patients come in fasted for regular care procedures. Fasted blood samples (total 55 mL) are collected after inserting an intravenous cannula in the holding area (regular care), to identify levels of CRP, IL-6, Hb and albumin, cytokine levels and micronutrient status. During the surgery, a biopsy (1 cm³) of the rectus abdominis muscle is taken and immediately aliquoted and frozen for 1) transcriptomics, 2) protein metabolism parameters, 3) histology and 4) single fibre measurements. For clarification of the cachexia process also biopsies from the subcutaneous and visceral fat are taken (0.5 cm³ each). Biopsies are taken as early as possible during the surgery to minimize influence of anaesthetics.

One year after surgery (Y1) patients are asked to come fasted to the hospital. Blood samples (total 55 mL) are collected to identify basal levels of CRP, IL-6, Hb albumin, cytokines and selected micronutrients. Muscle function (hand grip strength) will be measured. Patients will fill out a questionnaire on physical and functional wellbeing and on anorexia/cachexia (three subscales of the FAACT).

In figure 6.1 an overview of the participant timeline is visualised.

Figure 6.1: Graphical representation of the participant timeline in the COMUNEX study.
Sample size {14}

Since this is an observational study with gene expression as primary outcome, no exact power calculations could be performed. However, we did make a general estimation of the accuracy of gene expression comparison based on literature reports. It was reported that sample size of n=30 (both male and female) gave quite high prediction accuracy (0.8 – 0.9) for sex differences in muscle gene expression based on metabolic changes [32]. We expect larger differences between groups since we investigate pathology vs. no pathology. Therefore, we will include 15 control (10 male + 5 female) and 40 cancer patients (30 primary CC + 10 liver metastasis). We include more male controls as the incidence of CC is higher in male patients.

Recruitment {15}

Patients will be recruited at the Gelderse Vallei Hospital by the medical specialist.

Assignment of interventions: allocation

Sequence generation {16a}

Participants will get ascending numbers in the order of enrolment in the study, as we expect to include on average 1 participant per month. The identification number also includes a sequence which shows the group the patient belongs to, as we have no intervention.

Concealment mechanism {16b}

Allocation is not concealed and will be revealed to both the patient and the researcher.

Implementation {16c}

The participant identification number will be allocated by the researcher. No assignment to intervention arms is needed as this is an observational study.

Assignment of interventions: Blinding

Who will be blinded {17a}

Patients, researchers, and surgeons will not be blinded this is not needed, because of the observational nature of our study. Next to that our primary
outcome is transcriptomics, which is an absolute parameter and not objective data.

**Procedure for unblinding if needed {17b}**

The study design is open label, therefore there is no unblinding procedure.

**Data collection and management**

**Plans for assessment and collection of outcomes {18a}**

Data will be derived from electronic patient records and collected with a Case Report Form (CRF) on paper (Good Clinical Practice (GCP) Compliant). Participants will fill out a printed questionnaire which will be handed over or mailed to the researcher. CT scans, hand grip strength and most blood sample analysis will be done in at the hospital (Gelderse Vallei Hospital), other analysis will be performed at Wageningen University.

**Plans to promote participant retention and complete follow-up {18b}**

We expect very little drop out since we only measure during regular visits, so the burden for the patients is very low. Therefore, it is not needed to promote participant retention. If a subject is withdrawn from the study before the biopsy, a substitute will replace his or her place if possible. If the subject is withdrawn after the biopsies, no substitute will be sought. Due to the low safety risks in this study, it is not expected that the study has to be terminated prematurely.

**Data management {19}**

Data of patients participating in this study will be coded. This code will be linked with the personal details in a password-protected file. Only members of the project team can access this file. Only means and other statistical expressions based on individual data will be published. The informed consents will be stored separately from all other information. All tissue sampled during the study will be stored in coded format and will be destroyed 15 years after the study is finished. Samples can be used for additional analyses not mentioned in the protocol but within the scope of the study objective.

**Confidentiality {27}**

Communication about participant will only be done using the subject code number. Physical files are stored in a locked file system and computer files are only accessible after permission to the study folder.
Plans for collection, laboratory evaluation and storage of biological specimens for genetic or molecular analysis in this trial/future use {33}

Body composition: CT-scan

The assessment of skeletal muscle is based on method of prof. Baracos (Canada) as described in the Lancet Oncology [13]. In short: muscle cross-sectional area is measured by secondary analysis of electronically stored CT images of the patient. All present CT scans, including CT scans from previous surgery, that cover the area of the third lumbar vertebrae (L3) are analysed to measure muscle cross-sectional area (CSA), skeletal muscle index (SMI) and muscle radiodensity (SMD). Skeletal muscle is identified by use of Hounsfield unit (HU) thresholds (-29 to +150). Images are analysed using the Slice-O-matic software (Tomovision, Montreal, Canada).

Questionnaires physical and functional wellbeing and anorexia/cachexia

To get an indication about physical and functional wellbeing and anorexia/cachexia, corresponding three subscales of the Functional Assessment of Anorexia/Cachexia Therapy questionnaire are determined (together leading to the FAACT Trial Outcome Index (TOI)). This questionnaire is validated for the specific target group [33, 30].

In-vivo muscle function: hand grip strength

Hand grip strength is determined at D-1 and Y1. Three consecutive measures of handgrip strength (kg) at both hands are recorded to the nearest 1.0 kg using a hand dynamometer (Jamar®).

Blood markers

Haemoglobin and serum albumin are measured using routine clinical chemistry analysers. Cytokine and inflammation markers will be determined with ELISA and/or multiplex assays. Micronutrient status will be determined using routine clinical chemistry analysers and/or HPLC.

Collecting muscle and fat biopsies

Biopsies are taken directly after the first incision of the surgery to minimize possible effect of anaesthetics on gene expression. Most of the primary colon tumour resections are done using laparoscopic surgery. When it is not possible to collect enough material at once, the earliest biopsy is used for gene expression and metabolic markers, the later biopsy is used for the other parameters. In case of open surgery (most of the liver resections and surgery of controls) we take the muscle biopsy at once. Most biopsies are immediately
frozen in liquid nitrogen and stored at -80°C. Biopsies for muscle oxygen consumption, single fibre contractile properties and fat secretome are stored according to the procedures as described in the corresponding paragraph. Leftover material will be stored at Wageningen University according to the ATMP legislation.

**Muscle oxygen consumption**

Approximately 4-10 mg of muscle will be immediately placed in ice-cold biopsy preservation solution (BIOPS, 10 mM Ca-EGTA buffer, 0.1 µM free calcium, 20 mM imidazole, 20 mM taurine, 50 mM K-MES, 0.5 mM DTT, 6.56 mM MgCl₂, 5.77 mM ATP, 15 mM phosphocreatine, pH 7.1) [34] for determination of mitochondrial respiration. The biopsy will be kept on ice and within max 1hr of collection, muscle fibres will be mechanically separated using a pointed forceps under a binocular microscope in 2 mL ice-cold BIOPS on ice. Permeabilization of the plasma membrane will occur in BIOPS supplemented with 50 µg/ml of saponin (Sigma-Aldrich, St Louis, USA) for 30 minutes on ice. Mitochondrial respiration medium (MiR05) will be commercially obtained (MiR05 kit) and made following the manufacturer's instructions (Oroboros Instruments, Austria). Saponin incubation will be followed by rinsing the muscle fibres for 3 × 10 minutes in MiR05 on ice. Muscle tissue will be dried for 40 sec on whatman filter paper and subsequently weighed. Mitochondrial respiration will be measured in 2-5 mg of muscle tissue per chamber. Measurements will be done in duplicate in MiR05 at 37°C using the high-resolution Oxygraph-2k (Oroboros Instruments, Austria), with additional substrates. Oxygen concentration (mM) and flux (pmol × s⁻¹ × mg⁻¹) will be recorded using DatLab software. Reoxygenation by direct syringe injection of O₂ is assumed to be necessary to maintain O₂ levels between 275 and 450 mM and to avoid potential oxygen diffusion limitation. The substrates added (final concentration) can be found in table 6.1. Cytochrome c will be used to test the integrity of the outer mitochondrial membrane and if the respiration increases >10% upon addition, the values from that sample will be removed. Substrate and coupling control ratios will be calculated from the different titration steps.

A background calibration for the Oroboros machine is performed every 3 months, and air calibrations are performed before each experiment. The results are pasted into the excel spreadsheet supplied by the manufacturer (Oroboros Instruments, Austria). If air calibrations present more than 5% deviation in the results, membranes are changed, and new background calibration is done. Instrument backgrounds will be performed in MiR05, and oxygen levels will be kept at 450 nmol/mL. Highly variable graphs are indicative of poor quality, as shown on the O2K software, and will therefore be removed.
Table 6.1: Substrate injection protocol Oroboros

<table>
<thead>
<tr>
<th>Protocol COM1</th>
<th>Protocol H-0191 (SUIT-008)</th>
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<tbody>
<tr>
<td>1 5 mM pyruvate</td>
<td>5 mM pyruvate</td>
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<tr>
<td>2 mM Malate</td>
<td>2 mM Malate</td>
</tr>
<tr>
<td>10 mM Glutamate</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>5 mM ADP + Mg2+</td>
</tr>
<tr>
<td>3</td>
<td>10 μM Cytochrome C</td>
</tr>
<tr>
<td>4</td>
<td>10 mM Succinate</td>
</tr>
<tr>
<td>5</td>
<td>5 mM ADP + Mg2+</td>
</tr>
<tr>
<td>6</td>
<td>5 μM Oligomycin</td>
</tr>
<tr>
<td>7</td>
<td>Add O2</td>
</tr>
<tr>
<td>8</td>
<td>Titration with 1 μl steps of 0.5 mM CCCP</td>
</tr>
<tr>
<td>9</td>
<td>2.5 μM Rotenone</td>
</tr>
<tr>
<td>10</td>
<td>5 μM Antimycin A</td>
</tr>
</tbody>
</table>

Gene Expression Transcriptomics

Total RNA from the muscle and fat biopsies will be isolated using RNeasy kit (Qiagen, Venlo, The Netherlands). RNA concentrations will be measured by absorbance at 260 nm (Nanodrop). RNA quality will be checked using the RNA 6000 Nano assay on the Agilent 2100 Bioanalyzer (Agilent Technologies, Amsterdam, The Netherlands) according to the manufacturer’s protocol. RNA from fat biopsies will be analysed on whole genome level with real time PCR for genes specific for the lipid hydrolysis pathway. RNA from muscle or fat biopsies will be analysed on gene expression level using the standard method available when executing the experiments (for example micro-array or RNA sequencing). Data will be analysed using Rstudio [35, 36] and the best available methods. Further functional interpretation of the data will be performed using Ingenuity Pathway Analysis [37].

Kinase analysis muscle biopsy

To get an indication of the protein synthesis and protein breakdown, phosphorylation of essential kinases of the key pathways of the protein synthesis and breakdown will be analysed. Protein phosphorylation level and content will be measured by immune-blotting using antibodies for the phosphorylated and non-phosphorylated kinases [38].

Muscle Histochemistry

For the assessment of muscle function, histochemical analysis will be used. Muscle biopsies will be snap frozen in liquid nitrogen and stored in -80 until
use. Sections (10 \( \mu \)m) will be cut with a cryostat at -20°C, air-dried and stored at -80°C until use. Fibre type composition will be determined by immunohistochemical staining of myosin heavy chains (MHCs). Monoclonal antibodies will be used against myosin heavy chain I (MHC1 (BA-D5), MHCIIA (SC-71) and MHCIIIX (6H1) (Developmental Studies Hybridoma Bank, The University of Iowa)). Using monoclonal antibody against PAX7 (PAX7, Developmental Studies Hybridoma Bank, The University of Iowa) satellite cells will be visualized. To measure fibre size, cell membranes will be visualized by incubation of the sections with Wheat Germ Agglutinin (1:50, Invitrogen, USA). Slides will be mounted with Vectashield HardSet containing 4',6-diamidino-2-phenylindole (DAPI) to visualize myonuclei. Using succinate dehydrogenase staining, oxidative capacity of the myofibres can be determined.

**Single fibre contractile properties**

The fresh muscle biopsy is chemically skinned in skinning solution (100mM KCl, 10mM imidazole, 1mM MgCl2, 2mM EGTA, 4.5mM Na2ATP, Glycerol 50% v/v, pH 7 with KOH dissolved in MQ) for 24 hrs at 4°C. After skinning the myofibre bundles are transferred to 0.5 mol/L sucrose relax, 1 mol/L sucrose relax, 1.5 mol/L sucrose relax and 2.0 mol/L sucrose relax, for 30 min each. The relax solution (pH 7.0) contains, next to the sucrose, 100mM KCl, 10mM imidazole, 1mM MgCl2, 2mM EGTA, 5mM ATP. The bundles are frozen and stored in pre-cooled centrifuge tubes at -80°C. A single muscle fibre will be dissected from the fibre bundle, which will be permeabilized and connected to a motor arm and a force transducer. By submerging the permeabilized fibre in an ‘activating’ solution with high Ca2+ concentration (7mM EGTA, 20mM Imidazol, 5.25mM MgCl2, 64mM KCl, 7mM CaCl2, 5.75 mM ATP, CrP 0.5% w/v, pH 7), the fibre will contract maximally. By submitting the activated fibre to an isotonic shortening protocol, the relationship between force and velocity can be determined.

**Lipolysis fat biopsies**

To get an indication of cells size of and lipolysis in fat cells, fat cells will be isolated and extracted from the subcutaneous and visceral fat biopsies using collagenase treatment [39].

**Fat secretome**

Fat biopsies will be incubated in culture medium for 24hrs at 37°C and 5% CO2. Secretome can be analysed for excreted compounds or used for future *in vitro* experiments.
Statistical methods

Statistical methods for primary and secondary outcomes {20a}

Analysis of the whole genome expression data of both muscle and fat biopsies will be performed using a Bayesian linear regression model (implemented in the software package LIMMA [40]. Raw microarray data will be normalized using RMA quantile normalization. Statistically significantly different genes between control and CC patients will be identified by Student paired t-test, followed by a false discovery rate correction (FDR). The latter is used to correct for multiple testing.

All other parameters will be tested for normality. If data are normally distributed, differences in study parameters between control and CC patients will be tested with a one-way ANOVA. Differences in study parameters at Y1 between controls and CC patients in different stages of cachexia will also be tested with a one-way ANOVA. Relations between study parameters and relations between D-1/D0 and Y1 will be tested with correlation and regression analyses (for example mixed models). If data are not normally distributed, non-parametric equivalents of tests mentioned above will be used.

Interim analyses {21b}

There are no interim analyses planned.

Methods for additional analyses (e.g. subgroup analyses) {20b}

There are no subgroup analyses planned.

Methods in analysis to handle protocol non-adherence and any statistical methods to handle missing data {20c}

Missing data will be reduced to a minimum by using the appropriate measures described above. Mixed models do not require imputations for missing data. For ANOVA listwise exclusion will be used.

Plans to give access to the full protocol, participant level-data and statistical code {31c}

The datasets used and/or analysed during the current study can be made available by the corresponding author upon reasonable request. Genome expression data will be made available in the GEO database. Statistical methods (R scripts) will be shared.
Oversight and monitoring

Composition of the coordinating centre and trial steering committee {5d}

This is a monocentre study designed and coordinated by Wageningen University. Material collection is performed in the Gelderse Vallei Hospital and all other analysis will be at Wageningen University. Day to day support for the study is provided by:

- Principle investigator: takes supervision of the study
- Coordinating investigator: study registration, coordinate study visits, organize data collection, take informed consent, identify potential recruits, ensure follow-up according to protocol, perform laboratory experiments
- Medical specialist: identifies potential recruits
- Medical supervisor: takes medical responsibility of the patients

Composition of the data monitoring committee, its role and reporting structure {21a} In agreement with the advice from the central Data Safety Monitoring Board (DSMB), a DMC has not been appointed for this study. The decision was based on the lack of expected SAEs in this observational study. Moreover, since this is not a blinded study, there is no DMSB required to protect blinding of the researchers and physicians.

Adverse event reporting and harms {22}

Since the proposed study is an observational study that comprises no intervention, no benefits for the participants are expected. Additionally, the risks for the patients are also considered minimal, as the collection of biopsies will happen during the scheduled surgery. Research visits to the hospital will be combined with visits for regular care as much as possible. Time consumption for the patients is minor (+/- 3 hours). The extra load on the participants comprise, collection of in total 110 mL of blood, questionnaires and handgrip strength measurements.

All SAE’s will be reported through the Web portal ToetsingOnline to the accredited medical research ethics committee (METC) that approved the protocol, within 15 days after the principal investigator has first knowledge of the serious adverse events. All (S)AEs will be followed until they have abated, or until a stable situation has been reached. Depending on the event, follow up may require additional tests or medical procedures as indicated, and/or referral to the general physician or a medical specialist. If (S)AEs persist after the end of the study, follow up will be done by the medical specialist. SAEs need to be reported till end of study within the Netherlands, as defined in the
protocol. The follow-up of AE’s will also be reported in the annual progress report.

**Frequency and plans for auditing trial conduct {23}**

The investigator will submit a summary of the progress of the study to the accredited METC once a year. Information will be provided on the date of inclusion of the first subject, numbers of subjects included and numbers of subjects that have completed the study, serious adverse events, other problems, and amendments. Since this is an observational study, no independent audit is needed.

**Plans for communicating important protocol amendments to relevant parties (e.g. trial participants, ethical committees) {25}**

Amendments are changes made to the research after a favourable opinion by the accredited METC has been given. Non-substantial changes (such as typing errors, administrative changes like changes in names, telephone numbers and other contact details of involved persons mentioned in the submitted study documentation) will not be notified to the reviewing METC. All amendments will be notified to the METC that gave a favourable opinion.

The documentation that will be included in the submission should cover the following information:

1. Covering letter, including the reasons for the amendment in one or two sentences, a brief description of the changes that are included in the amendment and the name of the documents that are modified.

2. An extract of the modified documents, where applicable, showing both the previous and new wording.

3. The new version of the modified documents, where applicable, identified with updated number of version and date.

**Dissemination plans {31a}**

Results of this research will be disclosed completely in international peer-reviewed journals. Both positive and negative results will be reported. The investigator will notify the accredited METC of the end of the study within a period of 8 weeks. The end of the study is defined as the last subject’s last visit. In case the study is ended prematurely, the investigator will notify the accredited METC within 15 days, including the reasons for the premature termination. Within one year after the end of the study, the investigator/sponsor will submit a final study report with the results of the study, including any publications/abstracts, to the accredited METC.
Discussion

This observational study is designed to primarily investigate the effect of CC on muscle gene expression, body composition, muscle function and muscle metabolism. This will be investigated by comparing 40 cancer patients with 15 control patients.

Limitations

There are several limitations to consider. First, in the Netherlands we have a population-wide screening for CC. This causes discovery of tumours at a relatively early stage, which is beneficial for the patients. However, this also often implies that these patients often have not yet developed any other symptoms, including cachexia. As a consequence, our study population might be at a low or pre-cachectic level. We try to balance this by including the more advanced stage liver metastasis patients. Second, the timeframe from the discovery of the tumour until surgery is short (about two weeks). This is again beneficial for the patient but also means that eligible participants have a very short window to think about if they want to enrol themselves in the study. This could lead to a lower inclusion rate. Lastly, this is an observational study, this could mean that results do not allow to draw conclusions regarding causal relationships between cachexia and the endpoints assessed.

Strengths

This observational study has a unique study design, as we include age-matched controls that undergo a surgery in the abdomen which is not inflammation related. In this way we collect muscle biopsies of the rectus abdomens of both patients and controls which we can compare. Often weight stable cancer patients are considered as controls [28, 41, 42], which is not ideal as these patients could be pre-cachectic. Additionally, there is another great benefit of collecting biopsies during surgery, namely the size of the biopsy. The biopsies are large compared to needle biopsies, which allows us to do multiple measurements.

Trial status

Protocol version 5: 9th of August 2018
Study start date: 24th of February 2017
First inclusion: 28th of April 2017
Approximate date when recruitment will be completed: 31st December 2022
Abbreviations

(S)AE  (Serious) Adverse Event  
CC       Colon cancer  
CCMO     Central Committee on Research Involving Human Subjects; in Dutch: Centrale Commissie Mensgebonden Onderzoek  
CRP     C-reactive protein 
CT scan  Computed Tomography scan  
FAACT  Functional Assessment of Anorexia/Cachexia Therapy  
FDR     False Discovery Rate  
Hb       haemoglobin  
IC       Informed Consent  
METC    Medical research ethics committee (MREC); in Dutch: medisch ethische toetsing commissie (METC)  
WMO     Medical Research Involving Human Subjects Act (in Dutch: Wet Medisch-wetenschappelijk Onderzoek met Mensen)

Declarations

Acknowledgements

We thank all the patients who will and have participated in this study. Furthermore, we thank the students Saskia Rasser, Mina Jafarzadeh and Esmee Korver for their interest in writing an MSc thesis about this study. We thank all the staff of the Gelderse Vallei Hospital that helped or will help during this study. Specifically, Alina van de Vendel (research nurse), and Anne Venes, Hanneke Kleijwegt-Balk and Eveline de Grief (medical specialists). Furthermore, we thank Hilda van Staalduijnen, Floris van der Laan and Theresia Blok. We thank all surgeons and nurses of the surgery room especially Flip Kruyt, Tjarda van Heek, Colin Sietses and Gabie de Jong. For the inclusion of the inguinal hernia repair patients, we thank Bas Frietman and Willem Bökkerink. Next, at Wageningen University we thank Mieke Poland her help with the experiments and Koen Manusama for his help with the collection of the biopsies. Lastly, we thank Xiaolin Li and Hanneke Moonen for the continuation of the study.
## Authors’ contributions {31b}

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<td>Written or substantively revised manuscript</td>
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All authors read and approved the final manuscript.  
Corresponding author: KvN  
Xiaolin Li (XL) and Hanneke Moonen (HM) are not on the author list as they started recently with the continuation of the study.

## Funding {4}

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## Availability of data and materials {29}

The datasets used and/or analysed during the current study will be made available from the corresponding author upon reasonable request.

## Ethics approval and consent to participate {24}

Ethical approval was assigned by the CCMO of the Netherlands (NL58188.081.16). All participating patients will provide written informed consent.
Consent for publication {32}
This manuscript does not contain individual personal data from patients.

Competing interests {28}
The authors declare that they have no competing interests.
References


Chapter 7

COMUNEX; The effects of colon cancer on muscle gene expression, body composition, muscle function, and muscle metabolism - Preliminary data

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Preliminary data report
Abstract

BACKGROUND Cachexia is common among cancer patients, and effective treatment options are still lacking. An important cause of this is the absence of sufficient mechanistic insight into the complex processes underlying the muscle wasting that is typical for cachexia. The COMUNEX study was designed to contribute to unravelling these mechanisms in humans and the discovery of new treatment targets. The main aim of this study, which is ongoing since 2017, is to investigate differences in gene expression, body composition, muscle function and muscle metabolism in colon cancer patients compared to age-matched controls. The latter group consists of patients undergoing relatively simple surgery for a condition that is not believed to be associated with an elevated inflammatory status. Although inclusion is not complete, this preliminary data update will facilitate handing the study over to other researchers. METHODS At the moment of writing this report, 21 of the 30 primary colon cancer patients undergoing a tumour resection were included. Data on body composition (determined from available CT scans), parameters for muscle function (grip strength and O2 flux in permeabilized rectus abdominis muscle), FAACT questionnaires (Functional Assessment of Anorexia/Cachexia Therapy) and biochemical markers (in blood/serum) were collected. RESULTS About half of the included primary colon cancer patients showed sarcopenia as manifested by a skeletal muscle index below 55 cm²/m². Most other physical and blood values were within the reference range. The O2 flux in permeabilized rectus abdominis tissue, measured by OXPHOS complex I and II, showed an average of 23.05±6.27 pmol O2/(s*mg). Lastly, the cross sectional area of the visceral adipose tissue was found to be significantly correlated with body mass index, while the cross sectional area of the skeletal muscle was significantly correlated with the maximum power output delivered during a steep ramp test. CONCLUSION The patients participating in the COMUNEX study are a good representation of the Dutch male colon cancer population. Most participants are suffering from sarcopenia, however the comparison with the, still to be included, control and liver metastatic groups is needed for interpretation of other data regarding muscle wasting, including transcriptomics.
**Introduction**

The introduction and description of the methods of the COMUNEX study can be found in chapter 6. In the current chapter, preliminary data are presented and the direction of the study is described. Although the study is not finished yet, this update will facilitate handing over the study to other researchers. Unforeseen circumstances have been encountered; the low inclusion rate (due to a lower participation rate than anticipated) and restrictions in response to the SARS-CoV-2 pandemic have led to delays in the study. At this moment 21 (out of 30) primary Colon Cancer (CC) patients have been included. Unfortunately, no control and liver metastatic patients could be included. Of these 21 CC patients physical parameters, blood parameters and correlations between these parameters will be described. Additionally, the O$_2$ flux in the *rectus abdominis* muscle of seven patients will be presented.

It is expected that some of the patients in the CC group will have been pre-cachectic and some cachectic when they had surgery. The control group is expected to show no cachexia at all. In the liver metastatic group, part of the patients will be pre-cachectic and part of them will be cachectic, although it is expected that the percentage of cachectic patients is higher in this group than in the CC group.

**Results**

**Physical parameters**

The physical characteristics of the study population are shown in table 7.1. The population included thus far consists of 21 primary CC patients with an average age of 67±6.7 years, of which 95.2% is male. The average Body Mass Index (BMI) of this population is 26±3.9, of which 61.9% can be classified as obese (BMI≥30). The Functional Assessment of Anorexia/Cachexia Therapy (FAACT) [1, 2] questionnaire consist of the Physical Well-Being (PWB) (maximum 28 points), Functional Well-Being (FWB) (maximum 28 points) and the Anorexia/ Cachexia Subscale (A/CS) (maximum 48 points). Together, these form the Trial Outcome Index (TOI) for which a maximum score of 104 points can be attained. The higher the score, the better the Quality Of Life (QOL), there are no reference values for the FAACT. The average sub-scores in the study population were: for PWB 27±1.9 points, for FWB 22±5.6 points and for A/CS 41±4.1 points, which resulted in an average TOI score of 90±9.8 points, which is 13.5% (14 points) below the maximum score. Next, physical parameters were measured. Handgrip strength was on average 44±10.8 kgf (kilogram-force). Three patients had a handgrip strength under the reference values, which are dependent on age and height [3]. Maximal Inspiratory Pressure (MIP) was 84±10.8 cm H$_2$O, which is within the 95% confidence interval (84.6–100.8 cm H$_2$O) of the reference male age group (60-69 years) [4]. On the Time Up and
Go (TUG) test, all participants scored individually below the average of the reference groups (8.1 sec, 60-69 years) [5] with an overall average of 6.0±1.55 seconds. For the steep ramp test, the average maximum power output was 258±73.1 Watt with an average VO₂max reached of 29.5±4.82 mL/kg/min. The expected VO₂max was calculated with the formula: 
\[ \text{VO}_2\text{max (mL/kg/min)} = \left( (0.023 \times \text{height in cm}) + (0.0117 \times \text{body weight in kg}) - (0.031 \times \text{age}) - 0.332 \right) \times 1000 \] / body weight in kg [6]. This resulted in the 95% confidence interval of 30.5-33.8 mL/kg/min, which is slightly higher than our population. Lastly, Computerized Tomography (CT) scans were analysed. The average Cross Sectional Area (CSA) of Subcutaneous Adipose Tissue (SAT) was 146±69.0 cm² and of Visceral Adipose Tissue (VAT) was 172±120.0 cm². The Skeletal Muscle (SM) average attenuation (a measure for muscle density) [7] was 42±4.2 HU (Hounsfield Units, a scale for radiodensity in CT scans) and the CSA was 163±24.6 cm², which resulted in a Skeletal Muscle Index (SMI = L3 skeletal muscle CSA (cm²)/height²(m²)) of 52±8.1 cm²/m². The SMI was below the reference values of 55 cm²/m² (men) and 39 cm²/m² (women) [8].

**Blood parameters**

On the day of surgery seven blood parameters were measured (table 7.2). The average values for haematocrit, erythrocytes, Mean Corpuscular Volume of erythrocytes (MCV), calcium, glucose, albumin, cholesterol, triglycerides and magnesium all lie within the reference values. Levels for haemoglobin were slightly below the reference value for males, with an average value 8.3±1.47 mmol/L. The average level of C-Reactive Protein (CRP) is 18±25.4 mg/L, which is above the reference value. However, all but one (63 mg/L) patients have values within the reference.

**O₂ flux in rectus abdominis**

The O₂ flux in *rectus abdominis* muscle tissue was measured with high resolution oxygraphy (Oroboros, Innsbruck, Austria). All data were corrected by subtracting the O₂ flux after antimycin A addition, which represents non-mitochondrial O₂ flux. Data of only 7 CC patients was collected when writing this chapter (inclusion for Oroboros started after an amendment to the study, when some patients were already included), so no comparisons with controls could be made. In figure 7.1 the O₂ flux of *rectus abdominis* tissue is shown for the LEAK-N, OXPOS-N, OXPHOS-NS, ET-NS and ET-S. The LEAK-N state (0.31±1.45 pmol/(s*mg)) is achieved by adding the substrates pyruvate, malate and glutamate. After the addition of ADP, the OXPHOS-N state (8.33±1.25 pmol/(s*mg)) is reached. This is the state where the OXPHOS system mainly relies on complex I activity. OXPHOS-NS is reached by adding succinate, representing the state during which OXPHOS relies on complex I and II. In this state the average O₂ flux is 23.05±6.27 pmol/(s*mg). After adding oligomycin and an uncoupler, the ET-NS state (16.76±5.18 pmol/(s*mg)) is reached, which represents maximal uncoupled respiration.
<table>
<thead>
<tr>
<th>Table 7.1: Physical characteristics of participants of the COMUNEX study.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Colon cancer patients</strong></td>
</tr>
<tr>
<td><strong>Average</strong></td>
</tr>
<tr>
<td>N=21</td>
</tr>
<tr>
<td><strong>SD</strong></td>
</tr>
<tr>
<td><strong>Age (years)</strong></td>
</tr>
<tr>
<td>67</td>
</tr>
<tr>
<td><strong>Sex (male)</strong></td>
</tr>
<tr>
<td>95.2%</td>
</tr>
<tr>
<td><strong>Body weight</strong></td>
</tr>
<tr>
<td>Body mass index (BMI)</td>
</tr>
<tr>
<td>26</td>
</tr>
<tr>
<td>Overweight or obese</td>
</tr>
<tr>
<td>61.9%</td>
</tr>
<tr>
<td>Weight loss past 6 months</td>
</tr>
<tr>
<td>-1.1 kg</td>
</tr>
<tr>
<td><strong>Functional Assessment of Anorexia/Cachexia Therapy (FAACT) questionnaire</strong></td>
</tr>
<tr>
<td>Trial Outcome Index (TOI)</td>
</tr>
<tr>
<td>90</td>
</tr>
<tr>
<td>Physical Well-Being (PWB)</td>
</tr>
<tr>
<td>27</td>
</tr>
<tr>
<td>Functional Well-Being (FWB)</td>
</tr>
<tr>
<td>22</td>
</tr>
<tr>
<td>Anorexia/Cachexia Subscale (A/CS)</td>
</tr>
<tr>
<td>41</td>
</tr>
<tr>
<td><strong>Handgrip strength</strong></td>
</tr>
<tr>
<td>44 kgf</td>
</tr>
<tr>
<td><strong>Maximal Inspiratory Pressure (MIP)</strong></td>
</tr>
<tr>
<td>84 cm H₂O</td>
</tr>
<tr>
<td><strong>Time Up and Go (TUG)</strong></td>
</tr>
<tr>
<td>6.0 sec</td>
</tr>
<tr>
<td><strong>Steep ramp</strong></td>
</tr>
<tr>
<td>Maximum Power Output</td>
</tr>
<tr>
<td>258 Watt</td>
</tr>
<tr>
<td>VO₂ max</td>
</tr>
<tr>
<td>29.5 mL/kg/min</td>
</tr>
<tr>
<td><strong>Subcutaneous Adipose Tissue (SAT)</strong></td>
</tr>
<tr>
<td>CSA</td>
</tr>
<tr>
<td>146 cm²</td>
</tr>
<tr>
<td><strong>Visceral Adipose Tissue (VAT)</strong></td>
</tr>
<tr>
<td>CSA</td>
</tr>
<tr>
<td>172 cm²</td>
</tr>
<tr>
<td><strong>Skeletal Muscle (SM)</strong></td>
</tr>
<tr>
<td>Attenuation</td>
</tr>
<tr>
<td>42 HU</td>
</tr>
<tr>
<td>CSA</td>
</tr>
<tr>
<td>163 cm²</td>
</tr>
<tr>
<td>Skeletal Muscle Index (SMI)</td>
</tr>
<tr>
<td>52 cm²/m²</td>
</tr>
<tr>
<td><strong>Physical Well-Being (PWB)</strong></td>
</tr>
<tr>
<td>27</td>
</tr>
<tr>
<td><strong>Functional Well-Being (FWB)</strong></td>
</tr>
<tr>
<td>22</td>
</tr>
<tr>
<td><strong>Anorexia/Cachexia Subscale (A/CS)</strong></td>
</tr>
<tr>
<td>41</td>
</tr>
</tbody>
</table>
**Table 7.2: Blood parameters of participants of the COMUNEX study (n=21) at study day 0. pL = 10-12 liter. fL = 10-15 L**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Average</th>
<th>SD</th>
<th>Reference value [9]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemoglobin</td>
<td>8.3 mmol/L</td>
<td>1.47</td>
<td>Male: 8.5-11.0 mmol/L</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Female: 7.5-10.0 mmol/L</td>
</tr>
<tr>
<td>Haematocrit</td>
<td>0.39 L/L</td>
<td>0.060</td>
<td>Male: 0.41-0.51 L/L</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Female: 0.36-0.47 L/L</td>
</tr>
<tr>
<td>Erythrocyte</td>
<td>4.63 /pL</td>
<td>0.500</td>
<td>Male: 4.3-6.0 /pL</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Female: 3.8-5.5 /pL</td>
</tr>
<tr>
<td>Mean Corpuscular Volume of erythrocytes (MCV)</td>
<td>85 fL</td>
<td>6.6</td>
<td>2-98 fL</td>
</tr>
<tr>
<td>Calcium</td>
<td>2.31 mmol/L</td>
<td>0.101</td>
<td>2.10-2.55 mmol/L</td>
</tr>
<tr>
<td>Glucose</td>
<td>5.6 mmol/L</td>
<td>1.01</td>
<td>4.0-6.4 mmol/L</td>
</tr>
<tr>
<td>Albumin</td>
<td>36 g/L</td>
<td>3.9</td>
<td>35-55 g/L</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>4.5 mmol/L</td>
<td>0.84</td>
<td>0-6.5 mmol/L</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>1.13 mmol/L</td>
<td>0.477</td>
<td>0.6-2.2 mmol/L</td>
</tr>
<tr>
<td>Magnesium</td>
<td>0.83 mmol/L</td>
<td>0.085</td>
<td>0.7-1.0 mmol/L</td>
</tr>
<tr>
<td>C-Reactive Protein (CRP)</td>
<td>18 mg/L</td>
<td>25.4</td>
<td>&lt;10 mg/L</td>
</tr>
</tbody>
</table>
Lastly rotenone inhibits complex I and shows the ET-S state \( (12.51 \pm 2.86 \text{ pmol/(s*mg)}) \).

![Figure 7.1: O₂ flux in rectus abdominis tissues of CC patients. Mean ± SD n=7](image)

**Higher muscle CSA results in better steep ramp maximum power output reached**

The Pearson correlation coefficient was calculated to investigate associations between the measured parameters. The complete correlation table can be found in the table S7.1. O₂ flux measurements were not used to calculate correlations due to the low number of measurements (N=7). In table S3.3 correlations above 0.8 are displayed. The surface of adipose tissue (VAT CSA) correlates with BMI and the surface of muscle (SM CSA) correlates with the maximum power output (Watts) reached in the steep ramp test. Furthermore, correlations were found between blood parameters. Haematocrit (volume of red blood cells), haemoglobin (a protein of red blood cells), erythrocyte (red blood cells) and MCV (haematocrit divided by the concentration of red blood cells) correlate with each other.

**Discussion**

Strengths and limitations of the study design already have been discussed in chapter 6. Therefore, this discussion will focus on the preliminary results of
the COMUNEX study. The main discussion points are: Is the study population representative, what is the prevalence of cachexia and what are the future perspectives of the COMUNEX study?

In the Netherlands there is a population-wide screening for colon cancer (CC) between the age of 55 and 75. There is a higher incidence of CC in males (56%) then females (44%) and peaks between 80-84 years [10]. The COMUNEX study population is on average 67±6.7 years, these people most likely have been screened for CC, as they are younger than the age group with the highest CC prevalence. Remarkably, the COMUNEX study population so far consists of 95.2% male patients although screening was done in both male and female patients. In the Dutch male population 62.7% of the people above the age of 65 years has a BMI of 25 or higher [10], which is in line with the 61.9% we found in the COMUNEX study population. Therefore, it can be concluded that the study population is primarily a representation of the Dutch male population in whom CC was found by the population-wide screening. This sex disparity is mainly caused by the fact that more women apparently hesitate to participate in the study. We aim to correct this and focus on the inclusion of more women in the last part of the study.

Cachexia is often described as a certain degree of involuntary weight loss, but SMI should be taken into account as well [8, 11]. The diagnosis criteria for cachexia of Fearon *et al.* are weight loss of >2% over the past 6 months and an SMI of <55 cm²/m² (men) or <39 cm²/m² (women) [8]. In the literature it is reported that half of the colorectal cancer patients who received chemotherapy develop cachexia [12]. Additionally, a study in Norway showed that there was no large difference (55% vs 47%) in the prevalence of cachexia between cancer patients with metastasised CC and those with localised tumours [13]. On average the SMI of the COMUNEX study population is below the cut off value for men, while 11 of the 21 participants have an SMI below 55 cm²/m². However, involuntary weight loss of >2% was only reported in four participants and ten patients had weight loss between 0% and 2%. Therefore, the COMUNEX study population has a small degree of muscle wasting, reflected in the low SMI, but most patients are not considered cachectic, reflected by the low incidence of involuntary weight loss. For the FAACT questionnaire the participants score 86.5% of the points for the TOI. Additionally, for the A/CS 41±4.1 points were scored on average, which is above the cut-off value (≤37) to assess anorexia [1]. This means the study population is not suffering from anorexia. Other physical parameters were on average within the reference values. In conclusion, in general the COMUNEX study population so far included suffers from muscle wasting, but most of the individuals do not classify as cachectic under the definition of Fearon *et al.* [8], they either have a low SMI (N=11) or >2% weight loss (N=4). One possible explanation, also mentioned in chapter 6, is the early discovery of the tumour by population-wide screening.
Future perspectives

This chapter described only a subset of the COMUNEX study population. In the future, an additional 9 primary CC patients and 10 liver metastatic patients, as well as 15 control patients undergoing an inguinal hernia repair (men, 10) or an abdominal hysterectomy (women, 5) will be included, as planned in the protocol. The controls are patients of which a rectus abdominis muscle biopsy can be collected, as is done in the CC patients, but who have no inflammation, as inflammation could influence gene expression profiles. When this inclusion is completed, both control and liver metastatic patients will provide a more representative sample in which we expect a varying degree of muscle loss.

When the inclusion will be completed, the primary study parameter, muscle gene expression, will be analysed, as well as additional muscle function parameters (measured in a biopsy), metabolic markers (measured in fat and muscle biopsies), additional biochemical markers (measured in blood/serum) and gene expression in fat biopsies. The size of the muscle biopsy taken during surgery makes it possible to perform multiple measurements, which is an advantage of the COMUNEX study. Comparisons will be made between the different groups and with data collected one year after the surgery. The latter will be done using selected follow-up measurements (grip strength and blood/serum measurements). It is expected that significant changes in muscle gene expression will be found, and possible mechanisms which play a role in the cachectic muscle can be identified. Furthermore, the role of the muscle mitochondria could be further unravelled [14].

It could be considered to perform an analysis with a subset of patients who are cachectic and with those who do not meet the criteria of cachexia according to Fearon et al. The yet to be included control group will neither have cancer nor an elevated inflammatory status at surgery. It will therefore be very interesting to explore the differences between the individuals suffering from CC and the control group, independent of a clinically confirmed cachexia diagnosis. This might provide new insights in early genomic changes which are a sign of pre-cachexia. Furthermore, data obtained from the individuals with liver metastatic could provide the opportunity to do comparisons with those from patients having a more advanced stage of the disease. It is therefore expected that this study eventually will give valuable mechanistic insights in the genomic changes in muscle tissue under the influence of different degrees of tumour burden.

Acknowledgments

The authors would like to stress that the data described in this chapter is preliminary and that no definitive conclusions can be made about the outcomes of the COMUNEX study. The study is conducted according to the principles of the Declaration of Helsinki (64th WMA General Assembly, Fortaleza, Brazil, October 2013) and in accordance with the Medical
Research Involving Human Subjects Act (WMO). The authors would like to thank Mina Jafarzadeh and Esmee Korver for their help in analysing the CT scans. Next, we would like to thank everyone from the Hospital Gelderse Vallei for their help in recruiting patients and collection of the biopsies. We would also like to thank Koen Manusama and Mieke Poland for their practical help. Lastly, we would like to thank Xiaolin Li and Hanneke Moonen for the continuation of the study.
Table 7.3: Pearson correlation coefficient above 0.8 between the different study parameters.

<table>
<thead>
<tr>
<th></th>
<th>Pearson correlation coefficient</th>
<th>N</th>
<th>T statistic</th>
<th>DF</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>VAT CSA vs BMI</td>
<td>0.872819211</td>
<td>19</td>
<td>7.373778219</td>
<td>17</td>
<td>1.08719E-06</td>
</tr>
<tr>
<td>SM CSA vs Steep ramp power output</td>
<td>0.851244952</td>
<td>18</td>
<td>6.488536327</td>
<td>16</td>
<td>7.47745E-06</td>
</tr>
<tr>
<td>Haematocrit vs Haemoglobin</td>
<td>0.990314634</td>
<td>18</td>
<td>28.53082261</td>
<td>16</td>
<td>3.77707E-15</td>
</tr>
<tr>
<td>Erythrocyte vs Haemoglobin</td>
<td>0.881785382</td>
<td>18</td>
<td>7.478289077</td>
<td>16</td>
<td>1.31520E-06</td>
</tr>
<tr>
<td>Erythrocyte vs Haematocrit</td>
<td>0.904096512</td>
<td>18</td>
<td>8.462781043</td>
<td>16</td>
<td>2.65351E-07</td>
</tr>
<tr>
<td>MCV vs Haemoglobin</td>
<td>0.832505255</td>
<td>18</td>
<td>6.010682224</td>
<td>16</td>
<td>1.81521E-05</td>
</tr>
<tr>
<td>MCV vs Haematocrit</td>
<td>0.824633786</td>
<td>18</td>
<td>5.831231665</td>
<td>16</td>
<td>2.55300E-05</td>
</tr>
</tbody>
</table>
References


Supplemental material
Table S7.1: Pearson correlation coefficients parameters participants COMUNEX
study. SR = steep ramp.

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Chapter 8

General Discussion
Main findings

As introduced in chapter 1, one of the possible hallmarks of cancer is cachexia, which is a multi-organ syndrome with the wasting of skeletal muscle tissue as a profound consequence. The primary aim of the project ‘The Selfish Tumour: Cachexia associated changes in skeletal muscle’ was to investigate the crosstalk between tumour cells and skeletal muscle with a specific focus on changes in muscle micro-environment and energy homeostasis. The following research questions were addressed as further discussed in this chapter:

1. To what extent is skeletal muscle in the C26 cachexia mouse model responsive to whole-body vibration training?

2. What are the effects of conditioned media (CM) of different cachexia-inducing cancer cell lines on differentiating skeletal muscle cells and what is the role of individual mediators of the CM?

3. How is mitochondrial functioning in skeletal muscle changed during cancer cachexia?

4. Can the skeletal muscle gene expression results be confirmed in cancer patients?

Cachectic cancer patients experience fatigue and declined mobility, which hampers their ability to perform their daily activities and being physically active. A low impact exercise intervention could provide an option to enhance physical fitness and thereby improve quality of life and possibly treatment outcome. Therefore, the research presented in this thesis started with investigating whether a low impact exercise could improve muscle physiology (chapter 2). The study used whole-body vibration training (WBV) as low impact exercise intervention, and was performed in mice. Unfortunately, no effects of WBV on body mass and composition, daily activity, blood parameters and muscle histology in cachectic mice were found, but there was an improved contractility of soleus muscle (SOL). Additionally, the WBV prevented the upregulation of the proteasome pathway in the SOL of the cachectic mice, but not in the extensor digitorum longus muscle (EDL). This indicates a small response in the cachectic mice undergoing WBV, mainly at the level of altered gene expression profiles, towards the profile of the control mice. Therefore, for the time being, the answer to the first research question must be that on the basis of these results WBV is unlikely to represent a single therapeutic option for cancer cachexia. However, it could be part of a multimodal treatment plan, as WBV seemed to protect the SOL and a recent study reported that WBV improved walking performance in elderly [1]. In future research, WBV can be combined with nutritional and pharmacological therapies to create an additive effect. To be able to develop such new therapies, a better understanding of the signalling mechanisms that cause cancer cachexia is needed. This was investigated using an in vitro model in
which the effects of conditioned medium (CM) of different cachexia-inducing cancer cell lines on the differentiation of C2C12 myoblasts/myotubes (C2C12) were studied (chapter 3). Myotube differentiation, as part of the skeletal muscle regeneration process, was impaired by the CM. Furthermore, immune response processes were abundantly found in the top 20 most upregulated processes using the gene expression profiles of the C2C12 cells. A signature of immune signalling molecules was determined in the CM of the different cachexia-inducing cancer cell lines and used to create different mimic media containing immune signalling molecules. Only when prostaglandin E2 (PGE2) and prostaglandin F2 alfa (PGF2α) were present in the mixture the degree of differentiation of the C2C12 was lowered. However, the extent of this was smaller than when the C2C12 were incubated with the CM. Since polyunsaturated fatty acid eicosapentaenoic acid (EPA) inhibits the rise in muscle PGE2 content in response to serum from cachectic mice and also inhibits muscle protein degradation [2], this opens up the possibility of using PGE2 and PGF2α as a therapeutic target. Therefore, the answer to the second research question is that the CM has the ability to decrease the C2C12 differentiation and activate immune signalling pathways. Prostaglandins are playing a role, as shown by their ability to mimic the decrease in C2C12 differentiation. Although there are probably many other, not identified, signalling molecules present in the CM, which contribute to the decrease in differentiation, we decided to focus on the overlapping effects between the two studies (chapter 2 and chapter 3) described for the future research of this thesis.

In both the WBV study (chapter 2) and the in vitro study (chapter 3) a downregulation of the oxidative phosphorylation pathway was found when analysing the gene expression data. This seems logical as muscle function is dependent on mitochondrial function. For example, the ageing muscle shows that loss of muscle mass and dysfunction of mitochondria are going hand in hand [3, 4, 5]. Whether this is also the case in cancer cachexia is less clear. Therefore, an extensive overview of literature of animal models combined with analysis of micro array data of cachectic mouse models from the GEO database was performed (chapter 4). This analysis showed that during cancer cachexia the expression of genes involved in mitochondrial fusion, fission, ATP production and mitochondrial density are decreased, while the expression of genes involved in reactive oxygen species (ROS) detoxification and mitophagy are increased. This pointed towards dysfunctional mitochondrial processing. Similar results have been found when studying the MitoCarta gene set using RNA sequencing data of our C2C12 in vitro study from chapter 3 and using publicly available RNA sequencing data from the GEO database (chapter 5). For example, oxidative metabolism was found to be impaired in all models (in vitro and in vivo), based on decreased gene transcription of complex I, IV and V. This reduction in oxidative metabolism can explain the fatigue which is associated with cancer cachexia. Additionally, mitochondrial processes that are impaired in in vitro cancer cachectic models are for example, fission/fusion and pro-apoptosis. Between the in vitro models
and the in vivo models there was an overlap of eight MitoCarta genes (Dut, Ndufc1, Idh3a, Idi1, Cbr2, Coq7, Ndufa5 and Qdpr), which reflects a cachexia signature and can form a basis for future research. The answer to the third research question is: mitochondrial functioning is declined in both in vivo and in vitro models, which points towards a crucial role of mitochondria in cancer-induced cachexia. Therefore, it is highly relevant to further explore the role of mitochondria in cancer cachexia patients. To our knowledge no studies have been done to investigate mitochondrial oxygen consumption in human skeletal muscle during cancer cachexia. To this end, a human observational study was designed (chapter 6). This study could not be completed in time, due to measures associated with the SARS-CoV-2 pandemic, which prevented further inclusion of participants. Eventually, skeletal muscle gene expression of colon cancer patients will be investigated, which is the primary outcome, together with mitochondrial oxygen consumption, a measure for function. Together these results should give a better insight on how mitochondrial function is altered in cancer cachexia patients. The preliminary data of this study show that the population included to date is a good representation of the colon cancer patients as present in the general Dutch population (chapter 7). However, to finish this study more female patients and control patients need to be included. Therefore, the answer to research question 4 cannot be given yet.

In remainder of this chapter (chapter 8) the different models used in this thesis to study cancer cachexia will be discussed, as well as a possible new model. Next, cachectic inflammation and mitochondrial dysfunction are discussed as potential therapeutic targets, with whole-body vibration therapy as a possible add-on treatment option. Lastly, future perspectives are given.

Based on all results of presented in this thesis, one major conclusion is that immune signalling molecules from tumour cells directly impair muscle activity and myogenesis, since creatine kinase activity is affected and immune signalling pathways are upregulated in C2C12 cells incubated with CM (Figure 8.1). Although the various cachexia-inducing cancer cell lines produced similar effects when their CM were added to differentiating C2C12, their profiles of immune signalling molecules are different. Our results suggest at least a role for prostaglandins. Which other molecules are responsible and whether immune signalling or prostaglandins have a causal role remains to be established. Therefore, the specific common profile of immune signalling molecules that is responsible for the effects on muscle cells remains largely unknown. A second major conclusion is that the CM of cachexia-inducing cancer cell lines has a direct impact on mitochondrial function (Figure 8.1). Whole-body vibration training can influence muscle gene expression, and it modulates the expression of the eight identified MitoCarta cachexia signature genes positively (for results see below). This strengthens a possible role for whole body vibration training in the treatment of cachexia. Treatment is not only important to increase the quality of life of cancer patients, but also to increase the tolerance for anti-cancer treatment.
Figure 8.1: Concluding figure. Different tumour types have different profiles of immune signalling molecules, but also other unidentified molecules contribute to the development of cachexia in the muscle. This causes a downregulation of the RNA expression of MitoCarta genes which reduces the mitochondrial functioning of the muscle. Creatine kinase (CK) activity is decreased, which contributes to an impairment in the muscle activity and myogenesis. Whole body vibration (WBV) training can influence muscle gene expression positively.

Models to study cancer cachexia

Different models are in use to study cancer cachexia, which also applies to this thesis. It is very important to realise that every model comes with its advantages and disadvantages. In addition, it is important to realise that depending on the in vivo or in vitro model used to study cachexia, outcomes may differ to a certain extent. This will be further elaborated on using the results as presented in this thesis. Last, the potential of a possible future model which I investigated will be discussed with regards to the feasibility and opportunities.
“Why are controls important in a human observational cachexia studies?”

As the ultimate goal of cancer cachexia research is to find better treatment options for cachectic patients, studies in patients are often preferred. Most human studies on this subject published thus far are observational or are used to validate results obtained in *in vivo* or *in vitro* studies. This is also the case for the COMUNEX study (chapter 6). Perhaps the most challenging, but at the same time crucial aspect of designing this human observational study was to find an appropriate control group. Often, weight stable cancer patients are considered as controls [6, 7, 8, 9]. However, the process of changes ultimately leading to cachexia, pre-cachexia, could have started already, without any changes in weight becoming apparent. This is especially important when parameters like gene expression are studied, as this may be affected well before physical signs of cachexia emerge [10]. Next to that, different studies use different criteria for pre-cachectic patients, resulting in different control populations. This makes it hard to compare results across studies. Moreover, processes involved in initiation of cachexia can occur unnoticed. Therefore, in the observational COMUNEX study we selected a population without cancer and without any inflammatory diseases as control patients. These are patients that undergo a surgical intervention in which the abdomen is opened (inguinal hernia repair or abdominal hysterectomy), to limit the extra burden for the participants included. Crucially, this also allows us to collect a biopsy of the *rectus abdominis*, the same muscle used in the cancer patients. This makes these patients a very good control group for observational cachexia research. Standardisation and a consensus about how to design human studies and the characteristics of controls for cachexia research could help us to do quality research into the earlier events of cancer cachexia. All in all, human observational studies are important to gain knowledge of primary processes that are involved in cancer cachexia in the target population: humans. Since it can be difficult to perform research in humans, other models are used and needed in cachexia research.

“Why is using different models in parallel relevant to cachexia research?”

Animal models and *in vitro* models provide valuable tools to study cancer cachexia, as human studies are not suitable for all purposes (see following paragraph). In this thesis, the C26 mouse model was used to study the effects of whole-body vibration training (chapter 2). Next to this animal study, conditioned media (CM) of the cachexia-inducing KPC, 4662, LLC and C26 cell lines were used in the *in vitro* study (chapter 3). Although these *in vitro* models have a striking overlap in their results to induce cachexia and their underlying mechanisms regarding mitochondrial functioning (chapter 5), they differ in magnitude of their effects. *In vitro* models using KPC, 4662, LLC and C26 CM showed marked differences between cell lines in secreted molecules
inducing immune signalling (chapter 3), which could explain these differences. In line with this, it is understandable that the animal models based on these cachexia-inducing cancer cell lines also differ in their outcomes. Indeed, it is a common finding that these animal models do differ in the extent they represent the human situation [11]. This shows that overarching processes play a role, but also processes that are specific for the cachexia-inducing cancer cell line or model chosen. The results of the studies as presented in this thesis underline that it may be very risky to rely on single models to identify common treatment strategies. The individual cachexia models can initiate unique molecular pathways driving cachexia, which ultimately may only relate to subgroups of cancer cachexia patients [12]. For example, the muscle gene expression data of the LLC or C26 mouse model does not resemble that of pancreatic ductal adenocarcinoma patients [11]. Therefore, we should be careful with generalising the results found in one specific model for cachexia research. If we want to study the processes that are comparable between tumour types, multiple models are needed with different backgrounds. This will eventually lead to better translatability to the human situation. In addition to the differences between cachexia models, also different types of muscle tissue can react differently to the tumour [13]. For example, in chapter 2, we saw that the effects differ between EDL, which consists of mostly type II (fast) muscle fibres, SOL, which consists mostly of type I (slow) muscle fibres, and heart tissue. Improved contractility and prevention of the upregulation of the proteasome pathway were found under the influence of WBV in SOL, but not in EDL or heart muscle. These results show that it is important to apply multiple models and multiple skeletal muscle types (e.g. fast and slow fibre types) in cancer cachexia. When performing cell experiments, it is standard practice to do three biological replicates of each experiment. My results indicate that it may be more valuable to repeat experiments in at least three different in vitro models.

“Which models should we use in future cancer cachexia research?”

The models described above all have their own advantages and disadvantages. Human studies have as main advantage that results are directly applicable to the population studied. However, there are also disadvantages like difficulties to study molecular processes and the limited availability of tissue material to study. Another disadvantage is that patients present a very heterogenous sample. Also, interventions need solid preclinical proof before tests in humans can be performed. Animal models are therefore often used in cachexia research. The genetics of the animals and the environment they live in are very stable, and therefore these models are more likely to produce consistent results. Next to this, all organs of the animal can be studied in detail. Furthermore, interventions can be tested with the use of appropriate controls. Especially for the mouse many experimental tools are available, and, unlike humans, gene inactivation or overexpression
experiments can be done to validate specific targets. Furthermore, because of its relatively small size, maintenance costs and intervention costs (e.g. the amount of a compound needed is small) are relatively minor. An obvious disadvantage is that a mouse is not a human being and translation should be done with care. Nevertheless, in line with the high degree of conservation at the process level, responses are frequently highly comparable. The classic cachexia mouse models use injected cancer cell lines like C26, LLC or KPC cells (chapter 1). Other models use orthotopic tumours, patient-derived xenografts and genetically engineered cancer-prone mouse models [14]. In light of minimizing the use of animal and human studies, in vitro models should be used as well. In vitro models are especially well suited for detailed mechanistic studies. For example, a specific cell type can be studied and specific signalling molecules can be blocked, or genome modulation (e.g. knockdowns) can be applied. In vitro models are also highly suitable for cause-effect studies. For example, by using in vitro studies with CM I was able to show direct effects of tumour-secreted molecules on skeletal muscle mitochondria. In vitro models are also highly suited for screening, for testing many variables and to compare a variety of cachexia models as described above. Costs are relatively low and the in vitro models have a high turnover-time and therefore provide fast results. However, in vitro models also have disadvantages. During the research described in this thesis the C2C12 myoblast/myotubes were used to represent skeletal muscle (chapters 3 and 5). However, C2C12 myoblast/myotubes models can be challenging as C2C12 differentiation is a hard skill to master: protocols vary widely in seeding density and differentiation time [15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28]. Additionally, in vitro models are less representative of the in vivo situation, as the extracellular matrix, innervation and the micro-environment of the muscle are not present.

To obtain a more physiological in vitro model, I also aimed to develop an ex vivo primary muscle fibre culture method with intact muscle fibres and a myo-micro-environment. In order to mimic the wasting muscle in vivo these primary muscle fibres can be co-cultured with (primary) fat and tumour cells. For this technique, the hindlimb muscle (e.g. SOL or EDL) of a mouse was isolated from tendon to tendon after which single muscle fibres were isolated using a collagenase solution (Figure 8.2) [29, 30]. The main advantage of this ex vivo muscle fibre model over the C2C12 myoblast/myotubes is that the fibre structure is intact in all its complexity and thereby the uncertainties in seeding density and differentiation time described above when using C2C12 myoblast/myotubes can be tackled. Also the myogenesis process can be studied because the satellite cells remain in their micro-environment and are attached to the muscle fibres, similar to the in vivo situation. Moreover, both type I and type II muscle fibres can be studied. This approach leads to refinement and reduction compared to traditional in vivo models, because healthy mice instead of cachectic mice are used. Next to that, fewer animals are needed to yield results as muscle fibres of one mouse can be used for multiple conditions.

My results for this ex vivo model showed that intact adult muscle fibres
could be isolated and kept in culture for several days. The primary muscle fibres can be stained for different proteins and a test staining was successfully performed with DAPI to visualise the nuclei in the whole muscle fibre (Figure 8.3 A) and with PAX7 to visualize muscle satellite cells (Figure 8.3 B, C and D). The muscle fibres can also be used to measure muscle force using the permeabilized fibre system of Aurora Scientific [31]. Here, singles muscle fibres are tied down between two needles and put in an organ bath. The muscle fibre is moved to a calcium solution and isotonic muscle contractions can be measured. Using this method functional strength data can be obtained in an ex vivo experiment. At Manchester University, we successfully measured muscle force of the isolated single myofibres. However, the storage method of the single myofibres still needs to be optimized, as the myofibres are hard to recognise in the solution due to the refractive index.
To make this *ex vivo* model suitable to study cachexia, the approaches as used for C2C12, such as incubating primary muscle fibres in co-culture or exposing them to conditioned medium of cachexia-inducing cancer cell lines, should be applicable. Although I was able to culture, stain and measure the force of the muscle fibres, a method to determine whether the muscle fibres did become cachectic is still lacking. This was mainly due to several technical and biological challenges. For example, the equipment and methodology were not sensitive enough to detect effects in the tiny amount of material that is used in this model. Next to this, precision experiments to study protein or gene expression, oxygen consumption for mitochondrial activity, enzyme activity and specific proteins by staining, should be optimized. Additionally, the storage for the single muscle fibre force measurements needs optimisation. Especially experiments to measure gene expression or enzymatic CK activity, as done for the C2C12 experiments in this thesis, are needed to show if the muscle fibres indeed become cachectic. Despite these challenges significant progress has been made and this *ex vivo* model should definitely be considered for cachexia research in the future.
Potential therapeutic targets

In this thesis different potential therapeutic targets came to light. We identified a strong upregulation of inflammatory pathways in the *in vitro* cancer cachectic model, but also mitochondrial dysfunction both in the *in vitro* and *in vivo* cachectic models. Therefore, the contribution of immune signalling molecules is discussed first, after which the role of mitochondrial dysfunction as a potential treatment target is explored. Finally, the potential of whole-body vibration training as a therapy for cancer cachexia is addressed.

“Do immune signalling molecules contribute to cancer cachexia?”

In view of the potential role of inflammation in cancer cachexia, as proposed in chapter 3, studies have addressed the possible beneficial effects of anti-inflammatory and / or immunosuppressive compounds on the disorder. However, mixed effects were found with drugs modulating cytokine action [32]. Blocking TNF$_\alpha$ was found not to be effective in the treatment of cancer cachexia [33] and anti-TNF monoclonal antibodies (infliximab) did not give the results that were hoped for [34]. A natural IgG1k human monoclonal antibody targeting interleukin 1$_\alpha$ (MABp1) produced some positive changes on body mass, but these were not sufficient to produce significant effects [34]. Although literature indicated that TNF$_\alpha$ and IL6 play an important role in cancer cachexia [35, 36], I did not find these cytokines in the conditioned medium of the cachexia-inducing cancer cell lines as used in this thesis. For the immune signalling molecules that were investigated in this thesis, none of the candidate molecules were able to fully mimic the effects of the conditioned medium of cachexia-inducing cancer cell lines on differentiated C2C12 (chapter 3). However, the mimic medium containing prostaglandins, PGE2 and PGF2$_\alpha$, partly reproduced the effect. A rise in PGE2 level was also found in *gastrocnemius* muscle after incubation with serum from cachetic mice [2]. The precursor of PGE2 and PGF2$_\alpha$ is arachidonic acid, but supplementation with arachidonic acid has been found to induce muscle cell hypertrophy, which is opposed to what was expected [37]. However, when prostaglandins synthesis was targeted with cyclooxygenase-2 inhibitors, promising results in terms of improved body weight and appetite were obtained in the treatment of cancer cachectic patients [38]. The polyunsaturated fatty acid eicosapentaenoic acid (EPA) inhibited the rise in muscle PGE2 content in response to serum from cachetic mice and also inhibited muscle protein degradation [2]. On the other hand, as shown during ageing, too low levels of PGE2 also have detrimental effect on muscle mass, and inhibiting the prostaglandin degrading enzyme 15-PGDH rejuvenates aged muscle [39]. Together, these results suggest that the balance of prostaglandins is tightly regulated. Therefore, these kind of therapies should be focusing on re-establishing physiological homeostasis.

Despite our extensive investigations, there were also limitations to the
research methodology. A total number of 111 cytokines were measured. However, there are many more immune signalling molecules that could cause cachexia. Two prime examples are myostatin and activin A [40, 41, 42]. Additionally, recent research revealed that microRNAs can change the gene expression in skeletal muscle tissue and can contribute to muscle wasting [43, 44, 45]. The research of chapter 3 focused on the direct communication between the tumour and the muscle, but as stated in chapter 1, cachexia is a multi-organ syndrome. In the in vitro model, for example, the neuronal innervation and the immune system are missing. Because of these limitations, it beyond doubt that the contribution of the inflammatory signalling molecules to cancer cachexia has not been completely unravelled with my studies. Nevertheless, the possibility of prostaglandins as treatment target seems a relevant option to explore in the future.

“What is the role of mitochondrial dysfunction in cancer cachexia?”

Mitochondrial dysfunction not only plays an important role in cancer cachexia, but also in other chronic diseases, during cancer chemotherapy, ageing and during prolonged inactivity [46]. Mitochondrial dysfunction has also been reported on a functional level in cachectic mouse models (mitochondrial turnover, ROS production and mitochondrial respiratory control ratio) [47, 48]. We also found that mitochondrial dysfunction was present in both our in vitro and in vivo models (chapter 5). When compared to the data from literature that were used for chapter 4, we see in all studies performed a downregulation of oxidative phosphorylation. Complex I, IV, V and pyruvate dehydrogenase were found to be downregulated in both the studies of chapter 4 and chapter 5. Additionally, overlap was found in mitochondrial fusion and fission genes (Dnm1l, Opa1, Mfn2) which were downregulated. Since the CM of cachexia-inducing cancer cell lines are able to establish this effect in vitro, specific (combinations of) molecules secreted by these cell lines are involved.

Mitochondrial dysfunction and oxidative metabolism as treatment targets could be explored by for example pharmacological compounds targeting the mitochondria [49]. Elamipretide, a first-in-class cardiolipin-binding compound that is targeted to mitochondria can improve oxidative phosphorylation capacity. It has been described to increase mitochondrial capacity and to result in a clinically meaningful change in performance on the 6 min walk test in elderly [50, 51]. Furthermore, SS-31, a synthetic peptide that improves mitochondrial function, has been shown to improve cachectic symptoms in mice studies [52, 53]. These compounds are promising, however, both compounds should be investigated in more detail.

In the COMUNEX human study, which was not finished at the moment of writing this thesis, mitochondria are studied using rectus abdominis biopsies which will be compared to control patients in the future (chapters 6 and 7). Gene expression and the identified mitochondrial signature will be investigated
next to muscle oxygen consumption using the Oroboros O2k-FluoRespirometer. Here, we will probably gain more evidence for the role of muscle mitochondria in cancer cachexia. Collectively, this thesis provides considerable evidence for the prominent part mitochondrial dysfunction plays in cachectic muscle tissue in both in vitro and in vivo models.

The combination of the literature review (chapter 4) and the experimental data described in this thesis (chapter 5) show the importance to study the role of mitochondria in cancer cachexia in the future, as it appears in the in vitro and in vivo models and across the different cachexia-inducing cancer cell lines.

“Can whole-body vibration training be a part of anti-cachexia therapy?”

To target both inflammation and mitochondrial dysfunction, an exercise therapy could be part of a multimodal treatment strategy for cancer cachexia [49]. In chapter 2 the benefits of a low impact exercise for cancer patients suffering of cachexia are described. In our mice study, whole-body vibration training had a small impact on gene expression; the oxidative phosphorylation pathway was downregulated. Next to this, whole-body vibration training prevented upregulation of the gene expression of the proteasome pathway in the SOL in the cachectic C26 mice. In chapter 5 we found aerobic respiration to be the top downregulated pathway in the MitoCarta geneset of both the in vitro and in vivo cachexic models examined. Except complex II, all complexes of oxidative phosphorylation seem to be downregulated. This is remarkable as some subunits of these complexes are encoded on the mitochondrial DNA. Next to this, genes of the TCA cycle were also downregulated. This will lead to a low energy supply in the muscle and the muscle will not be able to generate energy to power contractions. Exercise could increase mitochondrial volume and oxidative capacity, and many animal studies have been performed in relation to cachexia treatment as has been reviewed by JM Memme and DA Hood [54]. Due to fatigue, cancer patients cannot always adhere to traditional exercise training. Therefore, it is interesting to explore the potential of WBV. A set of 8 MitoCarta genes was identified to be differentially regulated in both the in vitro general cachexia model (GCM) and the in vivo models from the GEO database (chapter 5). These cachexia signature genes were investigated as potential reporter genes in the microarray of the cachectic C26 mice with whole-body vibration training in both EDL and SOL muscle (table 8.1). All genes, except Idi1 which was absent on the microarray, were found to be downregulated in the tumour-bearing mice. Whole-body vibration training had a mild effect by attenuating the downregulation, especially in the EDL as the fold change values are closer to zero. The mild effect on gene expression and these additional results show that this form of low impact exercise training could be of added value in a multimodal therapy for cancer cachexia.
Table 8.1: Effect of WBV on the mitochondrial signature genes. FC = fold change, padj = adjusted p-value or false discovery rate, C = control mice, T = tumour-bearing mice, T + WBV = tumour-bearing mice + whole-body vibration training. * p<0.05, ** p<0.01, *** p<0.005, and **** p<0.001.

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Future perspectives

The studies described in this thesis show the complexity of cachexia and that the development of truly effective treatments for this serious syndrome will, unfortunately, take more time. On the other hand, the results confirm the direction in which the treatment seems to be developing, namely by applying a multimodal therapy targeting immune signalling molecules (prostaglandins) and restoring mitochondrial functioning.

Mitochondrial dysfunction (downregulation of mitochondrial energy processes and upregulation of ‘mitochondrial survival’) and a set of MitoCarta genes that were identified in the cell and mouse cachexia models, underlines the importance of altered mitochondrial processes in cancer cachexia. While the relevance of mitochondrial dysfunction during cancer cachexia have been studied, the eight overlapping MitoCarta genes that were found both in vitro and in vivo have not been studied in humans yet. This can be investigated using a similar approach as in chapter 5, using existing human datasets of the GEO database, but not much data is available. Therefore, the ongoing COMUNEX study described in chapter 6 could be of great value as this study has the appropriate controls. The study looks promising but could not yet be finished due to the practical limitations caused by the SARS-CoV-2 pandemic. However, this study will continue and hopefully confirm the results presented in the different chapters. Additionally, using existing animal or human datasets in which different treatments are tested, it could be explored whether effective anti-cachexia treatments change the gene expression of the identified MitoCarta genes.

Potential therapies involving the modulation of PGE2 and PGF2α synthesis and / or effects, mitochondrial function and WBV could be investigated in the future. In an ex vivo model in which cachexia is induced, drugs that modulate PGE2 and PGF2α or drugs that alter mitochondrial function (both described above) could be investigated. The ex vivo primary
muscle fibre model remains of great interest as the muscle can be studied in its micro-environment. The primary readout parameters would be myogenesis, using immunostaining, and mitochondrial dysfunction, by analysing MitoCarta genes, and mitochondrial oxygen consumption. The ex vivo model with primary muscle fibres can be used after the optimization steps stated above are performed. Different cachexia-inducing cancer cell lines should be used to investigate if the effects are transferable between cancer types. Furthermore, despite the high number of immune signalling molecules that were identified, only prostaglandins were able to partly mimic the effect on C2C12 in our hands, as described in chapter 3. An important question that remains is which other compounds present in the CM are responsible for the remainder of the effect on C2C12 cells. There are multiple options to investigate these other molecules: 1) analyse more compounds in the CM (for example including microRNAs, proteins and other inflammatory compounds); 2) block the release and/or activity of specific compounds (groups) in the tumour CM to find which one is essential; 3) identify differentially expressed receptors and do a ligand binding study. The identification of possible therapy targets and the signalling molecules responsible for inducing cachexia will lead to development of therapies for cancer cachexia. Research will teach us if this treatment can be applied broadly for multi cancer types or that a shift towards personalised medicine is needed.
References


Summary
Cancer cachexia is a systemic and multifactorial catabolic syndrome characterised by depletion of muscle mass, with or without concomitant loss of fat mass. It has profound consequences for patient prognosis and treatment outcomes. Moreover, quality of life due to the occurrence of fatigue, reduced appetite and declined mobility, is severely affected. Due to the ageing population, the prevalence of cancer and cancer cachexia increases, but also population-wide screening programs contribute to the increased prevalence. There are different guidelines for diagnosing cancer cachexia, which have loss of body weight in common as the important clinical feature. However, it becomes increasingly apparent that, especially in cancer patients suffering from obesity, CT scanning provides more detailed diagnosis information. This not only allows better detection of ‘hidden’ muscle depletion, but can also disclose the presence of intramuscular fat. Multiple organs are mechanistically involved in cancer cachexia, which makes it a complex condition to study. The molecular mechanisms that drive cancer cachexia are partly unknown and to date there are no effective therapies to manage the disorder. In the quest to understand these molecular mechanisms and to find potential treatment targets, multiple models are at hand. In this thesis an in vitro study, an in vivo animal study and a human observational study were used. In vitro models allow the study of direct interactions between the tumour and the muscle, while in vivo animal models have the advantage of studying the whole organism in a controlled environment. The human observational study described in this thesis will be used at a later stage to confirm results found in other models, as this study is currently not finished.

Exercise can often effectively increase skeletal muscle mass of healthy people. Unfortunately, this type of intervention is not always an option for cachectic patients due to fatigue and lessened mobility. For those situations, low impact exercise can be an option to explore, as compliance with this type of exercise could be higher. In chapter 2 of this thesis a cachectic mouse model was used to study the effects of whole-body vibration training (WBV). This is a type of intervention in which a vibration plate is used to cause reflexive muscle contractions. The cachectic model was created by using colon 26 carcinoma cells (C26) in CD2F1-mice. Body mass, lean mass, fat mass and extensor digitorum longus (EDL) mass were all lower in tumour bearing mice compared to controls, showing that the tumour bearing mice were indeed cachectic. The WBV (19 days, 15min/day, 45Hz, 1.0g acceleration) had no effects on body mass and composition, daily activity, blood parameters and skeletal muscle histology and function, apart from improved contractility in soleus muscle (SOL). However, analysis of transcriptome data clearly revealed that WBV reduced the tumour-related effects in EDL, SOL and heart muscle. These effects were associated with an attenuation of the upregulation of the proteasome pathway in SOL. Although these changes seem relevant, they were minor and do not (yet) provide clear directions for treatment strategies. In the tumour and tumour plus WBV groups we also found that the oxidative phosphorylation pathway was among the most abundant downregulated pathways in EDL, SOL and heart tissue.
The oxidative phosphorylation pathway is important for energy homeostasis in the muscle. The confirmation of these changes in mitochondrial functioning are described in chapter 4 and chapter 5.

To study the direct effects of different tumour types on skeletal muscle, an in vitro model was used in chapter 3. Next to the C26, which was also used in the animal model of chapter 2, three other cachexia-inducing cell lines were included; the Lewis lung carcinoma cell line (LLC) and two murine pancreatic ductal adenocarcinoma cell lines (KPC and the genetic variant 4662). The effects of the conditioned media (CM) of these cachexia-inducing cancer cell lines on differentiating murine C2C12 myoblasts/myotubes (C2C12) were investigated next to analysing several immune mediators present in the CM. As an endpoint outcome measure we used creatine kinase (CK) activity, an enzyme that is involved in regulating the concentration of adenosine triphosphate (ATP) within a cell. High ATP requirements are related to higher levels of CK and this indicates the muscle is growing. The CM of the different cachexia-inducing cell lines significantly reduced the CK activity in differentiating C2C12 cells. Global gene expression, measured by RNA sequencing, showed a significant upregulation of immune pathways on differentiation day 5. Additionally, oxidative phosphorylation was again found in the top downregulated pathways, which is in line with the results described in chapter 2. Based on the changes in immune-related pathways we decided to analyse a set of immune-related components present in the CM of the cancer-inducing cell lines. A total of 111 cytokines were screened and levels of selected cytokines and prostaglandins were quantified. Based on this information a mimic medium was created to further explore their effects on CK activity in differentiating C2C12 cells. Only mimic media containing prostaglandin E2 and prostaglandin F2-alpha decreased CK activity, although quantitatively the decrease was smaller than that caused by the CM. Using this in vitro model we showed that the CM of cachexia-inducing cancer cell lines is able to influence differentiation of C2C12 as a model for cancer cachexia. Additionally, there appears to be a role for prostaglandins in the direct induction of cachexia from the tumour to the muscle.

In the two cachexia models described in chapter 2 and chapter 3 a downregulation in the oxidative phosphorylation pathway was found. To review the current knowledge on muscle mitochondrial functioning during cancer cachexia a literature study was performed which is described in chapter 4. We aimed to fill existing knowledge gaps by integrating published data (94 research papers) on muscle protein or gene expression from 11 different cancer-induced cachexia animal models. This data was combined with four genome-wide transcriptome datasets of cancer-induced cachexia mouse models. Our analysis showed that the expression of genes involved in mitochondrial fusion, fission, ATP production and mitochondrial density is decreased, while that of genes involved in reactive oxygen species (ROS) detoxification and mitophagy is increased. This underlines the importance of muscle mitochondrial functioning and formed the basis for the research described in chapter 5. Here, we first aimed to explore comparability between
the CM of different cachexia-inducing cell lines (KPC, 4662, LLC and C26) to cause mitochondrial changes in global gene expression data, using the in vitro model of chapter 3 and the MitoCarta 3.0 dataset. About 70% of the significant differentially expressed MitoCarta genes were downregulated. In contrast, the whole genome expression consisted mainly of upregulated genes. The CM conditions of KPC, 4662 and LLC seemed comparable in their effect on C2C12 MitoCarta genes, while the effect of C26 CM was smaller. The gene expression results of the C2C12 incubated with CM of the KPC, 4662, LLC and C26 cell lines were combined to create a general cachexia model (GCM). Here, changes in MitoCarta genes were seen most abundantly with genes related to energy metabolism processes (oxidative phosphorylation, TCA cycle or pyruvate metabolism). Next, we aimed to study the comparability between our in vitro model and in vivo cachectic mouse models as present in publicly available databases. The in vivo data turned out to have an overlap with the in vitro data, eight MitoCarta genes were found in both the GCM and the in vivo models. These eight MitoCarta genes were also all downregulated in the C26 mouse model as described in chapter 2. Moreover, WBV training seemed to counteract this downregulation to a small extent. The translatability of these results to in vivo data shows the relevance of these cancer cachexia models.

To investigate muscle gene expression in humans, to gain more insight in the underlying processes and to confirm our results on skeletal muscle mitochondrial gene expression, the COMUNEX study was designed. In chapter 6 it’s study design was discussed. COMUNEX aims to investigate the differences in gene expression, body composition, muscle function and muscle metabolism in colon cancer patients compared to age-matched controls without colon cancer. We aim to include 40 colon cancer patients undergoing a tumour resection (30 patients with a primary tumour and 10 with liver metastases) and 15 control patients undergoing an inguinal hernia repair (men, n=10) or an abdominal hysterectomy (women, n=5). These control groups were chosen as these patients undergo relatively simple surgery for a condition that is not believed to be associated with an elevated inflammatory status. Like stated above, the primary study parameter will be muscle gene expression. Secondary parameters will be pre-surgery body composition (using CT scans), muscle function parameters (grip strength and selected biopsy tissue markers), metabolic markers (measured in fat and muscle biopsies taken during surgery), biochemical markers (measured in blood/serum taken during surgery) and gene expression in fat biopsies. Inclusion of participants started in 2017 and during the course of the study some setbacks were encountered. The inclusion rate was lower than anticipated and restrictions in response to the SARS-CoV-2 pandemic have led to delays in the study. To also facilitate handing the study over to other researchers an update of preliminary data was given in chapter 7. In this update, data of 21 of the 30 primary colon cancer patients undergoing a tumour resection were discussed. The patients participating in the COMUNEX study are a good representation of the Dutch male colon cancer
population. Most participants are suffering from sarcopenia. However, a comparison with the, still to be included, control and liver metastatic groups is needed for interpretation of other data regarding muscle wasting, including transcriptomics.

In chapter 8 the conclusions are presented and the main findings of this thesis are further discussed. In particular, the importance of models to study cancer cachexia is addressed. Additionally, the findings on immune mediators secreted by the cachexia-inducing cell lines and the muscle mitochondrial dysfunction is put into perspective and relevance to future therapies is discussed.
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The idea was to switch from my offices at HNH to an office at HAP during my PhD research. This did not go as planned due to experimental planning and COVID-19 measures. For a short time I was in the HAP office for at least one day a week, however soon after we started working from home. Despite this I always felt very welcome at the HAP office. **Bart Lagerwaard**, thank you for your help with the analysis of my RNAseq data. Also I liked having you as an Oroboros buddy, although it was only for a short time. **Evert**, thanks for the input on my manuscripts. Of course I also want to thank all other current and former HAP staff and PhD candidates **Arie**, **Vincent**, **Katja**, **Silvie**, **Sandra Tjabringa**, **Deli**, **Wenbiao**, **Jeske**, **Marianne**, **José**, **Lianne**, **Natasja**, **Taolin**, **Joelle**, **Anna**, **Dingyi**, **Jelle** and **Liangyu**. You were all great colleagues and I loved the HAPLABSTAP dag and other gatherings.

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About the author
Curriculum vitae

Miranda van der Ende was born on the 15th of August 1990 in Vlissingen, the Netherlands. She attended primary and secondary school in Middelburg. At the HAVO she enrolled in the *Nature and Health* track.

In 2008 she enrolled in the BASc program *Biological and Medical Laboratory Research* at Avans University of Applied Science (Breda, the Netherlands). At Avans she obtained two specialties, one in *Medical Research* and one in *Laboratory Animal Science* (article 13f, Dutch law on animal experiments). Her Bachelor internship and thesis consisted of a nine month project at University College Cork (UCC) in Ireland, where she studied satiety mediation through the modulation of GPCRs within the brain-gut axis. For this project, she obtained an Erasmus scholarship.

In 2012 Miranda’s education continued at the KU Leuven in Belgium, first with a 90 ECTS program to obtain an academic Bachelor. Then, in 2014, Miranda started the international *Biomedical Science* Master program from which she graduated *cum laude*. For her studies at the KU Leuven Miranda was awarded the VSBfonds scholarship. Miranda’s Master’s thesis at the laboratory of Clinical and Experimental Endocrinology was entitled “The importance of the macronutrient composition of a weight loss diet”. This study resulted in a publication in PLOS One.

Miranda was appointed as a PhD candidate in 2016 at the chair groups *Nutritional Biology* and *Human & Animal Physiology*. In her PhD project Miranda studied muscle wasting in cancer, also known as cachexia. Here she performed cell, animal and human studies. Miranda presented her work at the 4th Cancer Cachexia Conference in Philadelphia, USA and the 12th Cachexia Conference in Berlin, Germany. Next to her primary research activities, Miranda was involved in teaching, supervising MSc students, being a member of the PhD committee and being a member of the governance table. After 5 years Miranda finished her research, of which the results can be found in this thesis, meanwhile, two chapters of this thesis have been published in peer-reviewed journals.
List of publications


Roman Vangoitsenhoven, Miranda van der Ende, Katrien Corbeels, João Paulo Monteiro Carvalho Mori Cunha, Matthias Lannoo, Pierre Bedossa, Schalk van der Merwe, Ann Mertens, Ina Gesquiere, Ann Meulemans, Christophe Matthys, Chantal Mathieu, Lut Overbergh, Bart Van der Schueren. At similar weight loss, dietary composition determines the degree of glycemic improvement in diet-induced obese C57BL/6 mice. PloS one, 2018. DOI: 10.1371/journal.pone.0200779

* = Equal contributions

Expected publications

Miranda van der Ende, Rogier Plas, Sander Grefte, Renate Winkels, Dik Snijdelaar, Flip Kruyt, Tjarda van Heek, Marlieke Visser, Jaap Dronkers, Alessandro Laviano, Klaske van Norren. COMUNEX; The effects of colon cancer on muscle gene expression, body composition, muscle function, and muscle metabolism.

Miranda van der Ende, Mieke Poland, Fleur Jansen, Jocelijn Meijerink, Sander Grefte, Jaap Keijer, Renger Witkamp, Klaske van Norren. Prostaglandins can partly mimic the effects of cachexia-inducing cell line secretomes in differentiating muscle cell

Overview of completed training activities

**Discipline specific activities**

- Seminar and workshop: Opportunities for studying cellular metabolism in live cells with the Seahorse analyzer, 2017. Wageningen, the Netherlands
- Symposium Guts2Move, 2017. Wageningen, the Netherlands
- PUFA symposium, 2017. Wageningen, the Netherlands
- Summerschool Mitochondrial Physiology - From Organelle to Organism (poster), 2017. Copenhagen, Denmark
- 4th Cancer cachexia conference (poster), 2018. Philadelphia, United States
- Training at Manchester Metropolitan University, 2018. Manchester, United Kingdom
- 12th MIPschool Coimbra (poster), 2019. Coimbra, Portugal
- 12th Cachexia conference (poster), 2019. Berlin, Germany
- 5th Cancer Cachexia Conference, 2020. Online
- 13th Cachexia conference (poster), 2020. Online

**General courses and activities**

- Symposium Publish for Impact, 2017. Wageningen, the Netherlands
- VLAG PhD week. Baarlo, the Netherlands
- Course Data Management Planning (WGS), 2017. Wageningen, the Netherlands
- Effective behaviour in your professional surroundings (WGS), 2018. Wageningen, the Netherlands
- Teaching lab practicals (ESD), 2018. Wageningen, the Netherlands
- Supervising BSc and MSc thesis students (ESD), 2018. Wageningen, the Netherlands
- Reviewing a Scientific Paper (WGS), 2019. Wageningen, the Netherlands
- PhD workshop Carousel (WGS), 2019. Wageningen, the Netherlands
- GCP course (HNH), 2019. Wageningen, the Netherlands
- Chemometrics (Multivariate Statistics) (VLAG), 2020. Wageningen, the Netherlands
• Career Orientation (WGS), 2021. Wageningen, the Netherlands

• Writing propositions for your PhD (WGS), 2021. Wageningen, the Netherlands

• Last Stretch of the PhD Programme (WGS), 2021. Wageningen, the Netherlands

Other activities

• Preparation of research proposal, 2016. Wageningen, the Netherlands

• Weekly group meetings Nutrition and Pharmacology, 2016 -2021. Wageningen, the Netherlands

• Weekly group meetings Nutritional Biology group and Nutrition Metabolomic and Genomics group or group meetings Human & Animal Biology, 2016 -2021. Wageningen, the Netherlands

• Monthly muscle meetings, 2016 -2018. Wageningen, the Netherlands

• Journal club/literature reading, 2016 -2021. Wageningen, the Netherlands

• MOOC- Nutrition & Health, 2017. Wageningen, the Netherlands

• PhD study tour HNH to Canada, 2019
Colophon

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The selfish tumour: Cachexia-associated changes in skeletal muscle

Miranda van der Ende