

Skeletal muscle wasting:
Clinical implications and experimental treatment

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PART ONE

INTRODUCTION

Chapter 1

General introduction and aim and outline of the thesis

CHAPTER 1

GENERAL INTRODUCTION AND AIM AND OUTLINE OF THE THESIS

GENERAL INTRODUCTION

CACHEXIA

Cachexia is a clinical condition characterized by muscle wasting, anorexia and metabolic change.¹ The term derives from the Greek words 'kakos' and 'hexis', which translates into 'bad condition' and has for centuries been recognized as a state of deteriorating body habitus. It is associated with a wide variety of clinical conditions, e.g. cancer, chronic obstructive pulmonary disease (COPD), chronic heart failure (CHF), chronic kidney disease (CKD), acquired immune deficiency syndrome (AIDS) and sepsis.²⁻⁴ Individuals affected by cachexia undergo changes in body composition, characterized by a loss of skeletal mass with or without the loss of body fat.^{5,6} Affected patients suffer from reduced physical function.⁷ In some patients cachexia is associated with reduced caloric intake, yet conventional nutritional support cannot reverse the process of ongoing cachexia.^{6,8} This stands in contrast to e.g. a reduced caloric intake in patients suffering from dysphagia due to an underlying esophageal tumor, who may still show a beneficial response to aggressive nutritional support. Additionally, cachectic patients are more prone to reduced therapy effect and increased toxicity.⁹⁻¹¹ Cachexia is rarely recognized prior to end-stage disease.^{1,12,13} Up to 80% of patients with advanced cancer are affected by cancer associated cachexia (CAC) and it is estimated that as much as 30% of cancer-related deaths result from cachexia.¹⁴⁻¹⁷ Thus cachexia forms an important cause of mortality in the cancer patient. Cachexia is particularly common in patients with malignancies of the pancreas, esophagus, stomach, lung and colorectal tract. Weight loss is observed in up to 87% of these patients at initial diagnosis, before initiation of any form of therapy.^{12,18} To enable an early recognition of cachexia several attempts have been made to come to a consensus on its definition. At present, its criteria include weight loss greater than 5% over a six-month period, or BMI < 20 accompanied by any degree of weight loss greater than 2%, or a loss of lean body mass as assessed by dual-energy x-ray absorptiometry (DXA), bioelectrical impedance or using computed-tomography (CT) imaging.¹ Additionally with this consensus it is suggested that cachexia is, in contrast to historical belief, not just an indication of end-stage disease but rather a spectrum consisting of precachexia, cachexia and finally refractory cachexia.

FRAILITY AND SARCOPENIA

Frailty is defined as a biologic syndrome characterized by decreased reserve and resistance to stressors that results from cumulative declines across multiple physiologic systems, which causes vulnerability to adverse health outcomes.^{19, 20} A hallmark sign of frailty is sarcopenia, the involuntary loss of skeletal muscle mass.²¹⁻²³ The term sarcopenia is derived from the Greek words 'sarx' and 'penia', which translates into 'flesh' and 'poverty'. Originally, sarcopenia was considered to impair physical performance and survival in geriatric, non-cancer populations and to be characterized by a loss of skeletal muscle mass, skeletal muscle strength and physical performance.²⁴⁻²⁶ Later, sarcopenia was also found to impair survival in a variety of clinical conditions, e.g. cancer.²⁷ These findings were greatly facilitated by the introduction of computed tomography to quantify skeletal muscle mass. This allowed to interpret the impact of body composition in population-based studies, a method first described by Prado et al.²⁸

It is of importance to note however, such population based studies commonly refer to low skeletal muscle mass on computed tomography as sarcopenia, in contrast to earlier literature definitions which define sarcopenia as low skeletal muscle mass in combination with decreased skeletal muscle strength as indicated by functional parameters such as low gait speed.²⁹ However, it is likely that the cause of muscle wasting, e.g. sarcopenia or cachexia, may be indistinguishable in clinical practice or have an overlapping presence, e.g. the elderly patient suffering from malignant disease. Therefore, it has been suggested that new therapeutic approaches must target both conditions.³⁰

EXPERIMENTAL TREATMENT STRATEGIES

Muscle wasting in cachexia is the result of decreased protein synthesis, in combination with and perhaps more importantly, increased protein degradation.^{31, 32} It is suggested to be a gradual process aggravated by the chronic systemic inflammation found in cancer.^{1, 32} There is an important role for the activation of the ubiquitin-proteasome pathway (UPP).³¹ This pathway increases protein degradation due to elevated muscle specific ubiquitin (Ub) ligases Muscle atrophy F-Box (MAFbx, also known as atrogin-1) and Muscle RING Finger-1 (MuRF1).³³⁻³⁵ Myostatin, otherwise known as growth differentiation factor 8 (GDF8), is a key regulatory factor in the UPP.³⁶ Myostatin is part of the TGF- β family cytokines. It is mostly expressed in skeletal muscle. By binding the activin receptor type IIB (ActRIIB) it initiates two important signaling pathways. The aforementioned UPP which ultimately leads to increased protein degradation. Second, it causes an arrest in myoblast proliferation through interference with the Smad and ERK1/2

MAPK pathways. This leads to inhibition of key myogenic regulatory factors, such as MyoD.^{37,38} This ultimately leads to reduced protein synthesis. Therefore, ActRIIB inhibition could attenuate muscle wasting in cancer-associated cachexia.

DIETARY INTERVENTIONS

In addition to pharmaceutical strategies to limit the activity of catabolic cytokines in cancer cachexia, dietary interventions have sparked great interest.³⁹⁻⁴⁶ Such dietary interventions include, but are not limited to, long-chain omega-3 fatty acid eicosapentaenoic acid (EPA) and β -hydroxy- β -methylbutyrate (HMB), a leucine metabolite. EPA is one of the most frequently investigated supplements. However, systematic reviews since have been unable to support the clinical application of EPA for the treatment of cancer-associated cachexia.^{45,47} HMB on the other hand limits experimental muscle wasting in vivo^{40,42} as well as in limited clinical trials.^{48,49} Yet another dietary supplement, quercetin has been described to limit muscle wasting in vivo.⁴¹ Quercetin is a plant pigment (flavonoid). It is found in many vegetables, herbs, and fruits.⁵⁰ Antioxidant, anti-inflammatory, and anti-aging effects of quercetin have previously been described.⁵¹⁻⁵⁴ Moreover, quercetin was found to limit loss of muscle mass in an APC knockout cachexia model and obesity model.^{41,55} These data suggest that dietary supplementation with quercetin might limit muscle wasting and loss of muscle function in cancer cachexia.

Caloric restriction is another form of dietary intervention. The beneficial effects of CR on healthspan and longevity have been thoroughly established in model organisms, including reduced incidence of cancer, cardiovascular disease, and increased oxidative stress resistance⁵⁶⁻⁶³, and it has been reported to limit sarcopenia in rodents and nonhuman primates.⁶⁴⁻⁶⁷ Similarly as in cancer cachexia, catabolic pro-inflammatory cytokines are suggested to play an important role in the development of age related sarcopenia.^{68,69} Short-term CR improves insulin sensitivity, increases insulin/insulin-like growth factor 1 signaling, increases expression of markers of antioxidant defense, and reduces expression of markers of inflammation in mice.⁶¹ These data prompt the question whether CR could limit muscle wasting and loss of muscle function in cancer cachexia.

AIM AND OUTLINE OF THE THESIS

Although skeletal muscle wasting in cachectic cancer patients has long been recognized as detrimental for patient outcome, its detection is often limited to subjective clinical assessment. Due to a lack of reliable diagnostic tools early skeletal muscle wasting may easily go unnoticed in clinical practice. Body composition assessment on diagnostic CT imaging allows us to objectively determine skeletal muscle mass in population-based studies, and interpret its impact on treatment outcome. However standardization of software tools to analyze images obtained by CT is lacking. Therefore, the aim in **part one** of this thesis is to explore the accuracy of various software programs which have been used to quantify cross-sectional body composition using (diagnostic) CT imaging and investigate the impact of decreased skeletal muscle mass in patients undergoing curative intent treatment for underlying gastrointestinal and hepatopancreatobiliary malignancies, and patients considered candidates for liver transplantation.

Assessment of the impact of decreased skeletal muscle mass in these patient groups may help in preoperative risk stratification, i.e. select those patients who are deemed to have limited to no survival benefit from surgery. Additionally though, these patients may one day benefit from novel therapeutic treatment options countering the loss of muscle mass. Hence, in **part two** of this thesis we explore potential treatment strategies to attenuate muscle wasting in an experimental cancer-associated cachexia mouse model, via activin like kinase 4 and 5 inhibition, caloric restriction and quercetin supplementation.

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PART TWO

THE IMPACT OF LOW SKELETAL MUSCLE MASS IN SURGICAL ONCOLOGY & SOLID ORGAN TRANSPLANTATION

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| Chapter 2 | Systematic review and meta-analysis of the impact of computed tomography assessed skeletal muscle mass on outcome in patients awaiting or undergoing liver transplantation |
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CHAPTER 2

SYSTEMATIC REVIEW AND META-ANALYSIS OF THE IMPACT OF COMPUTED TOMOGRAPHY ASSESSED SKELETAL MUSCLE MASS ON OUTCOME IN PATIENTS AWAITING OR UNDERGOING LIVER TRANSPLANTATION

ABSTRACT

Liver transplant outcome has improved considerably as a direct result of optimized surgical and anesthesiological techniques and organ allocation programs. Because there remains a shortage of human organs, strict selection of transplant candidates remains of paramount importance. Recently, computed tomography (CT)-assessed low skeletal muscle mass (i.e. sarcopenia) was identified as a novel prognostic parameter to predict outcome in liver transplant candidates. A systematic review and meta-analysis on the impact of CT-assessed skeletal muscle mass on outcome in liver transplant candidates were performed according to the Preferred Reporting Items for Systematic Reviews and Meta-Analysis guidelines. Nineteen studies, including 3803 patients in partly overlapping cohorts, fulfilled the inclusion criteria. The prevalence of sarcopenia ranged from 22.2% to 70%. An independent association between low muscle mass and posttransplantation and waiting list mortality was described in 4 of the 6 and 6 of the 11 studies, respectively. The pooled hazard ratios of sarcopenia were 1.84 (95% confidence interval 1.11–3.05, $p = 0.02$) and 1.72 (95% confidence interval 0.99–3.00, $p = 0.05$) for posttransplantation and waiting list mortality, respectively, independent of Model for End-stage Liver Disease score. Less-consistent evidence suggested a higher complication rate, particularly infections, in sarcopenic patients. In conclusion, sarcopenia is an independent predictor for outcome in liver transplantation patients and could be used for risk assessment.

INTRODUCTION

2

As human organ shortage remains prevalent, strict selection of transplant candidates is of paramount importance. The combination of waiting list mortality and post-transplantation survival are key deciding factors in waiting list placement. Currently, the Model for End-stage Liver Disease (MELD) score, a validated risk-based system that predicts waiting list mortality, is used to allocate donor livers.¹ Although the introduction of the MELD-score has led to a decreased number of patients on the waiting list, shortened waiting time and decreased waiting list mortality despite increasing disease severity², objective parameters reflecting a patient's nutritional and functional status in particular are lacking and attempts have been made to modify and improve the MELD-score.^{3, 4} Frailty, the inability to adequately respond to stressors (i.e., surgery), for instance, has been identified as a prevalent syndrome in liver transplant candidates that strongly predicts waiting list mortality.⁵

Skeletal muscle wasting (i.e., sarcopenia), which is a common syndrome in chronic diseases such as liver failure, is a key feature of frailty. The association between sarcopenia and treatment outcomes, such as complications and survival, using single-slice computed tomography (CT) based measurements has recently been described in various patient groups.⁶ Sarcopenia is frequently found to be an independent predictor for treatment outcome, and is considered to be a stronger predictive marker than conventional risk factors, such as age and American Society of Anesthesiologists (ASA) classification.^{7, 8} However, study results remain inconclusive. Therefore, the aim of this study was to systematically review the impact of CT-based skeletal muscle measurements on outcome in patients awaiting or undergoing liver transplantation.

METHODS

The study was registered in the PROSPERO International prospective register of systematic reviews (CRD42015019086).⁷ A priori defined eligibility criteria were established. All original studies that investigated the influence of skeletal muscle mass by means of abdominal CT in patients who underwent liver transplantation or were registered on the waiting list were identified by a systematic search performed in EMBASE, PubMed, and Web of Science, which was limited to English papers published between January 2000 and February 2015. The following search terms were used: ('sarcopenia':de,ab,ti OR 'analytic morphomics':de,ab,ti OR 'body composition':de,ab,ti OR 'muscle depletion':de,ab,ti OR 'muscle mass':de,ab,ti OR 'psoas area':de,ab,ti OR 'myopenia':de,ab,ti OR 'core muscle':de,ab,ti OR 'lean body mass':de,ab,ti OR 'muscular atrophy':de,ab,ti) AND ('liver transplantation':de,ab,ti). Similar queries were used for PubMed and Web of Science. The systematic review was performed according to the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) guidelines.⁹

ELIGIBILITY OF STUDIES AND ASSESSMENT OF METHODOLOGICAL QUALITY

Duplicate records were removed and all abstracts were independently screened by two investigators to determine eligibility for further analysis. All abstracts describing the prevalence or predictive value for complications and survival of sarcopenia in patients awaiting or undergoing liver transplantation were further assessed. Studies that measured muscle mass with other means than CT were excluded. Only original studies were included. Case reports, review articles, opinion articles and experimental studies were excluded. The remaining full-text articles were subsequently retrieved and independently screened by two investigators. All articles within the inclusion criteria were included in the systematic review. The included full-text articles were screened for additional relevant references. The methodological quality of the included studies was independently assessed by two investigators using the Newcastle-Ottawa Quality Assessment Scale for Cohort Studies for each a priori defined outcome measure.¹⁰ This is a ten-point scale, with 0 being poorest quality and 9 being highest quality. Quality assessment was performed separately for short and long term outcomes.

DATA EXTRACTION

Two investigators independently extracted data regarding study design and results, including: age, gender distribution, patient selection, indication for liver transplantation or disease etiology, Body Mass Index (BMI), albumin level, MELD-score, presence of cirrhosis, details on skeletal muscle mass measurement methods, prevalence of sarcopenia, waiting list mortality, post-transplantation mortality and complications, length of intensive care unit (ICU) and hospital stay, graft survival, and overall survival. Relevant information for the meta-analyses that could not be extracted from the articles was requested from the corresponding authors and when provided, included in the review. If not stated otherwise, results from multivariable analyses were used for the interpretation of the data.

STATISTICAL ANALYSIS

All outcomes are reported as in the original articles. A meta-analysis was performed using Review Manager 5.3 (The Nordic Cochrane Centre, Copenhagen, Denmark). Data are presented as hazard ratios (HR) with 95% confidence intervals (CI). If not stated otherwise, results of adjusted analyses were used. Random effects models were used to calculate summary estimates and to adjust for potential heterogeneity. Studies were weighted according to the inverse of the variance of the log hazard ratio. Overall effects were assessed using the Z-test and heterogeneity was tested using Cochran's chi-square test. The I^2 statistic was used to assess heterogeneity, which was defined as low, moderate, or high with I^2 values above 25%, 50%, and 75%, respectively.¹¹ If a research group contributed multiple studies with (partly) overlapping cohorts or relevant data was missing in the articles, the research group was contacted to provide additional data. If this data could not be provided, only the most relevant study was entered into the meta-analysis. Two-sided p-values <0.05 were considered statistically significant.

RESULTS

Of the 470 records that were found on February 3rd, 2015, 28 full text articles were considered potentially relevant (figure 1). From these 28 records, eight studies assessed muscle mass with means other than CT and one study was performed in another population than patients awaiting or undergoing liver transplantation. The remaining nineteen studies, including 3803 patients, were included in this systematic review.¹²⁻³⁰ Cross-referencing yielded no additional records.

Table 1 shows the population characteristics and the quality of the enrolled studies. The main indications for liver transplantation were viral liver infections (i.e., hepatitis B and C), followed by alcoholic liver cirrhosis. Around 65% was male and the mean age was 52 to 62 years. The median MELD-score ranged from 9-21, the median albumin level from 2.8 to 3.4 g/dl, and median BMI from 24.0 to 29.4 kg/m². Eight studies included cirrhosis patients only^{12, 15, 17, 22-24, 27, 28}, of which one study Child Pugh A patients only.²⁸

Figure 1. PRISMA Flow Chart of included studies.

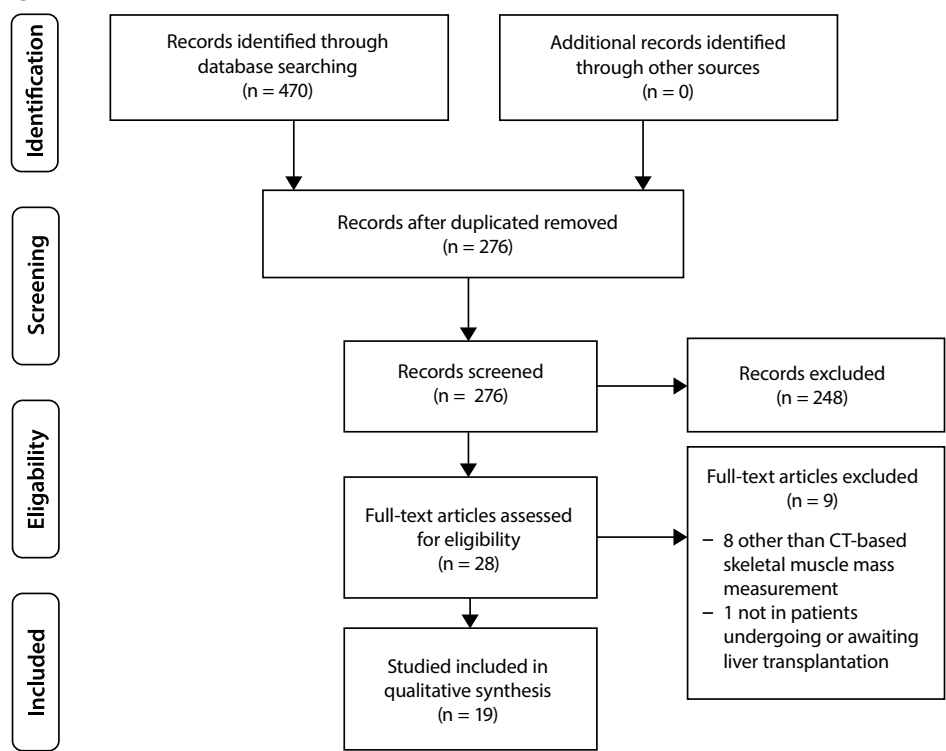


Table 1. Study population characteristics

Author, Year	Patient selection	n	Age (% f) yr	BMI kg/m ²	MELD score	Albumin g/dl	LT indication	Quality points [*]	
								C	S
Bergerson, 2014 ¹	All patients undergoing LT because of alcohol, NASH or PSC cirrhosis (2000 – 2012)	40 (35)	57 [†]	29 [†]	15 [†]	3.0 [†]	53% NASH 25% PSC	23% Alcoholic 35% HCC	n/a n/a
Cruz, 2013 ²	Adults evaluated for LT (Jan 2005 – Dec 2008)	234 (33)	55 [†]	28 [†]	21 [†]	3.0 [†]	25% HBV/HCV 24% Alcoholic 12% NASH 11% Autoimmune/PSC/PBC	11% Other n/a HCC 12% Alcoholic + HBV/HCV 5% Fulminant liver failure	n/a n/a 6/9
DiMartini, 2013 ³	First time LT without transplan- tation of other organs (Jan 2005 – Dec 2008)	338 (34)	55 [†]	28 [†]	20 [†]	3.0 [†]	27% HBV/HCV 23% Alcoholic 14% NASH 9% Alcohol + HBV/HCV	11% Other n/a HCC 12% Autoimmune/PSC/PBC 4% Fulminant liver failure	7/9 7/9
Durand, 2014 ⁴	All consecutive patients with cirrhosis listed for deceased donor LT (2002 – 2011)	562 (19)	53 [†]	26 [†]	16 [†]	N/a	42% Alcoholic 30% HCV 15% HBV	5% Biliary disease 8% Other 46% HCC	n/a 3/9
Englesbe, 2010 ⁵	Adult patients undergoing LT (2002 – 2008)	163 (37)	52 [†]	28 [†]	19 [†]	2.8 [†]	35% HCV 12% Alcoholic 10% PSC	6% 25% 13% HCC	PBC n/a Other 7/9
Giusto, 2015 ⁶	Adult patients with liver cirrhosis under evaluation for LT without acute liver failure and HCC beyo- nd Milan criteria (2011 – 2013)	59 (22)	59 [†]	25 [†]	HCC: 11 [†] No HCC: 16 [†]	N/a	56% HBV/HCV 22% Alcoholic	22% Other 41% HCC	n/a 7/9
Hamaguchi, 2014 ⁷	Adult patients undergoing LDLT (Jan 2008 – Oct 2013)	200 (53)	54 [†]	N/a	18 [†]	N/a	19% HBV/HCV 17% PSC/PBC	31% Other 34% HCC	n/a 5/9
Krell, 2013 ⁸	Adult patients undergoing LT (June 2002 – Aug 2008)	207 (38)	52 [†]	T1 [†] : 27 [†] T2 [†] : 28 [†] T3 [†] : 29 [†]	T1 [†] : 23 [†] T2 [†] : 19 [†] T3 [†] : 18 [†]	N/a	30% HBV/HCV 15% Alcoholic 4% NASH 23% Autoimmune/PBC/PSC	12% Other 25% HCC 2% Fulminant liver failure	5/9 n/a

Author, Year	Patient selection	n	Age (% f) yr	BMI kg/m ²	MELD score	Albumin g/dl	LT indication	Quality points*	
								C	S
Lee, 2014 ⁹	Adult patients undergoing LT (2000 – 2011)	325 (39)	52 [†]	T1 [†] : 27 [†] T2 [†] : 28 [†] T3 [†] : 30 [†]	18 [†]	T1 [†] : 2.9 [†] T2 [†] : 2.8 [†] T3 [†] : 2.9 [†]	40% Cirrhosis 29% HCV	39% HCC	6/9 6/9
Masuda, 2014 ¹⁰	Patients undergoing LDLT (Nov 2003 – Dec 2011)	204 (50)	54 [†]	24 [†]	≥20: S: 24% NS: 10%	N/a	63% HBV/HCV 13% PBC 5% Alcoholic	19% Other n/a HCC	6/9 5/9
Meza-Junco, 2013 ¹¹	Consecutive patients with HCC and cirrhosis evaluated for LT	116 (16)	58 [†]	29 [†]	9 [†]	3.4 [†]	60% HBV/HCV 11% Alcoholic 7% NASH	3% Other 100% HCC 20% Alcoholic + HCV	n/a 6/9
Montano-Loza, 2012 ¹²	Consecutive patients with cirrhosis evaluated for LT (10% underwent LT)	112 (30)	54 [†]	28 [†]	13 [†]	3.1 [†]	30% HBV/HCV 22% Alcoholic 19% Autoimmune/PBC/PSC	16% Alcoholic and HCV 13% Other n/a HCC	n/a 7/9
Montano-Loza, 2014 ¹³	Cirrhosis patients undergoing LT (2000 – 2012)	248 (32)	55 [†]	S: 25 [†] NS: 29 [†]	20 [†]	S: 3.3 [†] NS: 3.4 [†]	60% HBV/HCV 19% Alcoholic 6% NASH	15% Autoimmune/PBC/PSC 1% Other 39% HCC	5/9 7/9
Tandon, 2012 ¹²	Adult patients on the LT waiting list without HCC, acute liver failure, prior LT, multivisceral LT, LRLT (Feb 2005 – Nov 2009)	142 (40)	53 [†]	27 [†]	15 [†]	3.0 [†]	20% Alcoholic 7% Other 38% HCV + alcoholic	25% Autoimmune/PBC/PSC 11% Cryptogenic/NAFLD 0% HCC	n/a 6/9
Toshima, 2015 ¹⁴	LDLT recipients (Nov 2003 – Dec 2011)	143 (48)	S: 55 [†] NS: 55 [†]	24 [†]	S: 17 [†] NS: 13 [†]	N/a	N/a		6/9 n/a
Tsien, 2014 ¹⁵	Adult cirrhosis patients under- going LT (Jul 2009 – Jul 2011)	53 (23)	57 [†]	29 [†]	13 [†]	3.3 [†]	42% Viral 8% NASH 23% Alcoholic + viral	28% Other 64% HCC	4/9 4/9
Valero, 2015 ¹⁶	Child Pugh A patients under- going hepatic resection or OLT for HCC or ICC (2000 – 2013)	96 (39)	62 [†]	27 [†]	10 [†]	3.7 [†]	70% HCC 30% ICC	29% underwent LT (100% HCC)	6/9 6/9

Author, Year	Patient selection	n	Age (% f) yr	BMI kg/m ²	MELD score	Albumin g/dl	LT indication	Quality points*	
								C	S
Waits, 2014 ¹⁷	Adult patients who received liver transplants from deceased donors (2000 – 2011)	348 (38)	51† T1‡: 20† T2‡: 28† T3‡: 29†	T1‡: 27† T2‡: 28† T3‡: 29†	T1‡: 20† T2‡: 17† T3‡: 18†	T1‡: 2.9† T2‡: 2.8† T3‡: 2.8†	38% HCV 27% HCC	n/a	6/9
Yadav, 2015 ¹⁸	All patients listed for LT (Jul 2008 – Jul 2011)	213 (39)	55† S: 24† NS: 29†	29† S: 24† NS: 29†	16†	3.3†	44% HCV 16% Alcoholic 14% NASH 8% PBC/PSC	n/a	7/9

† mean, ‡ median. * Scored with the Newcastle-Ottawa quality assessment scale for cohort studies, on a scale of 0 to 9, with 0 being poorest quality and 9 being highest quality. Quality assessment was performed separately for short and long term outcomes. # Tertiles based on skeletal muscle mass. † Tertiles (young, middle, oldest) based on chronological age (psoas area, psoas density and abdominal aneurysmal calcifications).

Abbreviations: f: female, LT: liver transplantation, C: complications, S: survival, LDLT: Living Donor Liver Transplantation, OLT: Orthotopic Liver Transplantation, HCC: Hepatocellular Carcinoma (either primary etiology or concomitant); BMI: Body Mass Index (kg/m²), MELD: Model For End-Stage Liver Disease Score, S: patients with sarcopenia, NS: patients without sarcopenia, N/a: Not available. NASH: Nonalcoholic Steatohepatitis, PSC: Primary Sclerosing Cholangitis, HBV: Hepatitis B Virus, HCV: Hepatitis C Virus, PBC: Primary Biliary Cirrhosis, LRLT: living related liver transplantation.

Table 2. Methods used to measure skeletal muscle mass, definitions used to classify patients as sarcopenic and the prevalence of sarcopenia within studies.

Muscles					Sarcopenia	
Author, Year	measured	Software	Level	Cut off values / definition	Muscle area / density	prevalence
Bergerson, 2014 ¹	CSA (SMI)	MITK software package	L3	f 38.5 cm ² /m ² m 52.4 cm ² /m ²	f 41.9 cm ² /m ² m 52.2 cm ² /m ²	f 43% m 62%
Cruz, 2013 ²	CSA (SMI)	SliceOmatic	Closest to L3-L4 disc space	f 38.5 cm ² /m ² m 52.4 cm ² /m ²	43.0 cm ² /m ²	Nearly 70% m 76%
DiMartini, 2013 ³	CSA (SMI)	SliceOmatic	Closest to L3-L4 disc space	f 38.5 cm ² /m ² f 52.4 cm ² /m ²	43.8 cm ² /m ² f 38.5 cm ² /m ² m 46.5 cm ² /m ²	68%
Durand, 2014 ⁴	APMT, TPMT (right m. psoas)	N/a	N/A	N/a (continuous parameter used)	N/a	N/a
Englesbe, 2010 ⁵	TPA, PD	MATLAB	L4	Sex specific tertiles	TPA: 19.6 cm ² PD: 101.0HU [†]	33% (lowest tertile)
Giusto, 2015 ⁶	CSA (SMI)	Leonardo Syngo	Closest to L3-L4 disc space	f 38.5 cm ² /m ² m 52.4 cm ² /m ²	f 36.0 cm ² /m ² m 49.9 cm ² /m ²	76% f 69% m 78%
Hamaguchi, 2014 ⁷	TPA (PMI), IMAC	Aquarius NET server	At level of subfascial muscular tissue in multifidus muscle	PMI: f 4.1 cm ² /m ² m 6.7 cm ² /m ² MAC: f -0.2 HU m -0.4 HU	N/a	Low TPI: 44% High IMAC: 45%
Krell, 2013 ⁸	TPA	MATLAB	L4	Sex specific tertiles	High TPA: f 19.8 cm ² m 29.2 cm ² Low TPA: f 9.5 cm ² m 19.8 cm ²	33% (lowest tertile)
Lee, 2014 ⁹	DMG*, PA	MATLAB	T12, L4	Sex specific tertiles	DMG: f 25.3 cm ² m 33.9 cm ² TPA: Low: 12.8 cm ² High: 27.9 cm ²	33% (lowest tertile)
Masuda, 2014 ¹⁰	TPA	N/a	L3 (caudal end)	TPA: <5 th percentile per gender according to healthy donors; f 380 mm ² m 800 mm ²	531 mm ² f 423 mm ² m 761 mm ²	47%; f 36% m 58%
Meza-Junco, 2013 ¹¹	CSA (SMI)	SliceOmatic	L3	BMI ≥ 25: f 41.0 cm ² /m ² m 53.0 cm ² /m ² BMI < 25: 43.0 cm ² /m ²	54.0 cm ² /m ²	30%; f 28% m 31%
Montano-Loza, 2012 ¹²	CSA (SMI)	SliceOmatic	L3	f 38.5 cm ² /m ² m 52.4 cm ² /m ²	51.0 cm ² /m ²	40%; f 18% m 50%

Author, Year	Muscles measured	Software	Level	Cut off values / definition	Muscle area / density	Sarcopenia prevalence
Montano-Loza, 2014 ¹³	CSA (SMI)	SliceOmatic	L3	BMI ≥ 25 : f 41.0 cm ² /m ² m 53.0 cm ² /m ² BMI < 25: 43.0 cm ² /m ²	50.0 cm ² /m ^{2†}	45% f 30% m 52%
Tandon, 2012 ¹²	CSA (SMI)	SliceOmatic	L3	f 38.5 cm ² /m ² m 52.4 cm ² /m ²	f 44.9 cm ² /m ^{2†} m 50.8 cm ² /m ^{2†}	41%; f 21% m 54%
Toshima, 2015 ¹⁴	TPA	N/a	L3 (caudal end)	TPA: <5 th percentile per gender according to healthy donors; f 380 mm ² m 800 mm ^{2*}	S: 484.2 mm ^{2†¶} NS: 689.6 mm ^{2†¶}	46% f 30% m 52%
Tsien, 2014 ¹⁵	TPA (PMI)	Leonardo Workstation using Oncocare	L4	PMI < 50 years: f 10.5 cm ² /m ² m 12.3 cm ² /m ² PMI > 50 years: f 10.3 cm ² /m ² m 10.1 cm ² /m ²	PMI: 9.2 cm ² /m ^{2†} (33.0 HU [†])	62%
Valero, 2015 ¹⁶	TPA, TPV	ImageJ, AW Workstation Volume	L3	PMI: f 64.2 cm ² /m ² m 78.4 cm ² /m ² TPV: f 23.0 cm ³ /m m 34.1 cm ³ /m	TPA [†] : 784.4 mm ² /m ^{2†} TPV: 30.4 cm ³ /m ^{2†}	49%
Waits, 2014 ¹⁷	Morphometric age (including TPA, PD)	MATLAB	L4	Morphometric age (TPA, PD and AA calcification) as continuous variable	TPA: f 14.6 cm ^{2†} m 23.1 cm ^{2†} PD: f 49.1 HU [†] m 48.8 HU [†]	n/a
Yadav, 2015 ¹⁸	CSA (SMI)	SliceOmatic	L3	f 38.5 cm ² /m ² m 52.4 cm ² /m ²	54.3 cm ² /m ^{2†} SMI survivors (alive/LT) 54.6 vs non-survivors 53.1 (deceased on waiting list), p=0.40	22% [‡] f 13.1% m 28.1%

† mean, ‡ median. * Any muscle contained within the region posterior to the spine and ribs, and no more lateral than the lateral-most edges of the erector spinae muscles. † 743.1 mm²/m² for liver transplant patients. ‡ Reported as cm² in the original article. & Sarcopenia survivors (alive/LT) 22% vs non-survivors (deceased on waiting list) 24%, p=0.77.

Abbreviations: f, female; m, male; CSA: Cross Sectional Area, APMT: Axial Psoas Muscle Thickness; TPMT: Transversal Psoas Muscle Thickness; TPA: Total Psoas Area, IMAC: Intramuscular Adipose Content (defined as region of interest of multifidus muscle (Hounsfield units) divided by region of interest of subcutaneous fat (Hounsfield units)), TPV: Total Psoas Volume, PD: Psoas Density, N/a: Not available, L3: third lumbar vertebra; L4: fourth lumbar vertebra, T12: twelfth thoracic vertebra, HU: Hounsfield units, SMI: Skeletal Muscle Index (cm²/m²); PMI: Psoas Muscle Index (cm²/m²), BMI: Body Mass Index; AA: abdominal aneurysm, PSMA: Paraspinal Muscle Area, PSMI: Paraspinal Muscle Index, AWMA: Abdominal Wall Muscle Area, AWMI: Abdominal Wall Muscle Index.

DEFINITIONS AND PREVALENCE OF SARCOPENIA

A great variety in skeletal muscle measurement methods and definitions used to classify patients as sarcopenic or non-sarcopenic was observed. The methods of muscle measurement and sarcopenia definitions that were used are summarized in table 2. Nine studies reported the cross-sectional muscle area with corresponding skeletal muscle index^{12-14, 17, 22-25, 30}, whereas the psoas area was reported in eight studies^{15, 16, 18, 19, 21, 26-28} and the dorsal muscle group area in one study.²⁰ One study calculated the morphometric age (calculated with total psoas area, psoas density and abdominal aortic calcifications).²⁹ The mean skeletal muscle index ranged from 43.0 cm²/m² to 54.3 cm²/m².^{13, 30} The prevalence of sarcopenia was reported in seventeen studies.^{12-14, 16-28, 30} and ranged from 22.2%³⁰ to nearly 70%.¹³ The prevalence greatly depended on the definition used. All studies that reported the prevalence of sarcopenia separately for males and females, reported a higher prevalence among males.^{12, 14, 17, 21-26, 30}

WAITING LIST MORTALITY

Four^{15, 22, 23, 25} of the six^{15, 17, 22, 23, 25, 30} studies investigating the association between skeletal muscle mass and mortality among patients being evaluated for or awaiting liver transplantation found an independent association. All details about survival rates and times can be found in table 3. The forest plot in figure 2a shows the meta-analysis of the association between sarcopenia and waiting list mortality with a pooled hazard ratio (HR) of 1.72 (95% CI 0.99-3.00, p=0.05) and low heterogeneity between studies (I²=33%). Nevertheless, the evidence is limited, because three of the four studies with positive outcome were performed in one center.^{22, 23, 25}

In the study of Durand et al., an increasing transversal psoas muscle thickness corrected for height was associated with reduced mortality in both a pre-MELD cohort (HR 0.92 [95% CI 0.86-0.98], p=0.02) and MELD-era cohort (HR 0.86 [95% CI 0.78-0.94], p=0.001). Furthermore, the discrimination for waiting list mortality of the MELD-psoas area score was superior over the MELD-score and MELDNa-score (i.e., MELD-score with the addition of serum sodium), particularly in patients with a MELD-score ≤25 or refractory ascites.¹⁵ Waiting list mortality was also greater among sarcopenic patients compared with non-sarcopenic patients in the study of Tandon et al. (log-rank p = 0.04), and sarcopenia was an independent predictor of overall mortality in multivariable analysis (HR 2.36 [95% CI 1.23-4.53], p=0.009).²⁵ Remarkably, outcome in sarcopenic patients with a low MELD-score (<15) was similar as for patients with a high MELD-score (≥ 15) with or without sarcopenia.

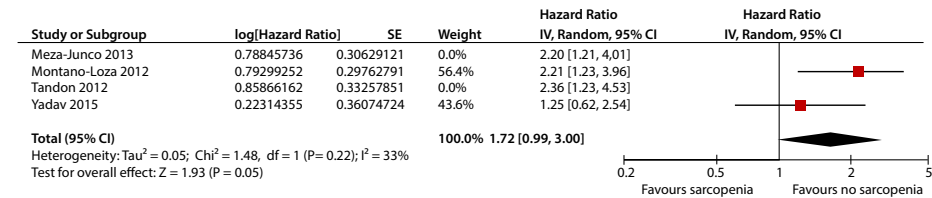
Table 3. Studies reporting the impact of sarcopenia on waiting list mortality in patients evaluated for liver transplantation or registered on the waiting list.

Author, Year	Survival
Durand, 2014 ⁴	Pre-MELD: HR 0.92 (0.86-0.98), p=0.02 MELD-era: HR 0.86 (0.78-0.94), p=0.001 (for increasing TPML)
Giusto, 2015 ⁵	HR 0.89 (0.79-1.00)*
Meza-Junco, 2013 ¹¹	Median survival: S 16 (95% CI 4-28) vs NS 28 (21-34) months, log rank p=0.003 6-month survival S vs NS: 67% vs 90% 1-year survival S vs NS: 52% vs 82% Sarcopenia (with MELD/CP): HR 2.20 (1.21-4.02), p=0.01 Sarcopenia (individual components MELD/CP): 2.53 (1.35-4.73), p=0.004
Montano-Loza, 2012 ¹²	Median survival: S 19 (7-30) vs NS 34 (14-55), log rank p=0.005 6-month survival S vs NS: 71% vs 90% 1-year survival S vs NS: 53% vs 83% Sepsis related death S vs NS: 22% vs 8%, p=0.02 Sarcopenia (with MELD/CP): HR 2.21 (1.23-3.95), p=0.008, Sarcopenia (individual components MELD/CP): 2.11 (1.13-3.94), p=0.02
Tandon, 2012 ¹²	1-, 2-, and 5-year survival rates S vs NS: (63%, 51%, 51% vs 79%, 74%, 70% respectively), log-rank p = 0.04; Low MELD (<15): log rank p=0.02; High MELD (≥15): log rank p=0.59 Sarcopenia: HR 2.36 (1.23-4.53), p=0.009
Yadav, 2015 ¹⁸	Sarcopenia: HR 1.25 (0.62-2.55), p=0.54*

Unadjusted data. * Provided by the authors after personal communication. Abbreviations: HR; Hazard ratio, S; sarcopenic patients, NS; non-sarcopenic patients, SMI; Skeletal Muscle Index, TPML; Transversal Psoas Muscle Index, TPA; Total Psoas Area, IMAC; Intramuscular Adipose Content, PMI; Psoas Muscle Index, DMG; Dorsal Muscle Group, MELD; Model for End-stage Liver Disease, CP; Child Pugh score, CI; confidence interval, MA; Muscle Attenuation, LT; Liver Transplantation.

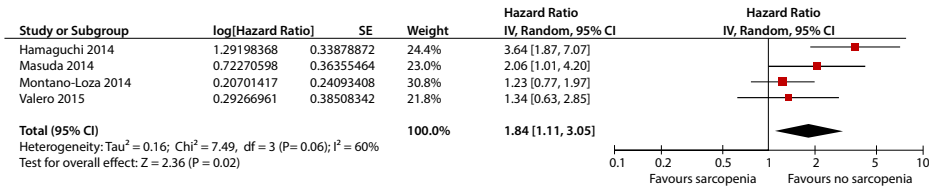
In subgroup analyses, sarcopenia remained associated with mortality in patients with a low MELD-score (log rank p=0.02), whereas it was not in patients with a high MELD-score (log rank p=0.59). None of the other included studies performed comparable subgroup analyses. Sarcopenia was also an independent predictor of mortality in patients evaluated for liver transplantation in the studies of Meza-Junco et al. and Montano-Loza et al.²² In both studies multivariable analyses were performed with MELD and Child Pugh scores on the one hand and with their individual components on the other hand, which all showed sarcopenia to be an independent predictor for mortality. Furthermore, Montano-Loza et al. reported a significantly higher sepsis related death in sarcopenic patients compared with non-sarcopenic patients (22% versus 8%, p=0.02), whereas no difference was found in liver failure related death.²³ ²⁵ Meza-Junco et al. reported a trend for higher liver failure related death in sarcopenic patients compared with non-sarcopenic patients (33% versus 15%, p=0.08.²²

Figure 2a. Forest plots of the association between sarcopenia and survival.



Forest plot showing studies that reported the association between sarcopenia and waiting list mortality. Due to data provided by authors that was more precise than the data published in the article or rounding off upwards or downwards by Review Manager, the confidence intervals can somewhat differ from the original confidence intervals. For the study of Yadav et al.¹⁸, unadjusted results were used because the multivariable analysis in the manuscript suggested a level of precision that did not correspond with the number of observed events. Because the studies of Meza-Junco et al.¹¹, Montano-Loza et al.¹², and Tandon et al.¹² were performed in overlapping cohorts and the first was performed in patients with HCC, only the most representative study was included in the meta-analysis (i.e., all consecutive patients with cirrhosis being evaluated for liver transplantation).¹² The authors of these studies stated that at most fifteen patients were included in the study of Tandon et al.¹² that were also included in the other studies. Including the study of Tandon et al.¹² in the meta-analysis, resulted in a pooled HR of 1.93 (95% CI 1.33-2.80, $p=0.0005$), Z of 3.48 and I^2 of 1%.

Yadav et al. investigated the relationship between sarcopenia, six-minute walk distance and health-related quality of life in liver transplant candidates and found no association between sarcopenia and overall mortality. The unadjusted HR was 1.25 (95% CI 0.62-2.55, $p=0.54$) and was used for the meta-analysis rather than the adjusted HR, as the multivariable analysis suggested a level of precision that did not correspond with the number of observed events.³⁰ Although the mean MELD-scores were comparable between these studies, the MELD-score of the study cohort of Yadav et al. varied from 9 to 40.³⁰ In the study of Giusto et al., CT-assessed muscle mass was compared with Dual-Energy X-Ray Absorptiometry (DEXA) and anthropometry. The skeletal muscle index was not predictive for mortality on the waiting list (HR 0.89 [95% CI 0.79-1.00], kindly provided by the authors after personal communication), whereas mid-arm muscle circumference and fat-free mass index were, also after adjusting for sex, MELD-score, age, and interaction between sex and mid-arm muscle circumference and fat-free mass index, respectively.¹⁷ However, the aim of this study was not to investigate the association between skeletal muscle mass and patient outcome and only 59 patients were included.

Figure 2b. Forest plots of the association between sarcopenia and survival.

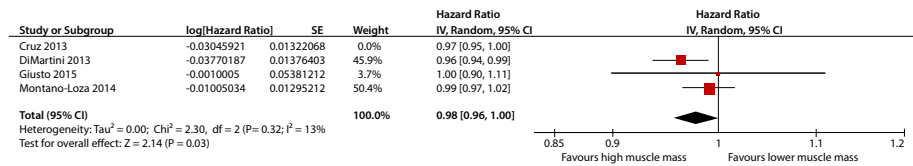
Forest plot showing studies that reported the association between sarcopenia and post-transplantation survival. Due to data provided by authors that was more precise than the data published in the article or rounding off upwards by Review Manager, the confidence intervals can somewhat differ from the original confidence intervals. Hamaguchi et al.⁷, Masuda et al.¹⁰, and Valero et al.¹⁶ performed measurements of the psoas muscle area, whereas Montano-Loza et al.¹³ performed measurements of the cross-sectional muscle area. A meta-analysis of studies that assessed skeletal muscle mass by measuring the psoas muscle area only resulted in a pooled HR of 2.21 (95% CI 1.25-3.90, $p=0.007$), Z of 2.72, and I^2 of 49%. A meta-analysis excluding the study of Valero et al.¹⁶, that included only few patients that underwent liver transplantation for hepatocellular- or cholangiocarcinoma, resulted in a pooled HR of 2.04 (95% CI 1.05-3.92, $p=0.03$), Z of 2.11, and I^2 of 71% and a meta-analysis of Hamaguchi et al.⁷ and Masuda et al.¹⁰ resulted in a pooled HR of 2.78 (95% CI 1.59-4.85, $p=0.0003$), Z of 3.60 and I^2 of 24%.

POST-TRANSPLANTATION SURVIVAL

In the eleven studies that investigated the association between skeletal muscle mass and post-transplantation survival, seven described an association^{13, 14, 16, 18, 20, 21, 29}, and three no association.^{17, 24, 27, 28} All details about median survival times, yearly survival rates and the association between skeletal muscle mass and overall survival are summarized in table 4. The forest plot in figure 2b shows the association between sarcopenia and post-transplantation survival (pooled HR 1.84 [95% CI 1.11-3.05], $p=0.02$) with moderate heterogeneity between studies ($I^2=60\%$). When studies that measured psoas muscle area were included only, this resulted in low heterogeneity ($I^2=49\%$) and a pooled HR of 2.21 (95% CI 1.25-3.90, $p=0.007$). When the study of Valero et al.²⁸, that included only few patients that underwent liver transplantation for hepatocellular- or cholangiocarcinoma, was excluded, the pooled HR was 2.03 (95% CI 1.05-3.92, $p=0.03$, $Z=2.11$, $I^2=71\%$). Finally, a meta-analysis of the studies of Hamaguchi et al.¹⁸ and Masuda et al.²¹ resulted in a pooled HR of 2.78 (95% CI 1.59-4.85, $p=0.0003$), Z of 3.60 and I^2 of 24%.

Meta-analyzing studies that reported the association between skeletal muscle index, as a continuous measure, and post-transplantation survival showed a pooled HR of 0.98 (95% CI 0.95-1.00, $p=0.03$) per incremental skeletal muscle index (figure 2c). Since DiMartini et al.¹⁴ and Cruz et al.¹³ performed studies in overlapping cohorts, only the latter was included in the meta-analysis. Additional results without stratification by gender were kindly provided by the authors of DiMartini et al.¹⁴, and these results were used in the meta-analysis. The results used for the study of Giusto et al. were also kindly provided by the authors after personal communication.¹⁷

Figure 2c. Forest plots of the association between sarcopenia and survival.



Forest plot showing studies that reported the association between skeletal muscle mass and post-transplantation survival. Only studies that reported the skeletal muscle index (cm^2/m^2) were included, as the other studies used different units of measurement. Due to data provided by authors that was more precise than the data published in the article or rounding off upwards or downwards by Review Manager, the confidence intervals can somewhat differ from the original confidence intervals. The hazard ratios shown represent an incremental increase in skeletal muscle index. Since DiMartini et al.³ and Cruz et al.² performed studies in overlapping cohorts, only the latter was included in the meta-analysis. Additional results without stratification by gender that were provided by the authors of DiMartini et al.³ and these results were used in the meta-analysis. The authors also provided the results used for the study of Giusto et al. after personal communication.⁶

Cruz et al. reported a protective effect of increasing skeletal muscle index on mortality (HR 0.97 [95% CI 0.94-0.99], $p=0.04$)¹³, whereas the protective effect of the psoas muscle index was only found significant for males (HR 0.95, $p=0.01$) and not for females (HR 0.98, $p=0.55$) in the study of DiMartini et al.¹⁴ Every standard deviation increase in dorsal muscle group area, as assessed by Lee et al., was also independently associated with increased overall (odds ratio [OR] 0.62 [95% 0.49-0.77], $p<0.001$), one-year (OR 0.53 [95% CI 0.36-0.78], $p=0.001$), and five-year (OR 0.53 [95% CI 0.38-0.70], $p<0.001$) survival, as well as total psoas area for one-year survival (OR 0.43 [95% CI 0.30-0.62], $p<0.001$).²⁰ In line with this, Englesbe et al. also found an independent association between total psoas area and survival (HR 0.27 [0.14-0.53], $p<0.001$ per increasing 1000 mm^2).¹⁶ The variously defined parameter sarcopenia was an independent predictor for mortality in the study of Masuda et al. (HR 2.06 [95% CI 1.01-4.20], $p=0.047$).²¹ High intramuscular adipose content (OR 3.90 [95% CI 2.03-7.76], $p<0.001$) and low PMI (OR 3.64 [1.90-7.17], $p<0.001$) have also been identified as independent predictors for impaired survival.¹⁸ Waits et al. showed that morphometric age (including total psoas area, psoas density and abdominal aortic calcifications) was a risk factor for mortality per year increase (HR 1.03 [95% CI 1.02-1.04], $p<0.001$).²⁹

Tsien et al. described a nonsignificant association between pretransplant sarcopenia and mortality ($p=0.06$) and higher mortality in patients with continued reduction in muscle area ($p=0.08$) in a relatively small cohort of 53 patients.²⁷

Table 4. Studies reporting the impact of sarcopenia on overall survival in patients undergoing liver transplantation.

Author, Year	Survival
Cruz, 2013 ²	SMI: HR 0.97 (0.94 – 0.99), p=0.04
DiMartini, 2013 ³	m PMI: HR 0.95 (p=0.01) f PMI: HR 0.98 (p=0.55)
Englesbe, 2010 ⁵	1-year survival S vs NS: 49.7% vs 87% 3-year survival S vs NS: 26.4% vs 77.2% (lowest vs highest tertile) TPA: HR 0.27 (0.14-0.53), p<0.0001 (per increasing 1000 mm ²)
Giusto, 2015 ⁶	HR 0.99 (0.90-1.11)*
Hamaguchi, 2014 ⁷	Median survival low vs normal TPA: 17.6 vs 33.9 months Median survival high vs normal IMAC: 21.9 vs 32.4 months High IMAC: OR 3.90 (2.03-7.76), p<0.001 Low PMI: OR 3.64 (1.90-7.17), p<0.001
Lee, 2014 ⁹	Overall survival: DMG: OR 0.62 (0.49-0.77), p<0.001 (per SD increase) 1-year survival: DMG: OR 0.53 (0.36-0.78), p=0.001 (per SD increase) TPA: OR 0.43 (0.30-0.62), p<0.001 (per SD increase) 5-year survival: DMG: OR 0.53 (0.38-0.70) p<0.001 (per SD increase)
Masuda, 2014 ¹⁰	Sarcopenia: HR 2.06 (1.01-4.20), p=0.047 3-year survival S vs NS: 74.5% vs 88.9% (p=0.02) 5-year survival S vs NS: 69.7% vs 85.4% (p=0.02)
Montano-Loza, 2014 ¹³	Median survival S vs NS: 117 (95% CI 84-151) vs 146 (95% CI 110-182) months, log rank p=0.4 1-year survival rate S vs NS: 89% vs 91% 5-year survival rate S vs NS: 74% vs 76% Sarcopenia: HR 1.23 (0.77-1.98), p=0.4 [#] SMI: HR 0.99 (0.96-1.01), p=0.3 [#] MA: HR 0.99 (0.96-1.02), p=0.5 [#]
Tsien, 2014 ¹⁵	Pre-OLT sarcopenia associated with mortality (p=0.06) [#] Non-significant association of continued reduction in muscle area with higher mortality (p=0.08) [#]
Valero, 2015 ¹⁶	Median survival S vs NS: 38.5 vs 69.1 months (p=0.32) 1-year survival rate S vs NS: 76.6% vs 87.8% (p=0.15) 3-year survival rate S vs NS: 61.7% vs 71.4% (p=0.31) 5-year survival rate S vs NS: 55.3% vs 69.4% (p=0.32) Sarcopenia: HR 1.34 (0.61-2.76), p=0.43
Waits, 2014 ¹⁷	Morphometric age: HR 1.03 (1.02-1.04), p<0.001 (per year) 1-year mortality morphometric age: OR 1.04 (1.03-1.06), p<0.001 (per year) 5-year mortality morphometric age: OR 1.03 (1.02-1.06), p<0.001 (per year)

Unadjusted data. * Provided by the authors after personal communication.

Abbreviations: m; male; f; female HR; Hazard ratio, S; sarcopenic patients, NS; non-sarcopenic patients, SMI; Skeletal Muscle Index, TPMI; Transversal Psoas Muscle Index, TPA; Total Psoas Area, IMAC; Intramuscular Adipose Content, PMI; Psoas Muscle Index, DMG; Dorsal Muscle Group, MELD; Model for End-stage Liver Disease, CP; Child Pugh score, CI; confidence interval, MA; Muscle Attenuation, LT; Liver Transplantation.

The median survival in the studies of Montano-Loza et al.²⁴ among cirrhosis patients undergoing liver transplantation and Valero et al.²⁸, among a relatively heterogeneous population of hepatocellular- and intrahepatic cholangiocarcinoma patients undergoing curative intent hepatic resection (70.9%) or liver transplantation (29.1%), did not significantly differ between sarcopenic and non-sarcopenic patients (117 versus 146 months, log rank $p=0.4$; and 38.5 versus 69.1 months, $p=0.32$, respectively). Neither sarcopenia^{24, 28} nor skeletal muscle index or muscle attenuation²⁴ were predictive for mortality in regression models. Although Montano-Loza et al. found no association overall in a population with a relatively high MELD-score and hepatocellular carcinoma prevalence, male patients undergoing liver transplantation in the lowest skeletal muscle mass sextile showed significantly impaired survival compared with patients in the other sextiles.²⁴ Finally, Giusto et al. found no significant association between skeletal muscle index and post-transplant mortality after adjustment for age, gender and MELD-score (adjusted HR 1.0 [95% CI 0.90-1.11], data provided by the authors after personal communication).¹⁷

POST-TRANSPLANTATION COMPLICATIONS AND TRANSPLANTATION RELATED MORTALITY

In both studies reporting overall post-transplantation complications, low skeletal muscle mass was associated with increased risk of postoperative complications (table 5).^{20, 28} The study of Lee et al. showed that an increase in both the total psoas area and the dorsal muscle group with one standard deviation was associated with an increased risk for complications within one year after transplantation (OR 0.48 [95% CI 0.32-0.72], $p<0.001$, and OR 0.67 [95% CI 0.50-0.90], $p=0.007$, per standard deviation increase in dorsal muscle group area).²⁰ In the study of Valero et al. post-transplantation complications occurred in 40.4% of sarcopenic patients compared with 18.4% in non-sarcopenic patients ($p=0.01$). Sarcopenia was an independent predictor for postoperative complications in multivariable analysis (OR 3.06 [95% CI 1.07-8.72], $p=0.03$).²⁸ All severe postoperative (23.4%) complications (i.e. Clavien-Dindo classification \geq IIIa) occurred in patients with sarcopenia. No differences were observed in 30- and 90-day mortality rates in sarcopenic and non-sarcopenic patients respectively (4.3% versus 0%, $p=0.24$ and 8.5% versus 2.0%, $p=0.20$).²⁸ The three-month mortality rate in the study of Montano-Loza et al. was 5% in sarcopenic patients compared with 2% in non-sarcopenic patients ($p=0.20$).²⁴ In the study of DiMartini et al., the relative risk for in-hospital mortality was 0.97 ($p>0.05$).¹⁴

Krell et al. reported that patients with a total psoas area in the lowest tertile had a 4.6-fold increased risk (95% CI 2.25-9.53) to develop any post-transplantation infection compared with patients in the highest tertile. In a multivariable model, pretransplant

total psoas area was an independent predictor for the occurrence of severe infections (HR 0.38 [95% CI 0.23-0.65], $p < 0.01$) together with age and pretransplant bilirubin level. These factors remained significant when infections were stratified by pathogen type (i.e. bacterial, viral or fungal).¹⁹ In line with this, Masuda et al. found that sarcopenia was an independent predictor for sepsis (HR 5.31 [95% CI 1.53-18.40], $p = 0.009$) in a cohort of 228 patients, which occurred in 17.7% of sarcopenic patients and 7.4% of non-sarcopenic patients.²¹ On the other hand, no independent association was found between sarcopenia and sepsis in a sub study in the same patient cohort ($n = 143$) of Toshima et al. (OR 1.72 [95% CI 0.67-5.03], $p = 0.263$).²⁶ However, both studies performed a multivariable analysis of risk factors for postoperative sepsis including ten and thirteen parameters respectively on only 25 (49%) and twelve events respectively. Therefore, the methodology of these studies could be questioned. Although the overall 90-day infection rate did not significantly differ between sarcopenic and non-sarcopenic patients (29% versus 20%, $p = 0.1$) in a study of Montano-Loza et al., bacterial infections in particular within 90 days after transplantation occurred significantly more in sarcopenic patients compared with non-sarcopenic patients (26% versus 15%, $p = 0.04$).²⁴ However, no multivariable analysis has been performed for bacterial infections. Only one study reported that five patients had confirmed acute graft rejection without specifying them as (non-)sarcopenic.²⁷

POST-TRANSPLANTATION LENGTH OF HOSPITAL STAY

Four studies reported on length of stay outcomes (table 5).^{14, 24, 27, 28} DiMartini et al. performed a Poisson regression analysis and found skeletal muscle mass to be predictive for length of both hospital and ICU stay, as well as intubation days (all $p < 0.001$).¹⁴ Furthermore, sarcopenic patients were more likely to be discharged to another hospital or nursing home rather than home ($p = 0.04$).¹⁴ A significantly increased length of ICU (12 days versus 6 days, $p = 0.001$) and hospital stay (40 days versus 25 days, $p = 0.005$) was also found in cirrhosis patients undergoing liver transplantation by Montano-Loza et al.²⁴, whereas Valero et al. found non-significant differences regarding hospital stay between sarcopenic and non-sarcopenic patients who underwent hepatic resection with curative intent or transplantation for hepatocellular- or intrahepatic cholangiocarcinoma (12.1 days versus 9.7 days, $p = 0.50$).²⁸ Obviously, these patients have a distinct postoperative recovery compared with transplant patients. Furthermore, Tsien et al.²⁷, who measured the psoas area in only 53 patients, found no association with length of hospital stay in contrast to Montano-Loza et al.²⁴ and DiMartini et al.¹⁴, who performed cross-sectional skeletal muscle measurements in larger cohorts.

Table 5. Studies reporting the impact of sarcopenia on short-term outcome in patients who underwent liver transplantation.

Complications			Length of Stay			
Author, Year	All	Clavien Dindo classification \geq IIIa	Mortality	Infectious	ICU	Intubation time
DiMartini, 2013 ³	N/a	N/a	In-hospital: f RR 0.97 (N.s.) m RR 0.97 (N.s.)	N/a	p<0.001	Discharge to hospital/nursing home: f RR 0.96 (p<0.05)m RR 0.95 (N.s.) Intubation days (p<0.001)
Krell, 2013 ⁸	N/a	N/a	N/a	Any infection: Low TPA vs high TPA OR 4.6 (2.25-9.53) Severe infections: decreasing TPA HR 0.38 (0.23-0.65) (p<0.01)	N/a	N/a
Lee, 2014 ⁹	Within 1 year TPA: OR 0.48 (0.32-0.72, p<0.001) per SD increase DMG: OR 0.67 (0.50-0.90, p=0.007) per SD increase	N/a	N/a	N/a	N/a	N/a
Masuda, 2014 ¹⁰	N/a	N/a	N/a	Sepsis: Sarcopenia 17.7% vs. no sarcopenia 7.4%; HR 5.31 (1.53-18.4), p=0.009	N/a	N/a
Montano-Loza, 2014 ¹³	N/a	N/a	3-month: S 5% vs NS 2%, p=0.2	Overall 90-day infections: S 29% vs NS 20%, p=0.1 Bacterial infections: S 26% vs NS 15%, p=0.04	S 12 days vs NS 6 days, p=0.001	S 40 days vs NS 25 days, p=0.005 N/a
Toshima, 2015 ¹⁴	N/a	N/a	N/a	Sepsis: OR sarcopenia 1.72 (0.67-5.03), p=0.263	N/a	N/a

Complications			Length of Stay			
Author, Year	All	Clavien Dindo classification ≥ IIIa	Mortality	Infectious	ICU	Hospital (days)
Tsien, 2014 ¹⁵	Psoas and paraspinal muscle mass reduction OR for DM 3.1 (1.01-9.38), p<0.05	N/a	N/a	N/a	TPA; p>0.1	TPA; p>0.1
Valero, 2015 ¹⁶	Sarcopenia 40.4% vs no sarcopenia 18.4%, p=0.01; OR sarcopenia 3.06 (1.07-8.72), p=0.03	All severe complications (23.4%) occurred in sarcopenic group.	30 day: S 4.3% vs NS 0% (p=0.24) 90 day: S 8.5% vs NS 2.0% (p=0.20)	N/a	N/a	S 12.1 vs NS 9.7 days, p=0.50

† mean. ‡ median. Abbreviations: m; male, f; female ICU; Intensive care unit, OR; Odds ratio, HR; Hazard ratio, NS.; Not significant, TPA; Total Psoas Area; DMG; Dorsal Muscle Group, DM; Diabetes Mellitus, S; sarcopenic patients, NS; non-sarcopenic patients, N/A; Not available.

DISCUSSION

In recent years, multiple narrative reviews regarding sarcopenia among transplantation patients have been published.³¹⁻³³ This is the first systematic review of studies that investigated the influence of skeletal muscle mass by means of abdominal CT in patients who were evaluated for or underwent liver transplantation or were registered on the waiting list. According to the current findings, there is consistent evidence that sarcopenia is associated with impaired survival, independent of other risk factors such as age and MELD-score or its individual components. This association was found both before and after the introduction of the MELD-score (i.e., in the United States in 2002).³⁴ Due to a substantial heterogeneity between reported outcome measures, less consistent evidence suggests that sarcopenia is associated with post-transplantation complications, which may be infectious complications in particular.

Some findings of this systematic review are conflicting and multiple reasons could be postulated. Definitions of sarcopenia greatly varied, as there currently is no consensus regarding adequate cut-off values. Although liver transplant patients greatly differ from cancer patients, most studies used cut-off values based on oncological studies, such as those defined by Prado and colleagues.³⁵ This could have led to inadequate classification of patients as (non-)sarcopenic. Therefore, one could wonder whether one set of cut-off values would be applicable for various populations and gender-, age-, ethnicity-, and disease-specific cut-off values may be needed. One study described the association between morphometric age, but the association between the individual skeletal muscle mass components (i.e., psoas area and psoas density) was not described.²⁹ Besides multiple definitions for sarcopenia, multiple methods to perform skeletal muscle mass measurements (e.g., psoas area, cross-sectional area) have been used throughout studies.

Sarcopenia was associated with waiting list mortality in four studies^{15, 22, 23, 25}, whereas two other studies reported no significant association.^{17, 30} Besides different cut-off values and methods used, an explanation of this difference could be a varying range of MELD-score within these studies. After all, the association between sarcopenia and waiting list mortality was found in patients with lower MELD-scores in particular.^{15, 25} No association between sarcopenia and overall post-transplantation survival was reported in three studies.^{17, 24, 28}

The varying study populations could be an explanation for these conflicting results, as well as the use of BMI- and sex-specific cut-off values that are used to predict survival in cancer patients, in transplant populations.³⁶ Meza-Junco et al. included hepatocellular cancer patients who underwent liver transplantation only and used the same cut-off values. They indeed found an independent association with survival.²² In all but one study with questionable methodology²⁶, describing the association between pre-transplantation sarcopenia and post-transplantation short-term outcome, sarcopenia was independently associated with complications and mortality.

This systematic review postulates that skeletal muscle mass is a prognostic factor, independent of MELD-score. Although it remains to be investigated whether skeletal muscle mass assessment is superior to the 'eyeball test', i.e. the subjective clinician's assessment of a patient's physical status or frailty, it could objectively underscore subjective assessments.¹⁶ The study of Tandon et al. showed that sarcopenic patients with a low MELD-score had a similar outcome compared with patients with a high MELD-score with or without sarcopenia.²⁵ Therefore, skeletal muscle mass assessment may be used to more accurately select liver transplant patients and allocate organs in the future. After all, a selection of patients that is at risk for early mortality is probably not adequately identified by the MELD-score, since 71% of the patients who died on the waiting list had a MELD-score ≤ 25 at registration in the study of Durand et al.¹⁵ Although this would be a challenge due to the current organ shortage, patients with a low MELD-score and low skeletal muscle mass could, for example, be prioritized on the waiting list or be selected for targeted treatment of muscle wasting. Currently, such trials are being performed³⁷ and potential drugs are being investigated³⁸ in cancer populations.

Despite the strong prognostic value of the MELD-score, the survival of 15-20% of the patients cannot accurately be predicted.³⁴ The most frequently reported limitation of the MELD-score is the lack of parameters reflecting patients' nutritional and functional status. Therefore, modifications of the MELD-score, such as the MELDNa-score and the 5-variable MELD-score, including serum sodium and albumin levels respectively, have been developed.^{3, 4, 39} Both scores improved mortality prediction. The outcome of this systematic review supports that sarcopenic liver transplant candidates face a worsened outcome. Besides the superiority of the MELD-psoas area score of Durand et al. over the MELD-score and MELDNa-score to predict waiting list mortality¹⁵, the predictive value of the MELD-Sarcopenia score was found superior compared with the MELD-score in a recent study.⁴⁰ However, validation of these scores is recommended. If validated, clinical trials are warranted to investigate whether transplantation in sarcopenic patients with lower MELD-scores may be preferential. Prospective trials are also needed

to investigate the natural course of sarcopenia following liver transplantation. Little is known on whether skeletal muscle mass fully normalizes, and how post-transplantation change in muscle mass impacts outcome, such as the development of post-transplantation diabetes mellitus.²⁷

Preoperative risk assessment remains of paramount importance in patients who have been allocated a donor liver. Some studies suggest that a poor nutritional status, for instance reflected by the subjective global assessment, impairs post-transplantation outcomes.⁴¹⁻⁴³ However, these measures are frequently considered as subjective.⁴⁴ Furthermore, limiting factors, such as fluid retention, could hamper nutritional assessment.¹⁵ Single-slice CT-assessed skeletal muscle measurements are considered an objective and easy-to-perform method with high inter-observer agreement that could be performed on routinely available CT scans.⁷ Up to now, no gold standard has been established to perform body composition measurements. However, CT imaging was the method of preference in an expert consensus meeting on cachexia⁴⁵, particularly in cirrhotic patients who frequently have ascites.¹⁷

Some limitations of the current review and included studies should be mentioned. First, all included studies were retrospective, observational cohort studies. Although this may have resulted in selection bias, the study cohorts consisted of non-selected, consecutive patients listed for or undergoing liver transplantation. Second, selective publication of data could have led to an underestimation of negative results. Third, four research groups from Canada²²⁻²⁵, Ann Arbor^{16, 19, 20, 29} and Pittsburg¹²⁻¹⁴, USA, and Japan^{21, 26} published multiple original articles including patient populations from their center that were all included in the current review. These four centers contributed to thirteen of the nineteen studies. Therefore, the number of patient cohorts studied is likely to be smaller than the number of original articles included in this systematic review.

In conclusion, sarcopenia impairs outcome in patients awaiting or undergoing liver transplantation. Skeletal muscle mass assessment may contribute to pre-transplantation risk assessment.

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CHAPTER 3

SYSTEMATIC REVIEW OF SARCOPENIA IN RESECTABLE GASTROINTESTINAL AND HEPATOPANCREATOBILIARY MALIGNANCIES

ABSTRACT

BACKGROUND

Preoperative risk assessment in cancer surgery is of importance to improve treatment and outcome. The aim of this study was to assess the impact of CT-assessed sarcopenia on short- and long-term outcomes in patients undergoing surgical resection of gastrointestinal and hepatopancreatobiliary malignancies.

METHODS

A systematic search of Embase, PubMed and Web of Science was performed to identify relevant studies published before 30 September 2014. PRISMA guidelines for systematic reviews were followed. Screening for inclusion, checking the validity of included studies and data extraction were carried out independently by two investigators.

RESULTS

After screening 692 records, 13 observational studies with a total of 2884 patients were included in the analysis. There was wide variation in the reported prevalence of sarcopenia (17.0–79 per cent). Sarcopenia was independently associated with reduced overall survival in seven of ten studies, irrespective of tumor site. Hazard ratios (HRs) of up to 3.19 (hepatic cancer), 1.63 (pancreatic cancer), 1.85 (colorectal cancer) and 2.69 (colorectal liver metastases, CLM) were reported. For esophageal cancer, the HR was 0.31 for increasing muscle mass. In patients with colorectal cancer and CLM, sarcopenia was independently associated with postoperative mortality (colorectal cancer: odds ratio (OR) 43.3), complications (colorectal cancer: OR 0.96 for increasing muscle mass; CLM: OR 2.22) and severe complications (CLM: OR 3.12).

CONCLUSIONS

Sarcopenia identified before surgery by single-slice CT is associated with impaired overall survival in gastrointestinal and hepatopancreatobiliary malignancies, and increased postoperative morbidity in patients with colorectal cancer with or without hepatic metastases.

INTRODUCTION

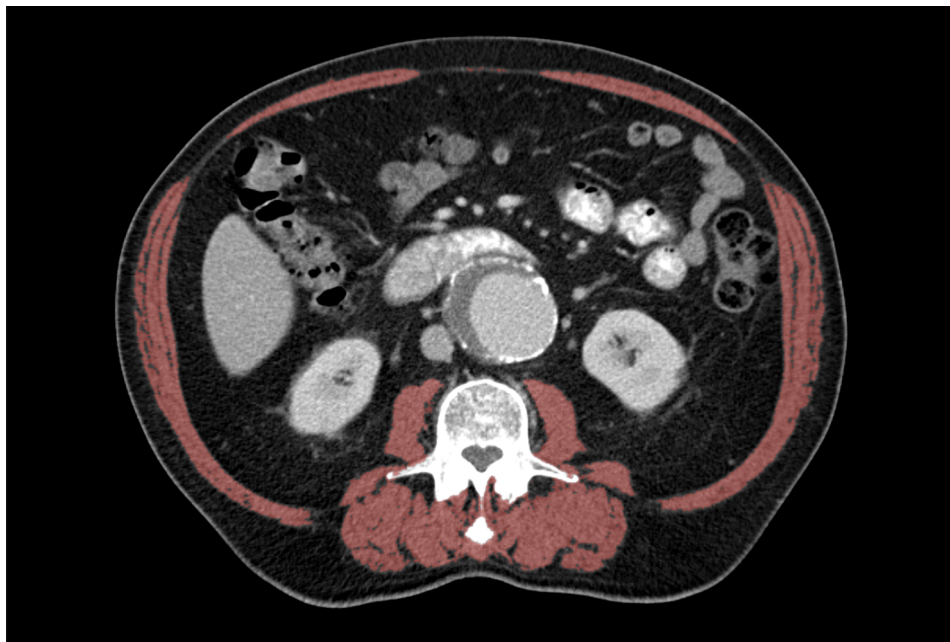
Advanced surgical techniques, developments in perioperative care and the introduction of enhanced recovery programs have improved surgical outcomes.¹⁻⁵ Nevertheless, risk assessment before major abdominal surgery remains of paramount importance to further improve outcomes after cancer surgery. Known factors that are predictive of short-term outcome include albumin levels, American Society of Anesthesiologists (ASA) classification and emergency surgery, whereas advanced age and disseminated disease determine long-term outcome.⁶⁻⁸ Outcomes of patients with similar age, tumor stage and ASA classification may be very different in clinical practice. Therefore, the risk factors commonly used to predict outcome after cancer surgery may reflect the patient's general health status and physiological reserves insufficiently. An important risk factor for worse outcome is frailty, which is poorly reflected by the traditional determinants of outcome⁹⁻¹³ Frailty is defined as a biological syndrome characterized by decreased reserve and resilience to stress factors across multiple physiological systems, and has been shown to be associated with adverse health outcomes^{14, 15} A hallmark sign of frailty is sarcopenia, the involuntary loss of skeletal muscle mass¹⁶⁻¹⁸ The prevalence of sarcopenia in healthy individuals increases with advanced age, ranging from 9 per cent at 45 years and up to 64 per cent in individuals aged over 85 years.¹⁹

Sarcopenia is characterized by a loss of skeletal muscle mass, skeletal muscle strength and physical performance.²⁰ It has been shown to impair physical performance and survival in geriatric, non-cancer populations^{21, 22}, and to impair survival in a variety of clinical conditions, such as cancer.²³ Up to 80 per cent of patients with advanced cancer are affected by cancer-induced cachexia, a clinical condition that also results in skeletal muscle wasting with or without loss of body fat.²⁴⁻²⁶ Cachectic patients are more prone to a reduced effect of therapy and increased chemotherapy toxicity²⁷⁻²⁹ It has been estimated that as many as 30 per cent of cancer-related deaths result from cachexia.³⁰⁻³³ One study²³ showed that sarcopenia was associated with decreased survival in obese patients with cancer by using CT to assess reduced skeletal muscle mass³⁴ (figure 1).

A systematic review was undertaken to investigate the influence of low skeletal muscle mass or skeletal muscle density assessed by CT on short- and long-term outcomes in patients undergoing surgery for gastrointestinal and hepatopancreatobiliary malignancies.

METHODS

Figure 1. A transversal computed tomogram at the level of L3 showing a cross sectional area of skeletal muscle mass



Highlighted in red, including the following muscles: psoas, paraspinal, transverse abdominal, external oblique, internal oblique and rectus abdominis.

Eligibility criteria were established a priori. A systematic search was performed to identify all original articles on patients undergoing surgical resection of malignancies of the gastrointestinal tract or hepatopancreatobiliary system, in which preoperative abdominal CT was used to assess skeletal muscle mass. The Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines were followed.³⁵

Included in the analysis were studies that reported on the prevalence of sarcopenia, and at least one of the following outcomes: postoperative mortality, postoperative complications, length of intensive care (ICU) stay, length of hospital stay, disease-free survival and overall survival.

The search was limited to papers in English with a publication date from January 2000 to September 2014. Three search strings with corresponding search terms were constructed (table S1, supporting information). The same search strings were used to develop queries in the EMBASE, PubMed and Web of Science databases.

The EMBASE database search was performed using the following query: ('sarcopenia':de,ab,ti OR 'analytic morphomics':de,ab,ti OR 'body composition':de,ab,ti OR 'muscle depletion':de,ab,ti OR 'muscle mass':de,ab,ti OR 'psoas area':de,ab,ti OR 'myopenia':de,ab,ti OR 'core muscle':de,ab,ti OR 'lean body mass':de,ab,ti OR 'muscular atrophy':de,ab,ti) AND ('cancer':de,ab,ti OR 'neoplasms':de,ab,ti OR 'malignancy':de,ab,ti) AND ('surgery':de,ab,ti OR 'resection':de,ab,ti OR 'esophagectomy':de,ab,ti OR 'gastrectomy':de,ab,ti OR 'hepatectomy':de,ab,ti OR 'colectomy':de,ab,ti OR 'pancreatectomy':de,ab,ti OR 'cholecystectomy':de,ab,ti). Similar queries were constructed for PubMed and Web of Science.

Duplicate records were removed and abstracts screened independently by two investigators to determine which records were eligible for further analysis. Abstracts were included for initial analysis if sarcopenia in patients undergoing surgical treatment with gastrointestinal or hepatopancreatobiliary malignancies was described. Abstracts that described sarcopenia determined by means other than abdominal CT or patients undergoing non-surgical treatment were excluded from further analysis. Records without abstracts, case reports, review articles, opinion articles and experimental studies were excluded.

ELIGIBILITY OF STUDIES AND ASSESSMENT OF METHODOLOGICAL QUALITY

Full-text articles of the remaining records were subsequently retrieved and screened independently by two investigators. All original articles that met the inclusion criteria were included. Additional relevant references were sought in the included full-text articles. Two investigators independently assessed the methodological quality of the included studies using the Newcastle–Ottawa quality assessment scale for cohort studies³⁶ for each a priori defined outcome measure.

DATA EXTRACTION

Data regarding study design and results were extracted independently by two investigators for each eligible study. Extracted data included age, sex distribution, patient selection, prevalence of sarcopenia, postoperative mortality, postoperative complications, length of ICU stay, length of hospital stay, disease-free survival and overall survival. If univariable and multivariable analyses had been performed to adjust for known risk factors, the latter was used for interpretation of the results.

Table S1. Search strings and terms.

Search Strings	Search Terms
Loss of muscle mass	Sarcopenia OR
	Analytic Morphomics OR
	Body Composition OR
	Muscle Depletion OR
	Muscle Mass OR
	Psoas Area OR
	Myopenia OR
	Core Muscle OR
	Lean Body Mass OR
	Muscular Atrophy AND
Malignancy	Cancer OR
	Neoplasms OR
	Malignancy AND
Surgical resection	Surgery OR
	Resection OR
	Esophagectomy OR
	Gastrectomy OR
	Hepatectomy OR
	Colectomy OR
	Pancreatectomy OR
	Cholecystectomy

STATISTICAL ANALYSIS

Outcomes are reported as originally shown. The prevalence of sarcopenia described in this review applies to the total population of each study. Therefore, rates could not be provided for subgroups (such as by cancer stage) separately. No meta-analysis was performed because there was great heterogeneity between studies.

RESULTS

The literature search was performed on 30 September 2014 and identified an initial 692 records, of which 27 were found to be potentially relevant (figure 2). From these 27 records, seven full-text articles were excluded as sarcopenia was assessed by means other than abdominal CT, four articles did not report relevant outcome data, and three articles reported on a population that received non-surgical treatment for the studied tumors. The remaining 13 studies matched the inclusion criteria.^{37–49} Cross-referencing yielded no additional results. The included studies provided data on patients with esophageal, gastric, pancreatic, primary liver and colorectal cancer, and resectable hepatic colorectal metastases (table 1). No studies reported on patients with bile duct or gallbladder cancer.

Figure 2. PRISMA Flow Chart (Selection Strategy) of Included Studies.

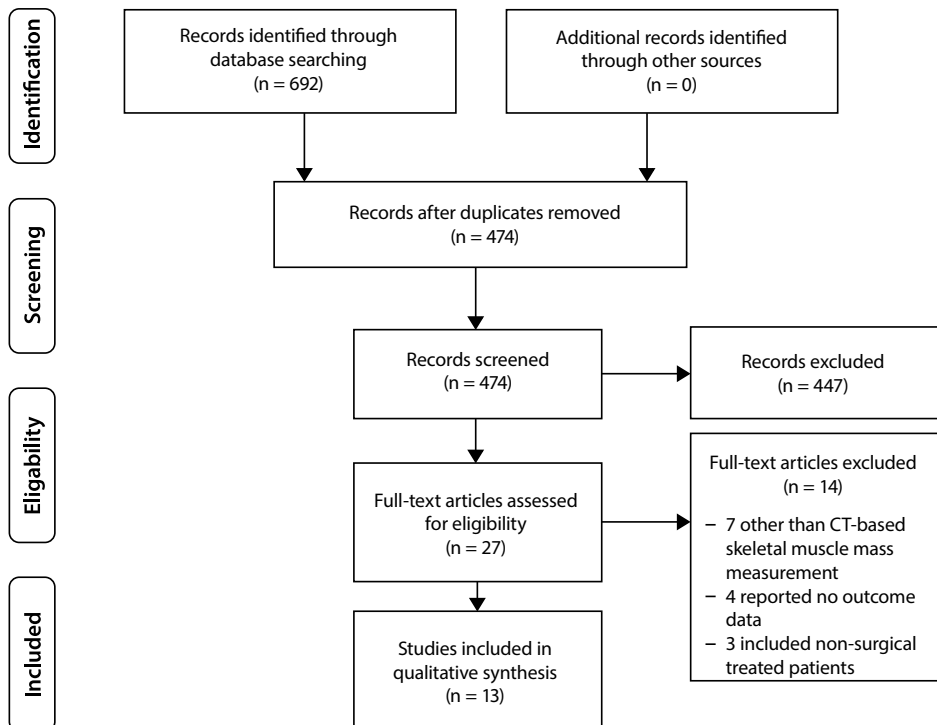


Table 1. Studies on the effects of sarcopenia in resectable gastrointestinal and hepatopancreatobiliary malignancies.

Author, year	Malignancy, patient selection	Disease stage	n (m)	Age	BMI	Muscle(s) measured (level), cutoffs	Outcome reported	Quality Points*
								ST DFS OS
Awad, 2012 ³⁶	Oesophageal and gastric cancer, WHO 0 - 2	Locally Advanced	47 (34)	63 [†]	Prior to NAC: 24.6 [†] Prior to resection: 23.8 [†]	CSAMM/m ² (L3) f 38.5 cm ² m 52.4 cm ²	ST, DFS, OS	5/9 4/9 4/9
Sheetz, 2014 ⁴⁵	Oesophageal cancer, all consecutive patients	I: 9.6% II: 24.3% III: 33.5% III: 27.4% IV: 5.2%	230 (202)	62 [†]	Overall: 28.6 [†]	TPA, PMD (L4) –	ST, DFS, OS	7/9 6/9 7/9
Yip, 2014 ³⁷	Oesophageal cancer	I: 5.7% I: 2.9% II: 51.4% III: 40.0%	35 (30)	63 [†]	Prior to NAC: 26.7 [†] Prior to resection: 25.8 [†]	CSAMM/m ² (L3) f 38.5 cm ² m 52.4 cm ²	ST, DFS, OS	5/9 5/9 5/9
Harimoto, 2013 ⁴⁰	Hepatocellular cancer, all consecutive patients	I: 15.6% II: 51.1% III: 26.3% IV: 7.0%	186 (145)	–	S: 20.5 [†] NS: 24.0 [†]	CSAMM/m ² (L3) f 41.1 cm ² m 43.75 cm ²	ST, DFS, OS	7/9 6/9 6/9
Itoh, 2014 ³⁹	Hepatocellular cancer, all patients without simultaneous procedures	N/a	190 (146)	–	< 18.5: 7.9% ≥ 18.5 - < 25: 68.4% ≥ 25 - < 30: 21.6% ≥ 30: 2.1%	CSAMM/m ² (L3) f 41.1 cm ² m 43.75 cm ²	DFS, OS	– 6/9 6/9
Voron, 2014 ³⁸	Hepatocellular cancer, all consecutive patients	N/a	109 (92)	62 [†]	Overall: 24.6 [†] S: 25.6 [†] NS: 26.9 [†]	CSAMM/m ² (L3) f 38.9 cm ² m 52.4 cm ²	ST, DFS, OS	7/9 6/9 6/9

Author, year	Malignancy, patient selection	Disease stage	n (m)	Age	BMI	Muscle(s) measured (level), cutoffs	Outcome reported	Quality Points*		
							ST	DFS	OS	
Peng, 2012 ⁴⁶	Pancreatic cancer, all consecutive patients	I: 0.2% I: 5.9% II: 16.9% III: 71.5% IV: 4.0% N/a: 0.6%	557 (296)	66†	≥30: 20.1%	TPA (L3) f 362 mm²/m² m 492 mm²/m²	ST, DFS, OS	6/9	5/9	5/9
Jung, 2014 ⁴⁸	Colorectal cancer	All stage III re-ceiving adjuvant chemotherapy	229 (134)	61†	S: 22.2† (<30: 87.8%) NS: 23.6† (<30: 71.1%)	TPA/m² (L4) –	DFS, OS	–	7/9	7/9
Lieffers, 2012 ⁴¹	Colorectal cancer	II: 31.6% III: 35.5% IV: 32.9%	234 (135)	63†	Overall: 28.5† S: 26.1† NS: 30.0†	CSAMM/m² (L3) f 38.5 cm² m 52.4 cm²	ST, DFS, OS	6/9	6/9	6/9
Reisinger, 2014 ⁴²	Colorectal cancer, all consecutive patients	II-I: 46.7% III-IV: 53.3%	310 (155)	69†	>25: 58.7% –	CSAMM/m² (L3) f 38.5 cm² m 52.4 cm²	ST	7/9	–	–
Sabel, 2013 ⁴⁷	Colorectal cancer, all consecutive patients	I: 24% II: 33% II: 30% IV: 11% N/a: 2%	302 (157)	68†	Overall: 28.7† –	PMD (L4) –	ST, DFS, OS	7/9	7/9	7/9
Peng, 2011 ⁴⁴	olorectal liver metastases, all consecutive patients	All stage IV	259 (155)	58†	≥30: 26.0%	TPA/m² (L3) 500 mm²	ST, DFS, OS	6/9	5/9	5/9
van Vledder, 2012 ⁴³	Colorectal liver meta-stases, all consecutive patients	All stage IV	196 (120)	65†	S: 23.7† NS: 26.7†	CSAMM/m² (L3) f 41.1 cm² m 43.75 cm²	DFS, OS	–	7/9	7/9

* Scored with the Newcastle-Ottawa quality assessment scale for cohort studies. † mean. ‡ median.

Abbreviations: BMI Body Mass Index (kg/m²). WHO World Health Organisation performance status. CSAMM Cross-sectional area of muscle mass. TPA Total psoas area. Squared body height. PMD Psoas mean density. HU Hounsfield Units. NAC Neoadjuvant chemotherapy. Sarcopenic patients. NS non-sarcopenic patients. ST Short term morbidity and/or mortality. DFS Disease free survival. OS Overall survival. N/a Not available. IS In situ. L3 At the level of the third lumbar vertebra. L4 At the level of the fourth lumbar vertebra.

PREVALENCE OF SARCOPENIA IN DIFFERENT MALIGNANCIES

The prevalence of sarcopenia as assessed by CT-based skeletal muscle mass measurement in patients undergoing surgery for gastrointestinal and hepatopancreatobiliary malignancies was reported in ten studies.^{37–45, 47} None of the studies^{39, 41, 42, 44, 45, 49} that compared characteristics in patients with and without sarcopenia reported on significant differences regarding cancer stage, differentiation grade or biomarkers. Despite comparable age and sex distribution between studies, there was a wide variation in the prevalence of sarcopenia, ranging from 17.0 per cent in a cohort of patients with hepatic colorectal metastases⁴⁵ to 79 per cent in a cohort with esophageal and gastric cancer.³⁷ In agreement, cohorts of patients with esophageal and gastric cancer reported a widespread prevalence of sarcopenia before surgery, ranging from 43 to 79 per cent.^{37, 38} Less variation in the prevalence of sarcopenia was observed among patients undergoing surgical resection of hepatocellular carcinoma (40.3–54.1 per cent)^{39–41}, colorectal cancer (38.9–47.7 per cent)^{42, 43} and hepatic colorectal metastases (17.0–19.4 per cent).^{44, 45} One study⁴⁷ reported a prevalence of sarcopenia of 25.0 per cent in patients with pancreatic cancer. Two studies^{37, 38} reported an increase in the prevalence of sarcopenia among patients with esophageal and gastric cancer following neoadjuvant chemotherapy. The impact of neoadjuvant therapy on the prevalence of sarcopenia was not assessed in the colorectal cancer studies included in the present analysis. A possible impact of age or sex on the prevalence of sarcopenia could not be discerned. Detailed information regarding the prevalence of sarcopenia is shown in table 2.

SHORT-TERM POSTOPERATIVE MORBIDITY AND MORTALITY

Data regarding complication rate, length of ICU stay, length of hospital stay, postoperative morbidity and postoperative mortality were reported in ten^{37–39, 41–43, 45–48} of the 13 studies included in the analysis (table 3).

An increased postoperative morbidity rate was found in patients with sarcopenia in all studies where this was reported among patients undergoing surgical resection of colorectal cancer^{42, 43, 48} and hepatic colorectal metastases.⁴⁵ One study⁴⁸ reported that an increase in psoas density protected against overall (odds ratio (OR) 0.96, 95 per cent c.i. 0.94 to 0.99; $P = 0.004$) and infectious (OR 0.95, 0.93 to 0.98; $P = 0.001$) complications in a cohort of 302 patients.⁴⁸ Another investigation⁴² observed an increase in infectious complications in patients with versus those without sarcopenia (23.1 versus 12.6 per cent; $P = 0.036$) in a cohort of 234 patients. Subgroup analysis revealed that the risk was especially pronounced in elderly patients (65 years or older) with sarcopenia (29.6 versus 8.8 per cent; $P = 0.005$). This difference remained significant in multivariable analysis (adjusted OR 4.6, 1.5 to 13.9; $P = 0.007$). The overall complication rate was not described.

Table 2. Studies reporting the prevalence of sarcopenia in gastrointestinal malignancies.

Author, Year	Malignancy	Prevalence
Awad, 2012 ³⁶	Oesophageal and gastric cancer	Prior to NAC: 57.4% Prior to resection: 78.7%
Yip, 2014 ³⁷	Oesophageal cancer	Prior to NAC: 26.0% Prior to resection: 43.0%
Voron, 2014 ³⁸	Hepatocellular carcinoma	54.1%
Itoh, 2014 ³⁹	Hepatocellular carcinoma	40.5%
Harimoto, 2013 ⁴⁰	Hepatocellular carcinoma	40.3%
Peng, 2012 ⁴⁶	Pancreatic cancer	25.0%
Lieffers, 2012 ⁴¹	Colorectal cancer	38.9%
Reisinger, 2014 ⁴²	Colorectal cancer	47.7%
van Vledder, 2012 ⁴³	Colorectal liver metastases	19.4%
Peng, 2011 ⁴⁴	Colorectal liver metastases	17.0%

† mean. ‡ median.

Abbreviations: NAC Neoadjuvant chemotherapy.

An increased risk of major postoperative complications (Clavien–Dindo grade IIIa or higher) among patients with sarcopenia compared with those without was reported among patients undergoing hepatic resection for colorectal metastases (22 versus 8 per cent respectively; OR 3.1 ; $P = 0.02$).⁴⁵ However, the study did not specify the type of complications. Another investigation⁴³ showed a strong association between sarcopenia and 30-day mortality combined with in-hospital mortality after elective colorectal cancer surgery (8.8 versus 0.6 per cent in patients with and without sarcopenia respectively; OR 43.3 , 2.74 to 685.2, $P = 0.007$).

No association between sarcopenia and postoperative morbidity and mortality was found in patients undergoing resection for esophageal or hepatocellular cancer.^{37,39,41,46} Specifically, in a cohort of 557 patients undergoing pancreatic cancer resection⁴⁷, there was no difference in the rate of any postoperative complication (44.6 versus 51.8 per cent in men with and without sarcopenia respectively, $P = 0.28$, 41.5 versus 43.4 per cent respectively among women, $P = 0.80$), major postoperative complications (20.6 versus 24.8 per cent for men, $P = 0.49$; 12.1 versus 20.5 per cent for women, $P = 0.15$) or 30-day postoperative mortality (1.4 versus 0.5 per cent for men, $P = 0.44$; 0 versus 0.5 per cent for women, $P = 1.00$). However, the 90-day mortality rate differed between men with and without sarcopenia (9.5 versus 2.7 per cent respectively; $P = 0.02$). Two studies^{43, 46} that reported on anastomotic leakage following surgical resection of colorectal and esophageal cancer did not demonstrate an association with sarcopenia.

Two studies adjusted for body mass index (BMI) in the multivariable analyses. One⁴³ reported that sarcopenia was a risk factor for 30-day mortality, whereas BMI was not. Similarly, in another investigation⁴⁵ sarcopenia, but not BMI, was a risk factor for post-operative complications.

LENGTH OF INTENSIVE CARE UNIT AND HOSPITAL STAY

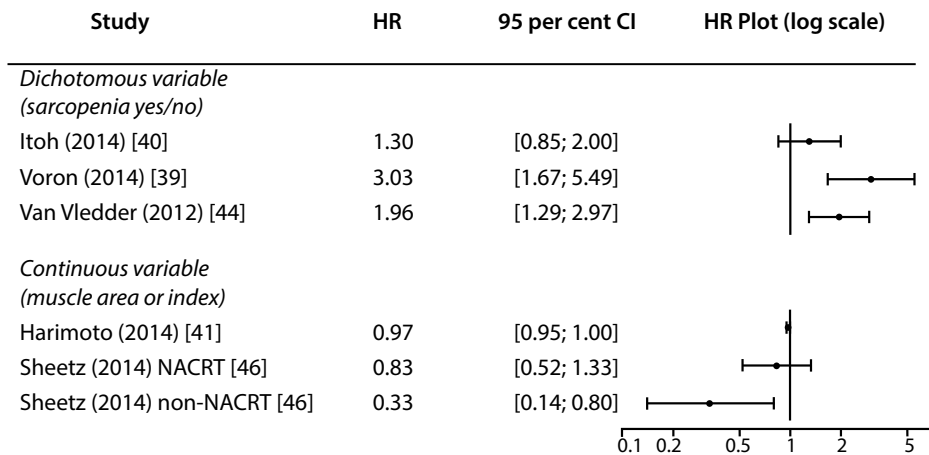
Peng and colleagues reported a prolonged ICU admission (more than 2 days) for patients with sarcopenia undergoing resection with curative intent for hepatic colorectal metastases compared with those without sarcopenia (15 versus 4 per cent respectively; $P = 0.004$)⁴⁵, but did not demonstrate a difference in the mean length of ICU stay in patients undergoing surgical resection of pancreatic cancer (mean (s.d.) 0.5(2.0) versus 0.5(1.7) days respectively for men, $P = 1.00$; 0.2(0.6) versus 0.2(0.6) days among women, $P = 0.74$).⁴⁷

In two^{42, 45} of five studies^{37, 38, 42, 45, 47} reporting length of hospital stay, patients with sarcopenia had a delayed discharge from hospital. Hospital stay was slightly prolonged in patients with sarcopenia undergoing resection with curative intent for hepatic colorectal metastases (6.6 versus 5.4 days; $P = 0.03$).⁴⁵ The impact of sarcopenia on length of hospital stay may be greater in conjunction with other patient characteristics. For instance, hospital stay was significantly longer in patients with sarcopenia than in those without for all patients undergoing surgery for colorectal cancer (15.9 versus 12.3 days; $P = 0.038$). The corresponding rates for patients aged 65 years or older were 20.2 versus 13.1 days ($P = 0.008$). In addition, sarcopenia was an independent factor for the need for rehabilitation in patients aged 65 years and older (OR 3.1, 95 per cent c.i. 1.4 to 9.4; $P < 0.040$).⁴² The two studies^{42, 45} that reported an increased length of hospital stay in patients with sarcopenia also observed an increased number of postoperative complications. Length of hospital stay did not significantly differ between patients with and without sarcopenia in studies of pancreatic cancer⁴⁷ and esophageal and gastric cancer.^{37, 38}

DISEASE-FREE SURVIVAL

Nine studies described the association between sarcopenia and disease-free survival^{38-41, 44-46, 48, 49} Data regarding disease-free survival rates and times in the individual studies are depicted in table 4, and figure 3.

Figure 3. Forest plots showing studies that reported the disease-free survival.



Only studies reporting hazard ratios with lower and upper 95 per cent confidence intervals are shown.

In patients with esophageal cancer, sarcopenia was found to be associated with impaired disease-free survival in those who underwent surgical resection without receiving neoadjuvant chemoradiation therapy independently of age, gender, and tumor stage (hazard ratio [HR] 0.33 [95 per cent CI 0.14-0.80], $p = 0.014$).⁴⁶ No association between sarcopenia and disease-free survival was observed in patients who underwent surgical resection following neoadjuvant chemoradiation therapy.^{38, 46}

Hepatocellular cancer patients with sarcopenia were found to have an increased risk of disease recurrence in two out of three studies.³⁹⁻⁴¹ One study reported a median disease-free survival of 10.1 months in sarcopenic patients versus 34.2 months in non-sarcopenic patients ($p < 0.001$) and an independent association between sarcopenia and disease free survival (HR 3.03 [95 per cent CI 1.67-5.49], $p < 0.001$).³⁹ Moreover, five-year disease-free survival rates of 13 per cent in sarcopenic patients compared with 33.2 per cent in patients without sarcopenia ($p = 0.013$) have been found in another study.

Table 3. Studies reporting the impact of sarcopenia on short-term outcome in gastrointestinal malignancies.

Author, year	Malignancy	Complications			Length of Stay			
		All	Clavien Dindo classification \geq IIIa	Postoperative/in-hospital mortality	Anastomotic Leakage	Infectious	ICU	Hospital (days)
Awad, 2012 ³⁶	Oesophageal and gastric cancer	-	-	N.s. (p=0.060)	-	-	-	N.s. (p=0.51)
Sheetz, 2013 ⁴⁵	Oesophageal cancer	LPA no complications 1993 mm ² versus LPA complications 1877 mm ² 1877 (p=0.12)	-	-	LPA no leakage 1922 mm ² versus LPA leakage 1953 mm ² 1877 (p=0.40)	-	-	-
Yip, 2014 ³⁷	Oesophageal cancer	N.s.	-	-	-	-	-	N.s.
Harimoto, 2014 ⁴⁰	Hepatocellular carcinoma	32.0% vs. 50.5%, p=0.61	-	-	-	-	-	-
Voron, 2014 ⁴⁸	Hepatocellular carcinoma	39.0% vs. 36.0%, p=0.749	20.3 vs. 16.0%, p=0.560	6.8% vs. 2.0%, p=0.372	-	-	-	-
Peng, 2012 ⁴⁶	Pancreatic cancer	OR 0.88 (0.60-1.29), p=0.51	OR 0.72 (0.43-1.21), p=0.21	HR 2.31 (0.78-6.77), p=0.13	-	-	0.4 vs. 0.4 days, p=0.92	12 vs. 12, p=0.98
Lieffers, 2012 ⁴¹	Colorectal cancer	-	-	-	-	12.6% vs. 23.1%,	-	10.6 vs. 13.2, p = 0.012
Reisinger, 2014 ⁴²	Colorectal cancer	-	-	OR 43.3 (2.74-685.2), p=0.007*	OR 0.57 (0.28-1.19), p=0.13	-	-	-
Sabel, 2013 ⁴⁷	Colorectal cancer	OR 0.96 (0.94-0.99), p=0.004* (for every unit of increased psoas density)	-	-	-	OR 0.95 (0.93-0.98), p=0.001* (for every unit of increased psoas density)	-	-

Author, year	Malignancy	Complications	Length of Stay					
			Clavien Dindo classification ≥ IIIa	Postoperative/ in-hospital mortality	Anastomotic Leakage	Infectious	ICU	Hospital (days)
Peng, 2011 ⁴⁴	Colorectal liver metastasis	All OR 2.22, p=0.02	22% vs. 8% OR 3.12 (1.14-8.49), p=0.02*	-	-	-	Prolonged stay (>2 days); 15% vs. 4% p = 0.004	6.6 vs. 5.4 p=0.03

† mean, ‡ median. * Using the fat free mass (FFM) assessed on the CT scans prior to resection. # Multivariable analysis performed. Abbreviations: ICU Intensive Care Unit. = No observed statistical significant difference between sarcopenic and non-sarcopenic patients. NS Non-sarcopenic patients. OR Odds ratio. N.s. Not significant. LPA Lean psoas area.

Table 4. Studies reporting the impact of sarcopenia on long-term outcome in gastrointestinal malignancies.

Author, Year	Malignancy	Disease-Free Survival	Overall Survival
Sheetz, 2014 ⁴⁵	Oesophageal cancer	NACRT: HR 0.83 (0.52-1.33), p=0.433 [#] Non-NACRT: HR 0.33 (0.14-0.80), p=0.014 [#] (for increasing LPA)	NACRT: HR 0.77 (0.46-1.28), p=0.311 [#] Non-NACRT: HR 0.31 (0.12-0.82), p=0.018 [#] (for increasing LPA)
Yip, 2014 ³⁷	Oesophageal cancer	N.s.	After chemotherapy: Median 25.6 vs. median not reached, p=0.063
Itoh, 2014 ³⁹	Hepatocellular carcinoma	HR 1.30 (0.85-2.00), p=0.215 [#]	HR 1.96 (1.06-2.83), p=0.031 [#]
Harimoto, 2014 ⁴⁰	Hepatocellular carcinoma	5-year: 71% vs. 83.7%, p=0.001. HR 0.97 (0.95-1.00), p=0.016 [#] (for increasing muscle mass)	5-year: 13.0% vs. 33.2%, p=0.013. HR 0.90 (0.84-0.96), p=0.002 [#] (for increasing muscle mass)
Voron, 2014 ³⁸	Hepatocellular carcinoma	HR 3.03 (1.67-5.49), p<0.00 [#]	HR 3.19 (1.28-7.96), p=0.013 [#]
Peng, 2012 ⁴⁶	Pancreatic cancer	N/a	Male 3-year: 39.2% vs. 20.3%, p<0.05 Female 3-year: 40.8% vs. 26.1%, p<0.05 3-year: HR 1.63 (1.28-2.07), p<0.001 [#]
Jung, 2014 ⁴⁸	Colorectal cancer	N.s. (p=0.946) [#]	HR 1.85 (1.10-3.13), p=0.022 [#]
Sabel, 2013 ⁴⁷	Colorectal cancer	HR 0.97 (0.95-1.00), p=0.03 (for increasing PD). In multivariable analysis: n.s.	HR 0.97 (0.95-1.00), p=0.04 (for increasing PD). In multivariable analysis: n.s.
Peng, 2011 ⁴⁴	Colorectal liver metastases	HR 1.07, p=0.78	HR 1.05, p=0.80
van Vledder, 2012 ⁴³	Colorectal liver metastases	HR 1.96 (1.29-2.97), p=0.002 [#]	HR 2.69 (1.67-4.32), p<0.001 [#]

[#] Multivariable analysis performed.

Abbreviations: NACRT Neoadjuvant chemoradiation therapy. HR Hazard ratio. LPA Lean psoas area. PD Psoas density. N.s. Not significant. N/a Not available.

In multivariable analysis, a high skeletal muscle was independently associated with a lower risk of disease recurrence (HR 0.97 [95 per cent CI 0.95-1.00], p = 0.016).⁴¹ Although another study found a reduced disease-free survival in patients undergoing hepatocellular cancer resection in univariable analysis (HR 1.62 [95 per cent 1.11-2.36], p = 0.012), this association did not remain significant in multivariable analysis (HR 1.30 [0.85-2.00], p = 0.215).⁴⁰

In patients with primary colorectal cancer, sarcopenia was found to impair disease-free survival in one of the two studies reporting on disease recurrence.^{48, 49} One study described a protective effect of high psoas muscle density (HR 0.97 (0.95-1.00), p = 0.03).⁴⁸ However, a significant difference in disease free survival between patients with normal and low skeletal muscle mass could not be demonstrated in another study.⁴⁹

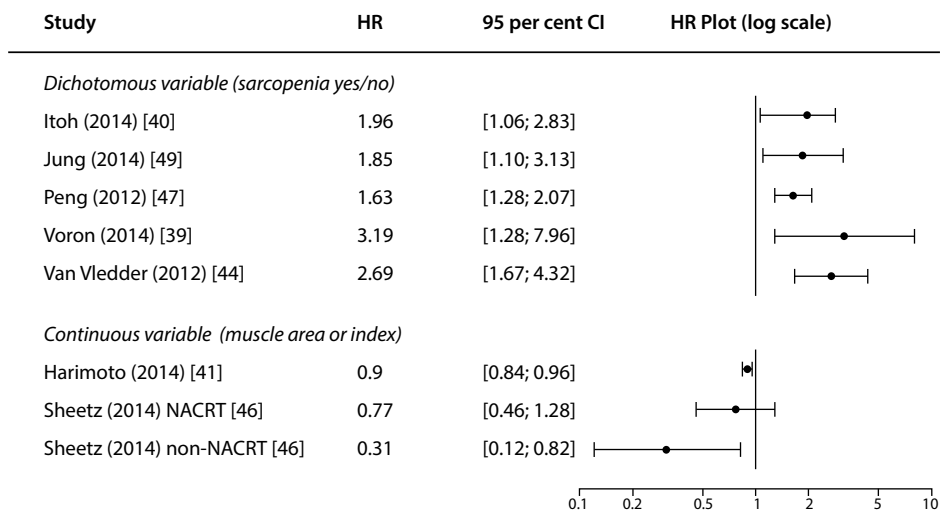
No median survival times and one-, three- or five-year survival rates were reported. In patients with hepatic colorectal metastases, one study reported a median disease-free survival time of 8.7 months in sarcopenic patients compared with 15.1 months in non-sarcopenic patients (HR 1.88 [95 per cent CI 1.25-2.82], $p = 0.002$).⁴⁴ However, another investigation found no association between sarcopenia and disease-free survival in patients with hepatic colorectal metastases: a 5-year recurrence free survival rate of 23% in sarcopenic patients versus 27% in non-sarcopenic patients was reported ($p = 0.078$).⁴⁵

Five studies adjusted the prognostic value of sarcopenia for BMI. Whereas sarcopenia was associated with disease-free survival in four out of nine studies as aforementioned, no association between BMI and disease-free survival was reported in patients with hepatocellular cancer, colorectal cancer, and hepatic colorectal metastases.^{39-41, 44, 49}

OVERALL SURVIVAL

Most authors reported a significant decrease in overall survival in sarcopenic patients compared with non-sarcopenic patients. This effect was observed irrespective of cancer site/origin.^{39-41, 44, 46-51} Data regarding survival rates and median survival times in the individual studies may be found in table 4 and figure 4.

Figure 4. Forest plots showing studies that reported the overall survival.



Only studies reporting hazard ratios with lower and upper 95 percent confidence intervals are shown.

A trend towards decreased survival in sarcopenic esophageal cancer patients was observed in one study (median overall survival 25.6 months versus not reached, $p = 0.063$).³⁸ As for disease-free survival, overall survival was impaired in esophageal cancer patients who did not receive neoadjuvant chemotherapy (HR 0.31 (0.12-0.82), $p=0.018$), whereas no significant association was found in patients who did receive neoadjuvant chemotherapy (HR 0.77 (0.46-1.28), $p=0.311$).⁴⁶

A study among hepatocellular carcinoma patients reported a median survival time of 52.3 months (sarcopenic) versus 70.3 months (non-sarcopenic) (log rank $p = 0.015$), with a particular steep decline in the one-year survival rate (69.8 per cent vs. 95.5 per cent, log rank $p = 0.015$).³⁹ Nevertheless, another investigation described a less severe impact of sarcopenia on survival in patients with hepatocellular carcinoma. A reduction in five-year survival rate from 83.7 per cent to 71 per cent (log rank $p = 0.001$) was found.⁴¹ A study among pancreatic cancer patients found a significantly lower three-year survival rate in sarcopenic patients compared with non-sarcopenic patients (male: 20.3 per cent vs. 39.2 per cent, $p = 0.003$; female: 26.1 per cent vs. 40.8 per cent, $p = 0.03$). In multivariable analysis, sarcopenia remained independently associated with an increased risk of death at three years (HR 1.63 [95 per cent CI 1.28-2.07], $p < 0.001$).⁴⁷

Median survival times or one-, three- or five-year survival rates have not been reported for patients with colorectal cancer. In patients with hepatic colorectal metastases, one study reported a median survival time of 23.8 versus 59.8 months (HR 2.53 [95 per cent CI 1.60-4.01], $p < 0.001$) in favor of non-sarcopenic patients.⁴⁴ Although in two studies no association between sarcopenia and overall survival was found in multivariable analyses, positive associations in univariable analyses were reported.^{38, 45}

Five studies adjusted the predictive effect of sarcopenia for BMI. Whereas sarcopenia was independently associated with overall survival in seven out of ten studies as aforementioned, no association between BMI and overall survival was reported in patients with hepatocellular cancer, and hepatic colorectal metastases.^{39-41, 44} Nevertheless, one study found BMI ≥ 25 as a risk factor for impaired survival, independent of sarcopenia, in stage III colorectal cancer patients receiving adjuvant chemotherapy.⁴⁹

DISCUSSION

This is the first systematic review describing the impact of CT-assessed sarcopenia on short- and long-term outcome in resectable gastrointestinal and hepatopancreatobiliary malignancies. Three conclusions may be drawn based upon the provided data. First, sarcopenia limits overall survival. Second, increased recurrence rates following surgical resection might be observed in patients with hepatic colorectal metastases and hepatocellular cancer. Third, impaired postoperative recovery in patients with colorectal cancer and hepatic colorectal metastases undergoing surgical resection was observed. Due to the heterogeneity of included studies, possible influence of age and gender on the prevalence of sarcopenia could not be assessed.

A previous review also described the relation between CT-assessed core muscle size and mortality, postoperative morbidity and length of stay after major abdominal surgery.⁵² Similarly, sarcopenia was found to be associated with increased morbidity, length of hospital stay and mortality. The relation between sarcopenia and recurrence was not described. Their systematic review included eight retrospective cohort studies, of which five investigated oncological populations. The other studies were performed among distinct surgical populations, such as transplantation and vascular surgery patients. The current review was aimed on abdominal malignancies in surgical patients in particular. Therefore this review could be considered more homogeneous. Furthermore, multiple studies among surgical oncology populations have been published in recent years, which have been united into this review.

Preoperative risk stratification is of utmost importance in patient selection for surgery, as it may help physicians to identify patients (un)fit for surgery. A tool suitable for use in daily care should be inexpensive, easily obtainable and reliable. Bioelectrical impedance analysis, Dual Energy X-ray Absorption (DEXA) scans and skinfold measurement are often not routinely performed during the oncological workup, whereas the majority of patients undergo abdominal CT-imaging as part of their preoperative (diagnostic) workup. Single-slice analysis of cross-sectional muscle area using abdominal CT-scans is a rapid method, linearly related to total body skeletal muscle mass⁵³ that offers a low level of inter-observer variability.^{43, 53} Computed tomography based skeletal muscle mass measurement in oncological patients may identify patients who could be in a pre-frail state. Such early stages of frailty may clinically remain undetected.⁵⁴ Surgical trauma, the presence of cancer or postoperative morbidity may act as a trigger

to further spiral downwards in the spectrum of frailty and lead to impaired overall survival, reduced resistance to stressors and increased morbidity.

Further research is required to determine whether treatment of sarcopenia may improve outcome. The understanding of muscle wasting in cancer has greatly increased over the past decade^{55, 56} and led to novel preclinical treatment options, e.g. myostatin inhibitors.^{57, 58} A phase II clinical trial on the effectivity of myostatin inhibitors in patients with advanced or metastatic pancreatic cancer receiving chemotherapy is ongoing with overall survival as the primary endpoint. This is just one of several ongoing clinical trials on halting or reversing muscle loss in cancer patients.⁵⁹

Some limitations of the included studies and this systematic review resulted in the inability to perform a reliable quantitative synthesis (i.e. meta-analysis) and should be acknowledged. First, the current data are based on predominantly retrospective, observational studies. Consequently, no causative relationship between sarcopenia and outcome is demonstrated. Second, this review is likely to be influenced by submission and/or publication bias. Moreover, negative results may have been omitted from publication. There was considerable heterogeneity in study design. As there is no golden standard definition of CT-based assessment of muscle mass, different methods have been used. Third, studies measuring total cross-sectional area of muscle mass used distinct gender specific cut-off values.^{23, 44} These cut-off values were obtained using the same method of optimal stratification in two different patient populations, yielding two sets of distinct cut-off values. As such, these cut-off values may not be interchangeable and applicable to all populations. On top of that, another study developed a third set of cut-off values, which are both gender and BMI specific and also includes muscle attenuation (based on Hounsfield units) as a marker for muscle fat infiltration.⁶⁰ However, these cut-off values have not been validated yet. Hence, larger (prospective) studies are warranted to define cut-off values associated with decreased survival for patients adjusted for gender, age, BMI, ethnicity, and tumor sort. Lastly, large disparities between reported outcome measurements were observed. Therefore, to improve comparability between future studies overall survival (median), disease-free survival (median), overall complications, minor complications (i.e. Clavien-Dindo grade \leq II), major complications (i.e. Clavien-Dindo grade \geq IIIa), and treatment related mortality (i.e. Clavien-Dindo grade V) should be included within their results.

In conclusion, sarcopenia impairs overall survival. A moderate association between sarcopenia and disease-free survival exists. Moreover, sarcopenia may increase postoperative morbidity, possibly due to postoperative infectious complications, in patients

undergoing surgical resection of colorectal cancer. Hence, CT-based muscle mass assessment may assist in preoperative decision-making, particularly for those patients who tend to be unfit for surgery or face a poor prognosis. However, larger and prospective trials are required to confirm this before any clinical recommendation can be made, and to evaluate whether treatment of sarcopenia may improve postoperative outcomes.

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CHAPTER 4

A COMPARATIVE STUDY OF SOFTWARE PROGRAMS FOR CROSS-SECTIONAL SKELETAL MUSCLE AND ADIPOSE TISSUE MEASUREMENTS ON ABDOMINAL COMPUTED TOMOGRAPHY SCANS OF RECTAL CANCER PATIENTS

ABSTRACT

BACKGROUND

The association between body composition (e.g., sarcopenia or visceral obesity) and treatment outcomes, such as survival, using single-slice computed tomography (CT) based measurements has recently been studied in various patient groups. These studies have been conducted with different software programs, each with their specific characteristics, of which the inter-observer, intra-observer and inter-software correlation are unknown. Therefore, a comparative study was performed.

METHODS

Fifty abdominal CT scans were randomly selected from 50 different patients and independently assessed by two observers. Cross-sectional muscle area (CSMA, i.e. rectus abdominis, oblique and transverse abdominal muscles, paraspinal muscles and the psoas muscle), visceral adipose tissue area (VAT) and subcutaneous adipose tissue area (SAT) were segmented by using standard Hounsfield unit ranges and computed for regions of interest. The inter-software, intra-observer, and inter-observer agreement for CSMA, VAT, and SAT measurements using FatSeg, OsiriX, ImageJ, and SliceOmatic were calculated using intra-class correlation coefficients (ICC) and Bland-Altman analyses. Cohen's κ was calculated for the agreement of sarcopenia and visceral obesity assessment. The Jaccard similarity coefficient was used to compare the similarity and diversity of measurements.

RESULTS

Bland-Altman analyses and intra-class correlation coefficients indicated that the CSMA, VAT, and SAT measurements between the different software programs were highly comparable (ICC 0.979-1.000, $p < 0.001$). All programs adequately distinguished between the presence or absence of sarcopenia ($\kappa = 0.88$ -0.96 for one observer and all $\kappa = 1.00$ for all comparisons of the other observer) and visceral obesity (all $\kappa = 1.00$). Furthermore, excellent intra-observer (ICC 0.999-1.000, $p < 0.0001$) and inter-observer agreement (ICC 0.988-0.999, $p < 0.0001$) for all software programs were found. Accordingly, excellent Jaccard similarity coefficients were found for all comparisons (mean ≥ 0.964).

CONCLUSIONS

FatSeg, OsiriX, ImageJ, and SliceOmatic showed an excellent agreement for CSMA, VAT and SAT measurements on abdominal CT scans. Furthermore, excellent inter- and intra-observer agreement were achieved. Therefore, results of studies using these different software programs can reliably be compared.

INTRODUCTION

Biological frailty and analytic morphomics (i.e. body composition) have increasingly gained interest in recent years in relation to treatment outcomes, such as complications and (disease-free) survival.^{1, 2} Frailty, a state of increased vulnerability towards stressors, leads to an increased risk of developing adverse health outcomes³ and is an important predictor of complications after interventional procedures, such as surgery and chemotherapy.⁴⁻⁷ For example, frail patients undergoing colorectal surgery have a fourfold increased risk to develop major postoperative complications.⁵ One of the hallmark signs of frailty is sarcopenia, the involuntary depletion of skeletal muscle mass.⁸⁻¹¹ It is estimated that up to 25% of persons under 70 years of age and over 50% of persons of 80 years and older experience sarcopenia.¹² In addition, up to 80% of patients with advanced cancer are affected by cancer-induced cachexia, a clinical condition that also results in skeletal muscle wasting with or without the loss of body fat.¹³⁻¹⁵ Patients with cachexia are more prone to a reduced therapy effect¹⁶ and patients with low skeletal muscle mass experience increased chemotherapy toxicity.^{17, 18} This ultimately results in death in nearly one third of all cancer patients.¹⁹⁻²²

Over the last years, numerous studies have used abdominal computed tomography (CT) scans to quantify skeletal muscle mass, for example in clinical^{17, 18, 23-25} and surgical oncology²⁶, vascular surgery²⁷, and transplantation surgery^{28, 29} patients. Furthermore, multiple studies measured visceral and/or subcutaneous adipose tissue on CT scans.³⁰⁻³³ However, different software programs have been used to perform these body composition analyses, such as FatSeg³³, OsiriX⁷, ImageJ²⁴, and SliceOmatic²³. To be able to adequately compare study results, the comparability of these various software programs should be known. Therefore, the aim of this study was to investigate the agreement of these four different software packages for the assessment of cross-sectional skeletal muscle and subcutaneous and visceral adipose tissue measurements on abdominal CT scans.

METHODS

PATIENTS

Fifty abdominal CT scans of patients who were scheduled for rectal cancer resection at Erasmus University Medical Center (Rotterdam, The Netherlands) between 2005 and 2012 were randomly selected. All CT scans were routinely performed as part of the pre-operative diagnostic work up or assessment of down staging after neo-adjuvant therapy. Only one CT scan was used per patient. None of the patients had an ostomy, abdominal wall deformity, abdominal wall tumor, or a CT scan with artefacts at the level of L3 that could potentially influence measurements. Self-reported weight and height in the preoperative work-up were retrospectively collected from electronic patient files.

SKELETAL MUSCLE AND ADIPOSE TISSUE AREA MEASUREMENTS

The cross-sectional skeletal muscle area (CSMA), subcutaneous adipose tissue area (SAT) and visceral adipose tissue area (VAT) (cm²), including renal adipose tissue, were measured at the mid third lumbar vertebra (L3) level on a slice showing both transversal processes. CSMA measurements included the following muscles: psoas, paraspinal, transverse abdominal, external oblique, internal oblique and rectus abdominis. All abdominal CT scans were assessed on identical slices in a random order by two medically trained observers (AG [observer A] and JLA^vV [observer B]), with great knowledge about radiological anatomy and extensive experience in skeletal muscle and adipose tissue area measurements using various software programs. Observer A performed measurements twice on identical a priori selected slices, whereas observer B performed a second reading without a priori selected slice numbers. The observers were blinded for each other's measurements and for patient details. For each observer the time interval between two readings in the same patient with different software programs was at least one week. This resulted in an interval of at least four weeks between two readings within one patient with the same software program. Only the first reading of observer B was used for the inter-software and inter-observer comparisons.

The CSMA was corrected for height squared (m²), resulting in the L3 muscle index (SMI, cm²/m²). Patients were classified as having sarcopenia or not having sarcopenia according to previously described cut-off values (52.4 cm²/m² for men and 38.5 cm²/m² for women).²³ Predefined cut-off values for visceral adipose tissue area to define visceral obesity of 163.8 cm² for men and 80.1 cm² for women were used.³⁴ For subcutaneous adipose tissue no cut-off values have been reported in the literature.

Four software programs were compared: FatSeg (developed by the Biomedical Imaging Group Rotterdam of Erasmus MC, Rotterdam, The Netherlands, using MeVisLab (Mevis Medical Solutions, Bremen, Germany)), OsiriX (Pixmeo SARL, Geneva, Switzerland), ImageJ (National Institutes of Health, Bethesda, Maryland, USA), and SliceOmatic (TomoVision, Magog, Canada). CSMA, VAT, and SAT were segmented using standard Hounsfield Unit (HU) thresholds in all four software programs. An intensity window between -30 HU and +150 HU was used for skeletal muscle tissue.³⁵ For adipose tissue an intensity window between -190 HU and -30 HU was used.³⁶ Since the tissue of interest is manually selected, competency in anatomic radiology is a prerequisite for these measurements.

FATSEG

FatSeg is an in-house developed software program to perform soft tissue measurements on CT scans and was developed using the MeVisLab development environment for medical image processing and visualization version 2.4 (available from <http://www.mevislab.de>). Inner and outer contours of aforementioned skeletal muscle and adipose regions were manually traced. The skeletal muscle and adipose tissue areas were computed automatically using the preset HU intensity thresholds, and expressed in cm². Intraluminal contents initially marked as adipose tissue were manually erased. Cutaneous tissue was included in the SAT measurement. Measurements were performed on a 3.2 GHz Intel® Core™ i5 Dell (Dell Inc., Round Rock, TX, USA) personal computer.

OSIRIX

The open-source 32-bit edition of OsiriX version 5.8.5 (available from <http://www.osirix-viewer.com>) was used. The "Grow Region (2D/3D Segmentation)" tool was used to semi-automatically select skeletal muscle and adipose tissue regions within our preset HU intensity thresholds. Non-skeletal muscle tissue regions adjacent to skeletal muscle were manually removed from the area selection using the brush option. The brush option was also used to manually erase intraluminal areas with contents having radiological density between -190 and -30 HU, resembling fatty content. Cutaneous tissue was not included in the SAT measurement. The skeletal muscle and adipose tissue areas were computed automatically and expressed in cm² using a 1.3 GHz Intel® Core™ i5 MacBook Air (Apple Inc., Cupertino, CA, USA) and computer mouse.

IMAGEJ

ImageJ version 1.48 is a freely downloadable public domain software program developed by the National Institutes of Health for image processing and analyzing (available from <http://rsbweb.nih.gov/ij/download.html>). First, manual delineation of the outer contour of the abdominal wall and paraspinal muscles was performed and the surface area of tissue with an attenuation between -30 and +150 HU was computed automatically (mm^2) and manually divided by 100, resulting in cm^2 . Second, delineation of the inner contour of the abdominal wall, paraspinal and psoas muscles was performed in a similar fashion to allow for subsequent correction of intra-abdominal content with attenuation between the preset HU intensity thresholds. The inner contour was manually subtracted from the outer contour surface area, resulting in the cross-sectional skeletal muscle area (cm^2).³⁷ The subcutaneous adipose tissue area measurements were performed in a similar manner as the muscle measurements, whereas visceral adipose tissue area measurements were performed by delineating a contour through the inner contour of the abdominal wall muscles, psoas muscles and vertebrae followed by manual erasing of intraluminal fatty content. Cutaneous tissue was included in the SAT measurement. A 3.2 GHz Intel® Core™ i5 Dell (Dell Inc., Round Rock, TX, USA) personal computer was used.

SLICEOMATIC

SliceOmatic (TomoVision, Magog, Canada) version 5.0 (64 bit; available from <http://www.tomovision.com/>) was used. Tissue was semi-automatically selected with the 'Region Growing' mode using the 'Grow 2D' and 'Paint' tools. Non-skeletal muscle tissue regions adjacent to skeletal muscle having radiological density between the predefined HU thresholds were manually erased using the 'Paint' tool. Cutaneous tissue was included in the SAT measurement. A 3.2 GHz Intel® Core™ i5 Dell (Dell Inc., Round Rock, TX, USA) personal computer was used.

CUTANEOUS TISSUE DISCLOSURE

In OsiriX, cutaneous tissue is not included in the SAT measurement, because this is not automatically selected using the "Grow Region (2D/3D Segmentation)" tool. SliceOmatic also allows to exclude encompassed skin. However, not all software programs allow to reliably exclude cutaneous tissue from SAT as a consequence of their measurement method: the delineation of tissue of interest using inner and outer contours. Consequently, to ensure highly comparable measurements in three rather than

two software programs, cutaneous tissue was included in the SAT measurements with SliceOmatic. A comparison of SAT measurements using SliceOmatic with and without the inclusion of cutaneous tissue resulted in a median difference of 2.3% (interquartile range 0.8-3.8) and was considered acceptable.

STATISTICAL ANALYSIS

Continuous data are presented as mean with the standard error of the measurement (SEM). Normality was tested using the Shapiro-Wilk test. Differences between the different software packages and within and between observers were compared using the paired samples t-test for normally distributed data and the Wilcoxon signed rank test for data that was not normally distributed. The inter-software and inter- and intra-observer agreement for the cross-sectional skeletal muscle, visceral adipose tissue and subcutaneous adipose tissue measurements were calculated using intra-class correlation coefficients (ICC) with 95% confidence intervals (CI) using a two-way mixed single measures model with absolute agreement. For the inter-observer correlation, the reading of observer B was compared with reading 1 of observer A. Ninety-five per cent limits of agreement were determined to investigate the agreement between the various software programs, according to the method described by Bland and Altman.³⁸ The presence of proportional systematic bias was determined by linear regression analysis of the difference and mean of two measurements. The inter-software and inter- and intra-observer agreement of the assessment of sarcopenia and visceral obesity were analyzed using Cohen's κ coefficients. The ICC and Cohen's κ coefficients were interpreted as poor (0.00-0.49), fair to good (0.50-0.74) and excellent (0.75-1.00), as proposed by Shrout and Fleiss.³⁹ The Jaccard similarity coefficient, ranging from 0-1, was used to compare the similarity and diversity of measurements by dividing the area of the intersection by the size of the union of two measurements.⁴⁰ An overlay of two measurements was created and the Jaccard similarity coefficient was calculated using MeVisLab version 2.7.1 (MeVis Medical Solutions AG, Bremen, Germany). A Jaccard similarity coefficient of 1 represents perfect overlap of two samples, whereas 0 represents no overlap. Two-tailed p-values <0.05 were considered statistically significant. All statistical analyses were performed using IBM SPSS Statistics for Windows version 21.0 (IBM Corp. Armonk, NY, USA).

RESULTS

PATIENTS

The study population consisted of 29 males (58%) and 21 females (42%) with a median age of 62 years (range 33 - 81) and a median body mass index (BMI) of 24.6 kg/m² (range 16.5 - 38.8). Ten patients had stage II (20.0%), 24 stage III (48.0%) and 15 stage IV (30.0%) rectal cancer. Tumor stage was unknown for one patient. The mean CSMA, VAT, and SAT for all measurements are provided in table 1 and table 2.

Table 1. Mean cross-sectional skeletal muscle and visceral and subcutaneous adipose tissue area (cm²) measurements and intra-observer agreement indices (i.e. ICC) using FatSeg, OsiriX, ImageJ, and SliceOmatic of observer A.

Observer A							
Software	Reading 1 (cm ²)	SEM	Reading 2 (cm ²)	SEM	Mean difference (95% CI)	p-value	ICC (95% CI)
Skeletal muscle area							
FatSeg	139.0	5.2	139.3	5.2	-0.3 (-0.6; 0.0)	0.072*	0.999 (0.999-1.000)
OsiriX	139.4	5.2	138.7	5.1	0.7 (0.4; 1.0)	<0.001*	0.999 (0.999-1.000)
ImageJ	139.0	5.2	139.3	5.1	-0.3 (-0.6; -0.1)	0.013*	1.000 (0.999-1.000)
SliceOmatic	138.7	5.2	138.6	5.2	0.1 (-0.2; 0.4)	0.441*	1.000 (0.999-1.000)
Visceral adipose tissue area							
FatSeg	149.9	13.1	149.2	13.1	0.7 (0.3; 1.0)	<0.001 [#]	1.000 (1.000-1.000)
OsiriX	147.6	13.0	147.3	13.0	0.3 (-0.3; 0.8)	0.220 [#]	1.000 (1.000-1.000)
ImageJ	148.6	13.0	150.8	12.8	-2.2 (-7.5; 3.1)	0.003 [#]	0.979 (0.964-0.988)
SliceOmatic	147.1	13.0	146.6	13.0	0.5 (0.2; 0.9)	0.004 [#]	1.000 (1.000-1.000)
Subcutaneous adipose tissue area							
FatSeg	158.9	11.2	158.9	11.2	0.1 (-0.2; 0.3)	0.359 [#]	1.000 (1.000-1.000)
OsiriX	155.9	11.2	155.7	11.3	0.2 (-0.1; 0.4)	0.137 [#]	1.000 (1.000-1.000)
ImageJ	158.9	11.2	159.1	11.3	-0.2 (-0.5; 0.0)	0.201 [#]	1.000 (1.000-1.000)
SliceOmatic	158.8	11.3	158.8	11.3	0.0 (-0.3; 0.2)	0.448 [#]	1.000 (1.000-1.000)

SEM, standard error of measurement; ICC, inter- and intra-class correlation coefficients; CI confidence intervals. Calculated with *paired-samples t-test and [#]Wilcoxon signed rank test

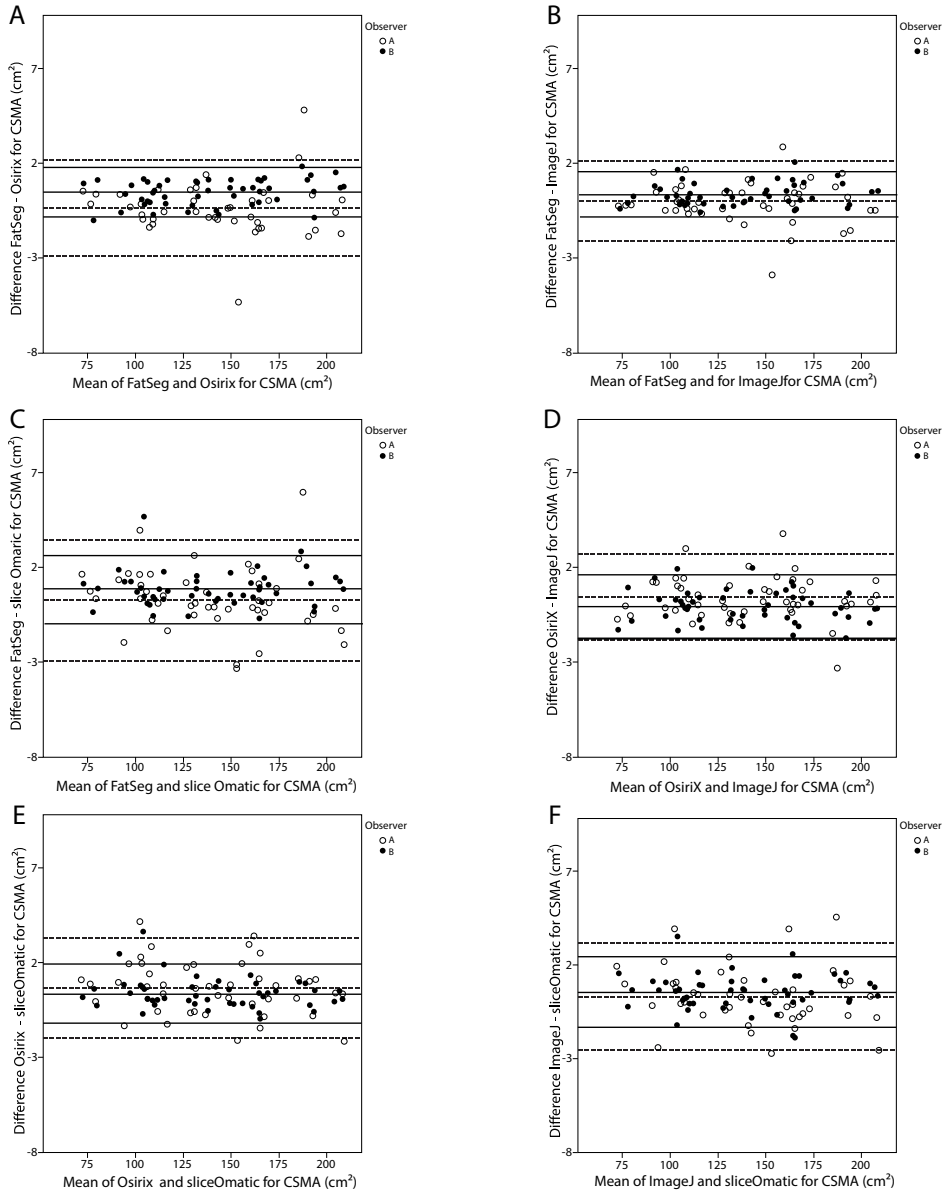
Table 2. Mean cross-sectional skeletal muscle and visceral and subcutaneous adipose tissue area (cm²) measurements and inter-observer agreement indices (i.e. ICC) using FatSeg, OsiriX, ImageJ, and SliceOmatic of reading 1 of observer A and observer B.

Observer A			Observer B				
Software	Reading 1	SEM	Reading 1	SEM	Mean difference (95% CI)	p-value	ICC (95% CI)
	(cm ²)		(cm ²)				
Skeletal muscle area							
FatSeg	139.0	5.2	140.1	5.2	-1.1 (-1.4; -0.8)	<0.001*	0.999 (0.989-1.000)
OsiriX	139.4	5.2	139.7	5.1	-0.3 (-0.5; 0.0)	0.047*	1.000 (0.999-1.000)
ImageJ	139.0	5.2	139.8	5.2	-0.8 (-1.0; -0.5)	<0.001*	0.999 (0.997-1.000)
SliceOmatic	138.7	5.2	139.3	5.2	-0.6 (-0.9; -0.2)	0.006*	0.999 (0.998-1.000)
Visceral adipose tissue area							
FatSeg	149.9	13.1	148.7	13.1	1.2 (0.8; 1.5)	<0.001*	1.000 (0.999-1.000)
OsiriX	147.6	13.0	147.3	13.0	0.3 (-0.3; 0.8)	0.133*	1.000 (1.000-1.000)
ImageJ	148.6	13.0	148.4	13.1	0.3 (-0.1; 0.6)	0.015*	1.000 (1.000-1.000)
SliceOmatic	147.1	13.0	146.9	13.0	0.2 (-0.1; 0.5)	0.412*	1.000 (1.000-1.000)
Subcutaneous adipose tissue area							
FatSeg	158.9	11.2	159.2	11.3	-0.3 (-0.5; -0.1)	0.005*	1.000 (1.000-1.000)
OsiriX	155.9	11.2	155.8	11.3	0.1 (-0.3; 0.5)	0.918*	1.000 (1.000-1.000)
ImageJ	158.9	11.2	158.7	11.2	0.2 (-0.2; 0.5)	0.306*	1.000 (1.000-1.000)
SliceOmatic	158.8	11.3	158.5	11.2	0.2 (0.0; 0.5)	0.183*	1.000 (1.000-1.000)

SEM, standard error of measurement; ICC, inter- and intra-class correlation coefficients; CI confidence intervals. Calculated with *paired-samples t-test and #Wilcoxon signed rank test

INTER-SOFTWARE AGREEMENT

The inter-software ICCs were excellent (≥ 0.999) for the CSMA, VAT, and SAT for all software programs with p-values < 0.001 (table 3). Figure 1 and supplementary figures 1 and 2 show the Bland-Altman 95% limits of agreement plots, with the mean difference and 95% limits of agreement for the CSMA, VAT, and SAT for both observers. All plots show a good agreement between the various software programs. Small limits of agreement are observed in the CSMA measurements, whereas these limits of agreement are greater for the VAT and SAT measurements. Proportional systematic bias was observed between FatSeg and OsiriX for CSMA ($p=0.049$) for observer B (figure 1a) and between FatSeg and SliceOmatic for SAT ($p=0.031$) for observer A (supplementary figure 2c).

Figure 1. Bland-Altman 95% limits of agreement plots for the agreement between the various software programs.

The dotted lines are the mean of the difference and the 95% limits of agreement (± 2 SD) between the CSMA (cm²) of reading 1 of observer A and the solid lines of reading 1 of observer B. (A) There was no proportional systematic bias for observer A ($p=0.908$), whereas there was significant bias for observer B ($p=0.049$). (B) There was no proportional systematic bias for any observer ($p=0.738$ and $p=0.359$). (C) There was no proportional systematic bias for any observer ($p=0.238$ and $p=0.704$). (D) There was no proportional systematic bias for any observer ($p=0.857$ and $p=0.363$). (E) There was no proportional systematic bias for any observer ($p=0.185$ and $p=0.228$). (F) There was no proportional systematic bias for any observer ($p=0.289$ and $p=0.843$).

Table 3. Mean cross-sectional skeletal muscle and visceral and subcutaneous adipose tissue area (cm²) measurements and inter-software agreement indices (i.e. ICC) using FatSeg, OsiriX, ImageJ, and SliceOmatic of reading 1 of observer B.

Software	Mean difference (cm ²) (95% CI)	SEM	p-value	ICC (95% CI)
Skeletal muscle area				
FatSeg – OsiriX	-0.4 (-0.8; 0.0)	0.184	0.047	0.999 (0.999-1.000)
FatSeg – ImageJ	0.0 (-0.3; 0.3)	0.151	0.992	1.000 (0.999-1.000)
FatSeg – SliceOmatic	0.3 (-0.2; 0.8)	0.230	0.207	0.999 (0.998-0.999)
OsiriX – ImageJ	0.4 (0.1; 0.7)	0.161	0.023	0.999 (0.999-1.000)
OsiriX – SliceOmatic	0.7 (0.3; 1.1)	0.189	0.001	0.999 (0.998-1.000)
ImageJ – SliceOmatic	0.3 (-0.1; 0.7)	0.208	0.165	0.999 (0.999-1.000)
Visceral adipose tissue area				
FatSeg – OsiriX	2.3 (1.6; 2.9)	0.326	<0.001	0.999 (0.995-1.000)
FatSeg – ImageJ	1.2 (0.8; 1.7)	0.203	<0.001	1.000 (0.999-1.000)
FatSeg – SliceOmatic	2.8 (2.3; 3.2)	0.238	<0.001	0.999 (0.971-1.000)
OsiriX – ImageJ	-1.0 (-1.5; -0.6)	0.237	<0.001	1.000 (0.999-1.000)
OsiriX – SliceOmatic	0.5 (0.0; 0.9)	0.229	0.044	1.000 (1.000-1.000)
ImageJ – SliceOmatic	1.5 (1.2; 1.8)	0.158	<0.001	1.000 (0.995-1.000)
Subcutaneous adipose tissue area				
FatSeg – OsiriX	3.0 (2.5; 3.6)	0.256	<0.001	0.999 (0.948-1.000)
FatSeg – ImageJ	0.1 (-0.3; 0.4)	0.180	0.698	1.000 (1.000-1.000)
FatSeg – SliceOmatic	0.2 (-0.1; 0.5)	0.141	0.240	1.000 (1.000-1.000)
OsiriX – ImageJ	-3.0 (-3.5; -2.5)	0.260	<0.001	0.999 (0.956-1.000)
OsiriX – SliceOmatic	-2.9 (-3.3; -2.5)	0.211	<0.001	0.999 (0.932-1.000)
ImageJ – SliceOmatic	0.1 (-0.2; 0.4)	0.139	0.485	1.000 (1.000-1.000)

The results of observer A are comparable with those of observer B. SEM, standard error of measurement; ICC, inter- and intra-class correlation coefficients; CI confidence intervals.

Furthermore, proportional systematic bias was frequently observed between programs for VAT measurements (supplementary figure 1). Comparable results were achieved when non a priori selected slices of observer B were analyzed (data not shown). The mean Jaccard similarity coefficients for the inter-software comparisons are summarized in table 4 and depicted in figure 2.

Table 4. Mean Jaccard indices for inter-software comparisons of reading 1 of observer A and reading 1 of observer B.

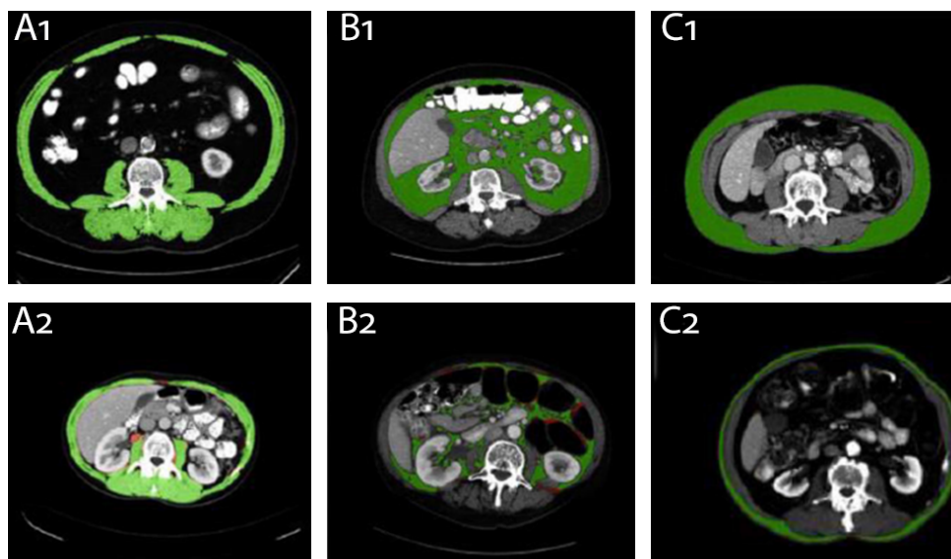
Software	Mean Jaccard similarity coefficients					
	Observer A (reading 1) (range)			Observer B (reading 1) (range)		
	CSMA	VAT	SAT	CSMA	VAT	SAT
FatSeg – OsiriX	0.978 (0.940-0.997)	0.964 (0.825-0.996)	0.965 (0.928-0.976)	0.983 (0.948-0.997)	0.973 (0.886-0.997)	0.965 (0.926-0.998)
FatSeg – ImageJ	0.982 (0.935-0.996)	0.981 (0.912-0.999)	0.988 (0.900-0.999)	0.987 (0.959-0.998)	0.981 (0.903-0.998)	0.990 (0.968-0.998)
FatSeg – SliceOmatic	0.978 (0.937-0.996)	0.970 (0.908-0.997)	0.989 (0.964-0.998)	0.981 (0.927-0.996)	0.972 (0.860-0.997)	0.987 (0.960-0.998)
OsiriX – ImageJ	0.982 (0.935-0.996)	0.968 (0.856-0.995)	0.964 (0.900-0.998)	0.983 (0.948-0.997)	0.976 (0.891-0.998)	0.966 (0.927-0.997)
OsiriX – SliceOmatic	0.979 (0.941-0.997)	0.974 (0.876-0.997)	0.988 (0.900-0.998)	0.985 (0.944-0.997)	0.973 (0.884-0.998)	0.967 (0.923-0.998)
ImageJ – SliceOmatic	0.979 (0.950-0.994)	0.967 (0.809-0.995)	0.966 (0.928-0.997)	0.983 (0.932-0.997)	0.975 (0.855-0.998)	0.988 (0.965-0.999)

CSMA, cross-sectional muscle area; VAT, visceral adipose tissue area, SAT, subcutaneous adipose tissue area.

INTRA-OBSERVER AND INTER-OBSERVER AGREEMENT

The ICCs for the intra-observer agreement of observer A were all 0.979 or higher for the different software programs, approaching perfect correlation (table 1). The ICCs for the inter-observer agreement also approached perfect agreement (all ≥ 0.999 , see table 2). The mean CSMA was significantly lower for observer A compared with observer B for all software programs. A significantly higher mean VAT of observer A was found using FatSeg (149.9 cm² versus 148.7 cm², $p < 0.001$) and ImageJ (148.6 cm² versus 148.4 cm², $p = 0.015$) compared with observer B, whereas the mean VAT of both observers did not significantly differ for OsiriX ($p = 0.133$) and SliceOmatic ($p = 0.412$). The mean SAT did significantly differ for FatSeg (158.9 cm² versus 159.2 cm², $p = 0.005$) between the observers. Comparable results were observed when non a priori selected slices of reading 2 of observer B were used for analyses (data not shown). The mean Jaccard similarity coefficients for the inter- and intra-observer comparisons are summarized in table 5 and depicted in supplementary figures 3 and 4. All remaining worst Jaccard similarity coefficients are provided in supplementary figure 5.

Figure 2. CSMA, VAT and SAT assessment.



(A) The CSMA measured with FatSeg and ImageJ (1) and FatSeg and SliceOmatic (2), resulting in Jaccard similarity coefficients of 0.998 and 0.927, respectively. (B) The VAT measured with ImageJ (1) and SliceOmatic (2), resulting in Jaccard similarity coefficients of 0.998 and 0.855, respectively. (C) The SAT measured with ImageJ and SliceOmatic (1) and OsiriX and SliceOmatic (2), resulting in Jaccard similarity coefficients of 0.999 and 0.923, respectively.

THE CLASSIFICATION OF SARCOPENIA AND VISCERAL OBESITY

The inter-software Cohen's κ 's of the first reading of observer A for the classification of sarcopenia were 0.96 (between FatSeg and Osirix, OsiriX and ImageJ, and ImageJ and SliceOmatic), 0.92 (between FatSeg and ImageJ, and Osirix and SliceOmatic), and 0.88 (between FatSeg and SliceOmatic). No inter-software differences were found in the classification of patients with and without sarcopenia for observer B. According to the cut-off values used, all software programs diagnosed sarcopenia in 16 men (55.2%) and 8 women (38.1%). This resulted in a Cohen's κ of 1.00 for all comparisons between software programs ($p < 0.001$).

The Cohen's κ for the intra-observer agreement of sarcopenia assessment of observer A was 0.96 using FatSeg and ImageJ and 1.00 for OsiriX and SliceOmatic (all $p < 0.001$).

The Cohen's κ for the inter-observer agreement (reading 1 of observer A versus observer B) of sarcopenia assessment was 0.92 for SliceOmatic, 0.96 for FatSeg and ImageJ, and 1.00 for Osirix (all $p < 0.001$).

THE CLASSIFICATION OF VISCERAL OBESITY

In total, 17 men (58.6%) and 9 women (42.9%) were classified as visceral obese using FatSeg, OsiriX, ImageJ, and SliceOmatic in all readings. This resulted in a Cohen’s κ of 1.00 for all comparisons (all $p < 0.001$).

Table 5. Mean Jaccard indices for inter-observer (reading 1 of observer A versus reading 1 of observer B) and intra-observer comparisons (reading 1 versus reading 2 of observer A).

Mean Jaccard similarity coefficients						
Software	Inter-observer (range)			Intra-observer (range)		
	CSMA	VAT	SAT	CSMA	VAT	SAT
FatSeg	0.981 (0.949-0.997)	0.976 (0.908-0.998)	0.991 (0.969-0.999)	0.982 (0.961-1.000)	0.984 (0.916-0.999)	0.991 (0.956-1.000)
OsiriX	0.985 (0.960-0.997)	0.973 (0.835-0.997)	0.989 (0.960-1.000)	0.984 (0.953-0.997)	0.975 (0.838-0.998)	0.990 (0.967-1.000)
ImageJ	0.982 (0.931-0.993)	0.980 (0.905-0.997)	0.988 (0.899-0.999)	0.985 (0.948-1.000)	0.982 (0.891-0.998)	0.990 (0.900-1.000)
SliceOmatic	0.981 (0.939-0.997)	0.976 (0.876-0.996)	0.989 (0.959-0.999)	0.986 (0.961-0.997)	0.980 (0.901-0.998)	0.993 (0.967-1.000)

CSMA, cross-sectional muscle area; VAT, visceral adipose tissue area, SAT, subcutaneous adipose tissue area.



Supplementary Figures. Web hyperlink for supplementary figures 1a-1f, 2a-2f, 3a-3c, 4a-4c, 5a-5c.

Supplementary figures 1a - 1f, 2a - 2f, 3a - 3c, 4a - 4c and 5a-5c may be found under the Supporting Information section of the publication in Journal of Cachexia, Sarcopenia and Muscle, doi: <http://doi.org/10.1002/jcsm.12158>.

DISCUSSION

This study shows that the inter-software agreement was excellent for all software programs. Furthermore, the inter-observer and intra-observer agreements were excellent for four distinct software programs to assess CSMA, VAT, and SAT on abdominal CT scans with high Jaccard similarity coefficients.

Body composition analyses using abdominal CT scans are increasingly being performed. In multiple surgical populations, such as vascular²⁷, gastrointestinal^{7, 33}, urological⁴¹⁻⁴³, gynecological⁴⁴ and transplantation surgery²⁹, the association between low skeletal muscle mass and an increased risk of postoperative complications, recurrent disease, or impaired survival has been shown. Low skeletal muscle mass is also related to discharge destination in elderly trauma patients⁴⁵, associated with an increased risk of dose-limiting chemotherapy toxicity^{17, 24, 25, 46} and with morbidity and mortality in various oncologic populations, such as lung cancer and melanoma patients.^{47, 48} Furthermore, CT-assessed visceral obesity is associated with worse short- and long-term outcome in distinct patient populations undergoing surgery.³⁰ Various software programs have been used to measure body composition in these studies. The current study shows that the results of these studies can reliably be compared. Based on our findings it is likely that this is also true for other software programs which similarly compute skeletal muscle area by quantifying selected voxels within preset HU intensity thresholds (e.g., studies that used software programs designed in MATLAB [MathWorks, Natick, MA, USA]⁴⁹).

Software programs for various body composition measurements on CT images, such as adipose tissue surface area, skeletal muscle tissue surface area and liver volumetric measurements, have been compared in multiple previous studies, demonstrating high levels of agreement.⁵⁰⁻⁵³ Excellent agreement levels between SliceOmatic and ImageJ⁵⁴, as well as between observers using SliceOmatic⁵⁵ for CSMA measurements have previously been reported. Furthermore, excellent agreement levels between OsiriX and ImageJ have been observed for paraspinal muscle measurements on magnetic resonance images.⁵⁶ Nevertheless, this is the first study to compare multiple software programs for the measurement of CSMA, VAT, and SAT, showing that previous studies investigating the association between skeletal muscle mass on the one side and visceral or subcutaneous adipose tissue on the other side, and patient outcomes can reliably be compared.

The skeletal muscle area (cm^2) measured at a single cross-sectional CT image at the level of the third lumbar vertebra (L3) is linearly related to total body skeletal muscle mass⁵⁷ and is therefore corrected for height squared (m^2), as is conventional for body composition measures. This results in the L3 muscle index (cm^2/m^2).¹⁸ Another frequently used method is measuring the total psoas area (TPA).²⁷ The principle of TPA measurements is identical to L3 muscle area measurements, using single cross-sectional CT images. Therefore, the findings of this study may be extrapolated to TPA measurements as well. Nevertheless, this should be confirmed in a future study.

Significant differences were observed between the mean skeletal muscle areas within and between observers. However, these mean differences are small and consequently not clinically relevant. Differences in individual measurements resulted, for instance, from the incorrect annotation of skeletal muscle tissue (see figure 2a2 for an example of an intra-observer difference). However, we decided not to correct measurements in retrospect to show inter- and intra-observer agreements. In our opinion, this study reflects daily practice, with observers who have excellent (radiological) anatomical knowledge performing body composition measurements. Regardless of these human errors and some inter- and intra-observer differences, high comparability between software programs was observed.

Significant differences between VAT measurements were also observed with greater mean differences between software. This could be due to the greater complexity of the measurement technique, as intraluminal content (i.e. fat in stool) needs to be manually erased. The greatest significant mean differences in SAT could partly be explained by the fact that in OsiriX the cutaneous adipose tissue is not included in the SAT, in contrast to the other software programs. Furthermore, every tissue of interest needs to be manually selected in OsiriX, in contrast to the other programs in which methods of delineating or a painting brush can be used to select regions of interest.

Significant differences in the mean VAT (FatSeg and ImageJ) within observer A and in the mean VAT (FatSeg OsiriX, and SliceOmatic) and SAT (FatSeg, OsiriX, and ImageJ) between observers (reading 2 of observer A with non a priori selected slices; data not shown) were found, whereas the CSMA did not significantly differ. One explanation for the differences in VAT and SAT could be the random slice selection. After all, the distribution of the intra-abdominal content (e.g., bowel) can greatly differ between slices. Consequently, single slice measurement of visceral adipose tissue would not be clinically applicable and should be reserved for clinical research of patient cohorts rather than individual patients. For SAT, the variance of subcutaneous adipose tissue

distribution could have led to the observed differences. Nevertheless, all differences are relatively small and could therefore be considered as not clinically relevant. The inter-observer agreement levels for OsiriX and SliceOmatic are in line with previous studies that showed a strong and significant correlation between CSMA measurements of two observers.^{7, 55} The inter-observer agreements for FatSeg and ImageJ have never been reported before, whereas a high agreement for the classification of patients with sarcopenia, as expressed in Cohen's κ , has previously been reported.⁷

Several limitations apply to the current study and the used software programs. First, both observers in the current study were experienced in quantifying skeletal muscle mass using these software programs prior to conducting this study. Therefore, the agreement rates that were obtained may not apply to less experienced users. Second, OsiriX is only compatible with Macintosh, which is less commonly used in clinical practice. Furthermore, FatSeg is not freely downloadable as it is an in-house developed software program that has not been made publically available, in contrast to OsiriX and ImageJ. A license is required for the use of SliceOmatic. Third, this study could only assess the agreement of the measurement with different software programs on the same data. Intra- and inter-scanner reproducibility of the measurement could not be assessed with the current study design. Lastly, previous studies reported an approximate time of eight minutes to quantify skeletal muscle, visceral and subcutaneous adipose tissue in liver transplant patients using SliceOmatic.⁵⁵ Although some differences in user-friendliness were observed while performing the measurements, these were not objectively observed and scored in the current study. Consequently, these are not described.

In conclusion, this study showed that four different software programs have an excellent agreement to measure VAT and SAT, and CSMA in particular on abdominal CT scans, which enables reliable comparison of results of studies that use these different software programs. Multiple slice analysis is preferred for VAT and SAT measurements.

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CHAPTER 5

BODY COMPOSITION AND OUTCOME IN PATIENTS UNDERGOING RESECTION OF COLORECTAL LIVER METASTASES

ABSTRACT

BACKGROUND

Recent evidence suggests that a depletion of skeletal muscle mass (sarcopenia) and an increased amount of intra-abdominal fat (central obesity) influence cancer statistics. This study investigated the impact of sarcopenia and central obesity on survival in patients undergoing liver resection for colorectal liver metastases (CLM).

METHODS

Between 2001 and 2009, patients who underwent hepatic resection for CLM in one center and had assessable peri-operative CT-scans, had their diagnostic imaging retrospectively analyzed. Total cross-sectional areas of skeletal muscle and intra-abdominal fat and their influence on outcome were analyzed.

RESULTS

Of the 196 patients who were included in this study 38 patients (19.4%) were classified as sarcopenic. Five year disease-free (28% versus 15%; $p = 0.001$) and overall (50% versus 20%; $p < 0.001$) survival rates were lower for sarcopenic patients at a median follow-up of 29 months (range, 1–97). Sarcopenia was an independent predictor of worse recurrence-free (HR 1.88; $p = 0.002$) and overall survival (HR 2.53; $p < 0.001$). Central obesity was associated with an increased risk of recurrence in men (HR 1.83; $p = 0.032$), but not in women ($p = 0.712$).

CONCLUSIONS

Sarcopenia negatively impacts on cancer outcomes following CLM resection.

INTRODUCTION

Complete resection or ablation of colorectal liver metastases (CLM) offers the best option for definitive cure.¹⁻⁴ Historically, clinicopathological factors have been incorporated into scores to stratify patients according to predicted outcomes. These risk scores do not consider whether a poor general condition of the patient (either pre-existent or cancer related) may predict worse outcomes. Current measurements of performance and overall condition such as the American Society of Anesthesiologists (ASA) score, weight loss and body mass index (BMI) are inadequate.⁵⁻⁶

Computed tomography (CT) based measurements of body composition such as an increased amount of intra-abdominal fat (central obesity) and depletion of skeletal muscle mass (sarcopenia) can be predictors of cancer survival.⁷⁻¹¹ Until now, no such data are available for patients undergoing liver resection for CLM. Therefore, the aim of the current study was to investigate the influence of the quantity of subcutaneous and intra-abdominal fat as well as the quantity of skeletal muscle mass on survival following CLM resection.

METHODS

PATIENTS

Within the Erasmus MC, a digital database was prospectively maintained including all patients who underwent hepatic surgery for CLM containing data with regard to the primary tumor, hepatic metastases, surgical and chemotherapeutic treatment, recurrence and survival. Only patients with peri-operative abdominal CT-scans available for review (no more than two months prior to surgery or one month after surgery) were included. Patients with only peri-operative MRI-scans available were excluded.

PRE-OPERATIVE WORK-UP AND CHEMOTHERAPY

All patients in the current study underwent pre-operative CT or MRI scanning in the Erasmus MC or in the referring hospital and were presented to a multidisciplinary liver board, including a hepatobiliary surgeon, medical oncologist, hepatologist, pathologist, radiologist and radiation oncologist. Indications for neoadjuvant chemotherapy included a marginal resectable status, bilobar disease or >3 metastases. Response to chemotherapy was assessed by CT or MRI scanning after 2 or 3 cycles. Administration of chemotherapy was stopped/ended in case of partial response or when initially unresectable metastases became resectable, and no more than 6 cycles of chemotherapy were administered. Resectability was defined as the ability to leave at least two consecutive liver segments in place with intact arterial, venous and biliary in- and outflow representing at least 25% of the total liver volume. RFA was applied for those lesions that could not be resected due to their location or spread.

POST-OPERATIVE FOLLOW-UP

Follow-up for disease recurrence was performed routinely every three months in the first year after surgery and every 6 months for the five years thereafter. Follow-up consisted of serum CEA levels and thoracic and abdominal CT-scans; additional diagnostics, e.g. colonoscopy was performed 2-3 year after resection of the primary tumor or on indication. No adjuvant chemotherapy was administered after liver resection.

ASSESSMENT OF ADIPOSE AND SKELETAL MUSCLE TISSUE

The quantity of intra-abdominal fat and skeletal muscle mass was determined using standard diagnostic CT-scans. For this purpose, a newly developed software application was used based on the MeVisLab (MeVis Medical Solutions AG, Bremen, Germany) software package. Cross-sectional areas (cm^2) of different tissue compartments were measured at the caudal end of the third lumbar vertebra based on their specific differences in attenuation (Hounsfield Units; HU). This was done by roughly manually outlining these compartments and segmenting the tissue of interest based on HU thresholds (-30 HU to + 150 HU for skeletal muscle and -190 HU to -30 HU for adipose tissue¹²⁻¹³) (figure 1). The total cross sectional area of the segmented tissue was then automatically calculated. Intra-colonic content initially marked as adipose tissue was manually corrected. The obtained body-mass indices were then normalized for stature (cm^2/m^2).



Figure 1. Assessment of adipose and skeletal muscle tissue.

A CT-Image showing highlighted areas of subcutaneous (green) and intra-abdominal (yellow) fat and skeletal muscle mass (red).

STATISTICAL ANALYSIS

Continuous data are presented as mean \pm standard deviation or median (range) as appropriate. Categorical data are presented as proportions. Differences between groups were investigated using the student t-test for continuous variables and the χ^2 test for categorical variables. To investigate the cut-off values for the cross sectional areas of skeletal muscle mass and adipose tissue at which the difference in survival was most significant, sex-specific cut-off values were determined using optimum stratification to find the most significant p-value by use of log-rank statistics. This method has been

previously described in literature as a method to solve the threshold value of the continuous covariate at which, based on log-rank statistics, patients of two categories (e.g. sarcopenic and non-sarcopenic) were best separated with respect to time to event outcome (e.g. mortality).⁸ Overall and disease-free survival were calculated and compared by the non-parametric Kaplan-Meier method and log-rank test. To investigate the correlation between sarcopenia, central obesity and survival, univariable and multivariable Cox regression analyses were performed and hazard ratio's (HR) and 95% confidence intervals were calculated. The following variables were included in the univariable analysis: Sarcopenia, age, gender, diabetes, BMI, ASA-score, primary tumor localization, synchronous staging, tumor number, tumor size, CEA, neo-adjuvant systemic therapy, and RFA. All variables were checked for interaction and confounding and were included in the multivariable model when significant. Introducing a time varying predictor variable in the model as well as calculating Schoefeld residuals indicated that the assumption of proportionality was met for this model. All statistical analyses were performed using SPSS 17.0 (SPSS, Inc, Chicago, IL), and Stata 11 (Statacorp, collegetown, TX). A p-value < 0.05 was considered to be statistically significant.

RESULTS

PATIENT CHARACTERISTICS

One-hundred and ninety-six patients qualified for the current study with a median follow-up of 29 months (range, 1–97). The clinicopathological features of these patients can be found in table 1. All patients were treated between 2001 and 2009.

Table 1. Demographic and clinicopathological characteristics of the 196 patients included in the study.

Sex (male to female ratio)		120 (61.2) : 76 (38.8)
Median Age, Years (range)		64.5 (31 – 86)
Primary tumor		
Location	Colon	116 (59.2)
	Rectum	80 (40.8)
T-Stage [†]	T1	0 (0)
	T2	25 (13.2)
	T3	148 (78.3)
	T4	16 (8.5)
	No data	7
N-Stage [†]	N0	80 (41.9)
	N1	77 (40.3)
	N2	34 (17.8)
	No data	5
Metastases	Synchronous	93 (47.4)
	Metachronous	103 (52.6)
Disease-free interval	< 12 months	129 (65.8)
	≥ 12 months	67 (34.2)
No. of metastases	≤ 3 tumors	147 (75.0)
	> 3 tumors	49 (25.0)
Maximum tumor size	< 5 cm	144 (73.5)
	≥ 5 cm	52 (26.5)
ASA physical status score	ASA 1	46 (23.5)
	ASA 2	122 (62.2)
	ASA 3	28 (14.3)

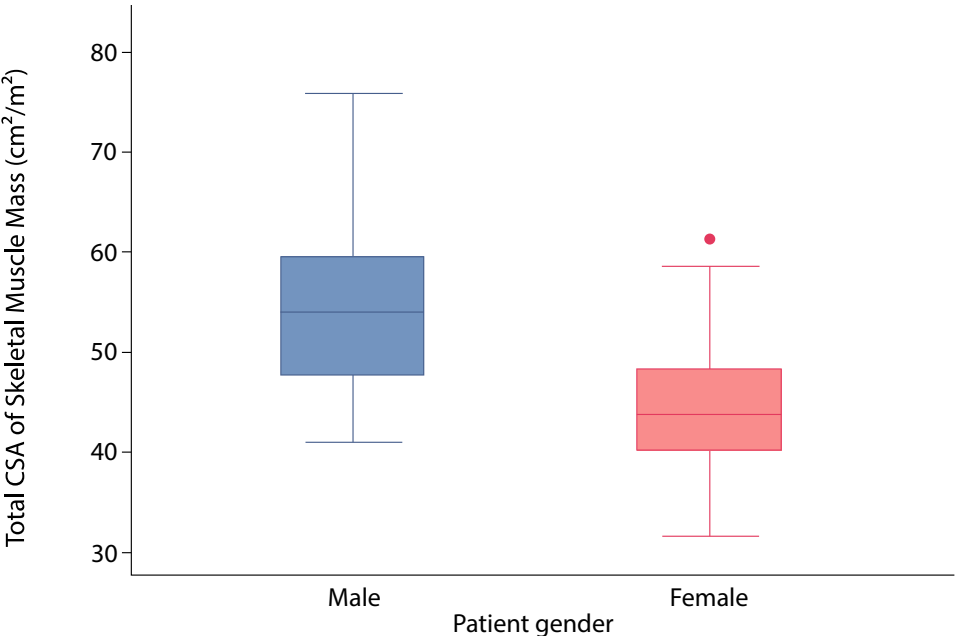
Values in parentheses are percentages unless indicated otherwise. † Missing data for some patients.
Abbreviations: ASA American Society of Anesthesiologists.

The systemic therapy (administered to 91 patients) were mostly oxaliplatin-containing combination regimens (86%). A major hepatic resection (three segments or more) was performed in 63 (32%) patients, whereas a segmentectomy of two or less segments and non-anatomic resections were in 133 (68%) of the patients. Radiofrequency Ablation (RFA) was applied in 39 (20%) of the patients, of which 5 received open RFA alone.

MEASUREMENTS OF BODY COMPOSITION

A wide range of body compositions was found in the analysis of the CT-images (figure 2). Intra-abdominal fat ranged from 7.0 cm²/m² to 171.6 cm²/m² with a mean of 58.8 cm²/m². For skeletal muscle mass the range was found to be smaller, with a minimum of 31.5 cm²/m² and a maximum of 75.9 cm²/m², with a mean of 50.4 cm²/m² for skeletal muscle mass.

Figure 2. Box-and-whisker plot showing the correlation between gender and total cross-sectional area (CSA) of skeletal muscle mass.



Male patients had a median skeletal muscle mass of 54.02cm²/m² (40.94 – 75.91), whereas female patients were found to have a significantly lower median skeletal muscle mass of 43.79cm²/m² (31.50 – 61.32) ($p < 0.001$).

SKELETAL MUSCLE MASS AND SURVIVAL

Sex-specific cut-off values for skeletal muscle mass associated with overall mortality obtained by means of optimum stratification were 41.10 cm²/m² for female patients and 43.75 cm²/m² for male patients. By these definitions, 38 patients (19%) were found to be sarcopenic. Demographic and clinical characteristics are compared between these groups in table 2. Sarcopenia significantly correlated with the female gender, low BMI and a lower quantity of intra-abdominal fat (table 2). No difference in subcutaneous fat was found between the sarcopenic and non-sarcopenic population. No statistical differences were found between the two groups with regard to known risk factors and pre-operative systemic therapy.

In total, 126 (64%) patients had a disease recurrence after a median follow-up of 29 months (range, 1–96). The median disease-free survival was 11.8 months with 1-, 3- and 5-year disease-free survival rates of 49%, 33% and 26% respectively. Eighty-four (43%) patients died during follow-up, with a median overall survival of 50.3 months and corresponding 1-, 3- and 5- year survival rates of 94%, 58% and 43% respectively.

Patients who were found to be sarcopenic had a significantly shorter disease-free survival when compared to patients without sarcopenia. In sarcopenic patients the median disease-free survival was 8.7 months and corresponding 1, 3 and 5-year disease-free survival rates were 31%, 20% and 15% respectively. For non-sarcopenic patients the median disease-free survival was 15.1 months and corresponding 1, 3 and 5-year disease-free survival rates were 54%, 36% and 28% respectively ($p=0.002$)(figure 3). Similarly, overall survival was worse in patients with sarcopenia, when compared to non-sarcopenic patients. The median survival was 23.8 months for patients with sarcopenia with corresponding 1, 3 and 5-year survival rates of 84%, 34% and 20% respectively. For patients without sarcopenia the median survival was 59.8 months with corresponding 1, 3, and 5-year survival rates of 96%, 65% and 50% respectively ($p<0.001$) (figure 4). Moreover, when adjusting for well known risk-factors, sarcopenia was found to be an independent predictor of worse disease-free (HR 1.88 95% CI 1.25 – 2.82; $p = 0.002$) and overall survival (HR 2.53 95% CI 1.60 – 4.01; $p < 0.001$)(table 3 and 4).

To investigate whether the impact of sarcopenia might be different for patients who did and who did not receive pre-operative chemotherapy, recipients of chemotherapy were compared to patients who did not receive systemic therapy. In both groups, sarcopenia was found to have a negative impact on overall survival (HR 2.56 95% CI 1.27 - 5.18; $p = 0.009$ and HR 2.44 95% CI 1.32 - 4.48; $p = 0.004$ respectively). Also, sarcopenia negatively impacted on disease-free survival in both groups, although this did not reach statistical significance in the chemotherapy group, likely as a result of smaller numbers (HR 1.72 95% CI 0.96 - 3.08; $p = 0.070$ and HR 1.95 CI 1.11 - 3.43; $p = 0.021$ respectively).

CENTRAL OBESITY AND SURVIVAL

Optimum stratification did not detect any correlation between central obesity and survival. Also, no statistical significant correlation between intra-abdominal fat, disease-free and overall survival was found when the total cross sectional area of intra-abdominal fat was treated as a continuous variable (table 3 and 4). However, subgroup analysis showed a significant impact of intra-abdominal fat on disease-free survival in male patients, using a cut-off of 94.00 cm²/m² obtained by means of optimum stratification (log-rank $p = 0.032$) which for purpose of this study we defined as central obesity. By this definition, 20 (17%) of all male patients were found to have central obesity.

Figure 3. Disease-Free survival in the sarcopenic vs non-sarcopenic population.

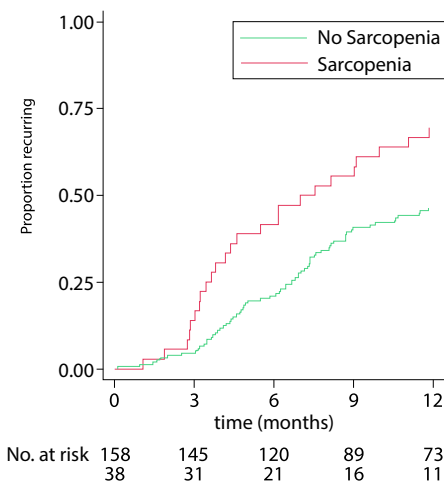


Figure 4. Overall survival in the sarcopenic vs non-sarcopenic population.

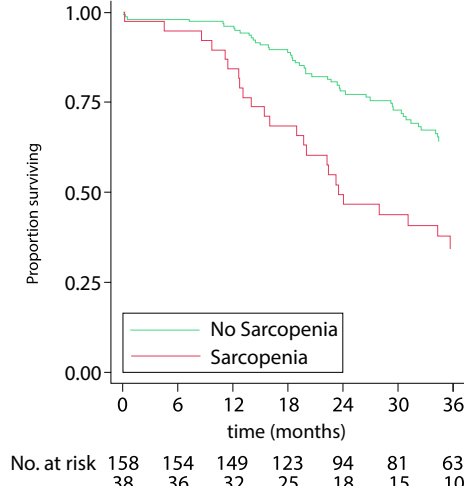


Table 2. Comparison of demographic and clinical characteristics among sarcopenic and non-sarcopenic patients using the cut-off values obtained by means of optimum stratification; 41.10 cm²/m² for female patients and 43.75 cm²/m² for male patients.

		Sarcopenic n=38 (19.4%)	Non-sarcopenic n=158 (81.6%)	p
Median Age, Years (range)		65.50 (47 – 84)	65.00 (31 – 86)	0.229
Sex	Male	11 (28.9)	109 (69.0)	< 0.001
	Female	27 (71.1)	49 (31.0)	
Mean BMI		23.66 ± 3.01	26.66 ± 3.53	< 0.001
Primary Tumor Site	Colon	21 (55.3)	95 (60.1)	0.587
	Rectum	17 (44.7)	63 (39.9)	
Primary Tumor Node	Negative	14 (37.8)	66 (42.9)	0.711
	Positive	23 (62.2)	88 (57.1)	
	No data	1	4	
Disease-free interval	< 12 months	27 (71.1)	102 (64.6)	0.554
	≥ 12 months	11 (28.9)	56 (35.4)	
Detection	Synchronous	21 (55.3)	72 (45.6)	0.366
	Metachronous	17 (44.7%)	86 (54.4)	
CEA	< 200	30 (78.9)	133 (84.2)	0.471
	≥ 200	8 (21.1)	25 (15.8)	
Max. Tumor Size	< 5cm	26 (68.4)	118 (74.6)	0.421
	≥ 5cm	12 (31.6)	40 (25.4)	
No. of Tumors	≤ 3	29 (76.3)	118 (74.6)	1.000
	> 3	9 (23.7)	40 (25.4)	
Pre-operative	Yes	18 (47.4)	73 (46.2)	1.000
chemotherapy	No	20 (52.6)	85 (53.8)	
ASA	1	9 (23.7)	37 (23.4)	0.755
	2	25 (65.8)	97 (61.4)	
	3	4 (10.5)	24 (15.2)	
Mean Intra-abdominal Adipose Tissue		42.23 ± 23.89	62.77 ± 30.92	< 0.001
Mean Subcutaneous Adipose Tissue		61.54 ± 22.65	57.29 ± 23.35	0.387

Abbreviations: CEA Carcinoembryonic Antigen, ASA American Society of Anesthesiologists, BMI Body-Mass Index

Table 3. Impact of muscle mass and other clinical characteristics on disease-free survival.

	Univariable Analysis			Multivariable Analysis		
	HR	95% CI	p	HR	95% CI	p
Sarcopenia [†]	1.880	1.252 – 2.822	0.002	1.957	1.290 – 2.969	0.002
Age	1.003	0.986 – 1.021	0.694			
Gender (female)	1.195	0.837 – 1.706	0.328			
BMI ≥ 25	1.076	0.750 – 1.543	0.692			
Diabetes	0.821	0.503 – 1.337	0.428			
Primary tumor site (colon)	0.861	0.604 – 1.225	0.405			
Synchronous*	1.345	0.948 – 1.909	0.096			
Tumor No. (> 3) [†]	1.975	1.341 – 2.907	0.001	1.750	1.088 – 2.815	0.021
Tumor size (≥ 5cm)	1.047	0.709 – 1.546	0.818			
CEA (≥ 200) [†]	1.739	1.125 – 2.689	0.013	1.749	1.117 – 2.739	0.015
Pre-operative chemotherapy	1.302	0.918 – 1.848	0.139			
Radio-frequency ablation [†]	1.843	1.225 – 2.772	0.003			NS
ASA physical status (≥ ASA 3)	0.875	0.537 – 1.425	0.591			
Resection margin (≤ 1mm) [†]	1.629	1.101 – 2.410	0.015			NS
Visceral adipose tissue CSA	1.001	0.994 – 1.007	0.829			

Abbreviations: BMI Body-Mass Index, ASA American Society of Anesthesiologists, CEA Carcinoembryonic Antigen, CSA Cross-Sectional Area. † Included in the multivariable analysis. * Synchronous indicates detection of hepatic metastases within three months of diagnosis of the primary tumor.

Figure 5. Disease-Free survival in the obese vs non obese subpopulation of male, non-sarcopenic patients.

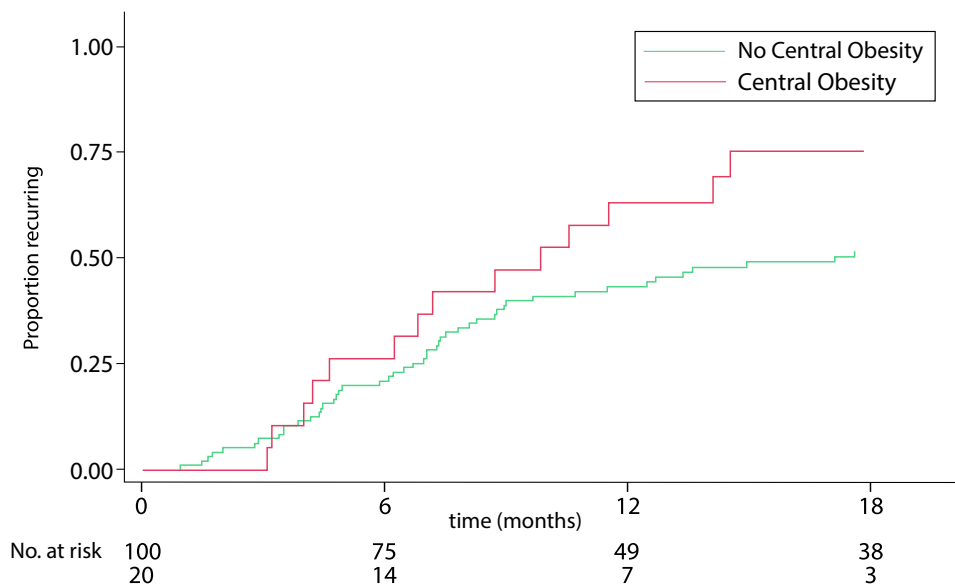


Table 4. Impact of muscle mass and other clinical characteristics on overall survival.

	Univariable Analysis			Multivariable Analysis		
	HR	95% CI	p	HR	95% CI	p
Sarcopenia [†]	2.531	1.596 – 4.012	< 0.001	2.685	1.671 – 4.315	< 0.001
Age	1.006	0.984 – 1.028	0.621			
Gender (female)	1.264	0.820 – 1.948	0.289			
BMI ≥ 25	0.832	0.539 – 1.282	0.404			
Diabetes	0.742	0.402 – 1.368	0.339			
Primary tumor site (colon)	0.770	0.501 – 1.183	0.232			
Synchronous*	1.111	0.723 – 1.707	0.632			
Tumor No. (> 3) [†]	1.652	1.036 – 2.633	0.035			NS
Tumor size (≥ 5cm)	1.015	0.628 – 1.640	0.952			
CEA (≥ 200)	1.155	0.639 – 2.091	0.633			
Pre-operative chemotherapy	0.904	0.586 – 1.396	0.694			
Radio-frequency ablation [†]	1.711	1.020 – 2.870	0.042			NS
ASA physical status (≥ ASA 3)	0.620	0.320 – 1.201	0.156			
Resection margin (≤ 1mm) [†]	1.795	1.135 – 2.840	0.012	1.853	1.154 – 2.972	0.011
Visceral adipose tissue CSA	0.996	0.989 – 1.004	0.348			

Abbreviations: BMI Body-Mass Index, ASA American Society of Anesthesiologists, CEA Carcinoembryonic Antigen, CSA Cross-Sectional Area. [†] Included in the multivariable analysis. * Synchronous indicates detection of hepatic metastases within three months of diagnosis of the primary tumor.

Male patients with central obesity had a significant worse DFS compared to non-obese males. Male patients with central obesity had a median disease-free survival of 9.8 months and corresponding 1, 3 and 5-year disease-free survival rates of 38%, 19% and 0% respectively. Non-obese male patients had a median disease-free survival of 18.0 months and corresponding 1, 3 and 5-year disease-free survival rates of 57%, 40% and 28% respectively ($p = 0.032$) (figure 5). Overall survival was found not to be affected by central obesity in men ($p = 0.837$). For female patients no association could be found for central obesity and disease-free survival ($p = 0.712$) and overall survival ($p = 0.566$).

DISCUSSION

A wide variety of body composition can be found in patients with CLM. Sarcopenia was a strong indicator of a worse prognostic outcome for disease-free survival and overall survival. The occurrence and effect of sarcopenia was independent of physical status (ASA classification) and tumor related factors known to impact on survival after liver resection for CLM.¹⁴ These findings emphasize the importance of the assessment and potential treatment of sarcopenia in cancer patients scheduled for curative intent surgery.

Nineteen percent of patients with CLM were sarcopenic. Sarcopenia was not associated with known risk factors for recurrence and death such as pre-operative CEA level, synchronous diagnosis and the number of metastases.¹⁴ Regardless, sarcopenia may still reflect increased metabolic activity of a more aggressive tumor biology leading to systemic inflammation, causing muscle wasting.¹⁵ Sarcopenia should not be confused with “clinical cachexia” or simple weight loss, since many sarcopenic patients have a normal or elevated BMI.¹⁵⁻¹⁷ Sarcopenia can therefore only be uncovered by precise quantification of skeletal muscle mass. CT analysis has been shown to be a widely available and highly precise method for this.¹³

Patients with sarcopenia did not have a worse physical status according to the ASA classification when compared with non-sarcopenic patients. Also, a worse ASA score did not predict worse long term outcome. While the ASA score gives some estimation of organ disease and functional status, it has been criticized for being subjective and imprecise.¹⁸ Frailty has been reported to allow for a more global assessment of a patient's health status and physiological reserve. However, most frailty scores include measurements of weakness and physical activity assessed by patient questionnaires and can therefore be potentially subjective and susceptible to bias as well. Sarcopenia has been described as a more robust measure of frailty and might therefore give a more objective assessment of a patient's functional reserve than currently used scoring systems.

Sarcopenia was an independent predictor of worse disease-free and overall survival. Prado et al showed an impaired survival in sarcopenic obese patients with gastrointestinal and respiratory tract malignancies.⁸ Other studies have confirmed the impact of sarcopenia on survival in patients with pancreatic cancer and patients undergoing liver transplantation.⁹⁻¹⁰ The correlation between the amount of intra-abdominal fat and long term outcome was less defined in the current study.

Others have found reduced disease-free survival in patients with colorectal cancer where there were high visceral / subcutaneous fat ratios.⁷ Intra-abdominal adiposity has also been associated with an increased incidence of malignant and pre-malignant tumors of the gastro-intestinal tract¹⁹ The exact mechanisms by which sarcopenia and central obesity affect survival in cancer patients have yet to be unraveled, although both conditions have been described to both impact on the risk of cancer development as well as short- and long term post-operative outcomes.

Several limitations apply to the current study. Patients were excluded from analysis due to unavailability of CT scans. This has undoubtedly caused a selection bias with regard to pre-operative chemotherapy, disease status and post-operative morbidity. Second, the inclusion of post-operative CT-scans created a potential bias since patients with post-operative CT-scans might have suffered from more complications with a negative impact on long term outcome. However, no difference in the incidence of sarcopenia was found between the pre-operative and post-operative CT scan groups and long term outcome was similar for both groups. Patients in the post-operative group tended to have slightly more skeletal muscle mass than patients with pre-operative scans only (data not shown). The cut-off values for sarcopenia may not be directly applicable to another population set and should be further validated.

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CHAPTER 6

SARCOPENIA IMPAIRS SURVIVAL IN PATIENTS WITH POTENTIALLY CURABLE HEPATOCELLULAR CARCINOMA

ABSTRACT

BACKGROUND

A reduction in skeletal muscle mass (sarcopenia) independently predicts poor survival in patients with hepatocellular carcinoma (HCC) undergoing treatment with curative intent. Whether this is due to an increased risk of recurrence and disease specific death, or due to an increased risk of postoperative morbidity and mortality is currently unclear. In this study, we investigate the association between sarcopenia and death in a cohort of HCC patients undergoing treatment with curative intent.

METHODS

Patients undergoing surgical resection or radiofrequency ablation for lesions ≤ 3 cm between 2002 and 2013 were identified. Clinicopathological characteristics, CT-assessed sarcopenia and outcomes were analyzed.

RESULTS

Among 90 patients, 52 (57.8%) were found to be sarcopenic. Sarcopenic patients had a limited overall survival (median: 33 months vs. non-sarcopenic median: 105 months; $p = 0.002$), but not disease-free survival. Sarcopenia was an independent predictor for overall survival in multivariate Cox-regression analysis (HR 3.756; $p = 0.001$). Major complications (32.7% vs. 13.2%, $p = 0.033$) and treatment-related mortality (17.3% vs. 2.6%, $p = 0.029$) were more frequent in sarcopenic patients.

CONCLUSIONS

Sarcopenia impairs survival in patients with potentially curable hepatocellular carcinoma, mainly due to an increase in treatment-related mortality.

INTRODUCTION

Sarcopenia as pre-operative risk assessment indicator has increasingly gained interest in recent years.¹⁻³ Sarcopenia is characterized by a loss of skeletal muscle mass, strength and physical performance.⁴ Initially sarcopenia has been described to limit survival and physical performance in geriatric populations.^{5,6} With advanced age an increase in prevalence of sarcopenia may be observed in healthy individuals.⁷ Prado et al. first showed that sarcopenia decreases overall survival in obese cancer patients. In this study, sarcopenia was defined as a loss of skeletal muscle mass measured on a single CT slice. Other studies have since then shown that CT-assessed sarcopenia is associated with reduced overall survival, shortened disease-free survival, increased post-treatment morbidity and treatment toxicity in patients with a variety of malignancies including colorectal cancer, colorectal liver metastases, lung cancer, breast cancer and melanoma.^{1-3, 8-10}

Recent studies have shown that sarcopenia plays an important role in the prognosis of patients with hepatocellular carcinoma. Hepatocellular carcinoma is one of the most common types of cancer worldwide and often develops in patients with underlying liver disease. This population is especially interesting since chronic liver disease is a risk factor for sarcopenia itself.¹¹ A recent study by Harimoto et al. identified sarcopenia as a predictor of poor survival and recurrence-free survival in a Southeast Asian cohort of patients undergoing liver resection for HCC.¹² Three other studies have reported similar results.¹³⁻¹⁵

The reduction in survival in these studies might be attributed in part by the decreased ability to withstand the surgical trauma associated with HCC treatment and increased risk of early death in these patients. Results with regard to the association between sarcopenia and the risk of disease recurrence are conflicting, as only half of the studies found such an association. Only one of these studies addressed the interaction between body mass index (BMI), sarcopenia and survival. These studies have focused exclusively on patients undergoing liver resection for HCC, while sarcopenia might impact on the survival of HCC patients undergoing liver transplantation or local ablation with curative intent as well.

Thus, the aims of the current study were (1) to investigate the impact of sarcopenia on survival in patients undergoing curative intent therapy for HCC, (2) to investigate the possible interaction between BMI and sarcopenia on survival and (3) to assess the impact of sarcopenia on the risk of early death after surgery.

METHODS

PATIENT SELECTION

Between January 2002 and March 2013 consecutive patients who underwent invasive treatment with a curative intent consisting of resection or radiofrequency ablation (RFA) for lesions ≤ 3 cm at the Erasmus MC - University Medical Center Rotterdam, the Netherlands, were evaluated for this study. Patients were included in this study if an abdominal CT scan was available for review (performed less than 3 months prior to or 3 days after treatment) and corresponding data regarding body weight and length. Patients with Child-Pugh B or Child-Pugh C liver cirrhosis have been excluded from this study. Demographic, clinical, radiologic, clinical chemistry and pathologic characteristics were retrieved from patient clinical records.

ASSESSMENT OF SKELETAL MUSCLE TISSUE

A CT slice at the level of the third lumbar vertebra where both transverse processes were clearly visible was selected for each patient. Image analysis was performed independently by two trained investigators (RM and SL). The outer and inner contours of the abdominal wall and paraspinal muscles (rectus abdominis, external and internal obliques, transversus abdominis, quadratus lumborum, erector spinae and psoas) were manually traced and segmented using FatSeg version 4.0 (Erasmus MC – Biomedical Imaging Group Rotterdam (BIGR), Rotterdam, Netherlands). An intensity window between -30 HU and +150 HU was used for the identification of skeletal muscle mass.¹⁶ The cross-sectional area of the delineated skeletal muscle mass (cm^2) in the selected slice was determined by semi-automatic segmentation using the HU thresholds (figure 1). The obtained cross-sectional areas of muscle were subsequently normalized with respect to squared body height to obtain a lumbar skeletal muscle index (lumbar SMI, cm^2/m^2), which was used for further analysis.

TREATMENT AND FOLLOW-UP

All patients were presented to a multidisciplinary team, which included a hepatobiliary surgeon, hepatologist, oncologist and radiologist to determine selection of best available treatment. Comorbidity, underlying liver disease, tumor characteristics (e.g. size, segmental location) and liver remnant volume were considered when assessing

Figure 1. Computed Tomogram showing delineated cross-sectional area of skeletal muscle mass at the level of the middle of the third lumbar vertebrae.



6

surgical resectability. Patients were considered for (percutaneous) RFA treatment when no more than 3 tumors were present, the largest tumor did not exceed 3 cm in diameter and tumor location allowed for RFA approach. Treatment related complications and mortality were scored in patient clinical records. Complications were categorized according to the Clavien-Dindo classification.¹⁷ Overall and disease-free survival rates were computed from the date of treatment. Recurrence data was obtained through clinical records. Patients had a follow up on an outpatient basis by periodic 6-months abdominal ultrasonography, CT imaging, magnetic resonance imaging (MRI) and serum α -Fetoprotein (AFP) determination if the initial tumor was AFP positive. Follow up included at least 5 years if no recurrence was found. Survival data were obtained from the national civil registry.

STATISTICAL ANALYSIS

Continuous data are presented as mean \pm SD if normally distributed. Continuous data are presented median (range) if the distribution is skewed. Categorical data are presented as proportions. Differences between groups were investigated using Student's t-test for continuous variables and χ^2 or Fisher's exact test for categorical variables

where appropriate. All p-values are derived from two-tailed tests. Gender-specific cut-off values for lumbar SMI at which the difference in survival was most significant were determined using optimum stratification. Overall and disease-free survival rates were calculated and compared with the non-parametric Kaplan–Meier method and the log rank test. To investigate the association between sarcopenia and survival, univariate and multi-variable Cox regression analyses were performed. Hazard Ratios (HR) with 95% confidence intervals (95% CI) were computed. The following variables were used for inclusion in univariate Cox regression analysis based on clinical relevance: age, body-mass index (BMI), therapy (resection, liver transplantation, RFA), BCLC classification, WHO performance status, portal thrombosis, number of tumors, maximum tumor size, clinical presence of cirrhosis prior to treatment as well as sarcopenia. Interaction between various variables was checked by introducing interaction terms in the regression analysis. All variables were included in the multivariate analyses for both overall survival and disease-free survival using the backward selection method. Univariate and multivariate Cox regression analyses were stratified for gender to account for gender specific differences in sarcopenia. To investigate the association between sarcopenia and treatment related mortality, univariate and multivariate logistic regression analyses were performed. Odds ratios (OR) with 95% CI were computed. The variables used for univariate Cox regression analysis, including gender, were used for inclusion in univariate and multivariate logistic regression analysis. All variables were included in the multivariate analysis using the backward selection method. All statistical analyses were performed using SPSS version 21.0 (SPSS, Chicago, Illinois, USA). A p-value < 0.05 was considered statistically significant.

RESULTS

CLINICAL CHARACTERISTICS AND BODY COMPOSITION

A total of 90 patients, with a median follow-up of 22.5 months (range: 0 - 120) were found eligible for inclusion in the study. Seventy-one patients underwent surgical resection of the hepatocellular carcinoma (67.8%). Twenty-nine patients (32.2%) underwent treatment by RFA of which twenty-four (82.8%) were treated by percutaneous approach. Five patients (17.2%) were treated by open RFA. Baseline demographics and clinical characteristics are shown in table 1. A wide variation in body composition was observed. Thirty-three (36.7%) patients were overweight (BMI 25-30) and 14 (15.6%) patients were obese (BMI >30). Nine patients (10.0%) had a BMI of less than 20. Mean lumbar SMI was $49.7 \pm 8.8 \text{ cm}^2/\text{m}^2$ for male patients and $39.9 \pm 5.8 \text{ cm}^2/\text{m}^2$ for female patients ($p \leq 0.001$). Gender-specific cut-off values for lumbar SMI associated with overall survival obtained by means of optimum stratification were $52.0 \text{ cm}^2/\text{m}^2$ for male patients and $39.5 \text{ cm}^2/\text{m}^2$ for female patients. Using these gender-specific cut-off values 52 patients (57.8%) were found to be sarcopenic. No differences were found between sarcopenic patients and non-sarcopenic patients with regard to age, gender, clinical presence of cirrhosis prior to treatment, Child-Pugh status, hepatitis B status, hepatitis C status, performance status, BCLC classification, number of tumors, maximum tumor size, α -fetoprotein, portal thrombosis, therapy group or muscle attenuation (table 2). Sarcopenia was associated with a reduction in body mass index, which was 24.5 ± 3.9 in the sarcopenic patients versus 27.3 ± 5.0 in non-sarcopenic patients ($p = 0.003$).

OVERALL SURVIVAL

Sarcopenic patients had a reduced overall survival (median: 33 months, range: 17 – 48) when compared to non-sarcopenic patients (median: 105 months, range: 28 – 181; log rank test $p = 0.002$). Kaplan-Meier survival curves are shown in figure 2. As can be seen in table 3, advanced age, tumor diameter and sarcopenia were associated with a reduction in overall survival in multi-variable Cox regression analysis. In addition, a strong interaction was found between BMI and sarcopenia. Therefore, survival analysis was stratified for patients with a BMI less or more than 25, respectively (figure 3).

Table 1. Baseline demographic and clinical characteristics of the 90 patients included in the study.

	Overall (n = 90)
Age (years)	62 (22 – 86)
Gender	
Male	63 (70.0%)
Female	27 (30.00%)
BMI (kg/m ²)	25.2 (17.6 – 37.7)
Chronic Obstructive Pulmonary Disease (COPD)	1 (1.1%)
Cardiovascular Disease (Excluding hypertension)	11 (12.2%)
BCLC Classification	
Very Early	15 (16.7%)
Early	30 (33.3%)
Intermediate	36 (40.0%)
Advanced	9 (10.0%)
Therapy group	
Resection	61 (67.8%)
RFA	29 (32.2%)
WHO Performance Status	
Fully active (0)	84 (93.3%)
Able to do light activity (1)	6 (6.7%)
Hepatitis B	15 (16.7%)
Hepatitis C	22 (24.4%)
Cirrhosis (Child-Pugh A)	45 (50.0%)
No. of tumors	1 (1 – 5)
Maximum tumor size (cm)	3.6 (0.9 – 20.0)
Portal Thrombosis	3 (3.3%)
α -fetoprotein	13 (2 – 2.745 x 10 ⁶)
MA (HU) ^a	34 (13 – 61)
Lumbar SMI (cm ² /m ²) ^b	45.9 (21.8 – 71.7)

a Mean muscle attenuation as assessed at the third lumbar vertebrae.

b Mean cross-sectional area of skeletal muscle mass assessed at the third lumbar vertebrae, and standardized for patient height.

Table 2. Comparison of demographic and clinical characteristics of patients with and without sarcopenia.

	Sarcopenia (n = 52)	No Sarcopenia (n = 38)	p
Age (years)	61 (22 – 86)	62 (25 – 77)	0.481
Gender			0.226
Male	39 (75.0%)	24 (63.2%)	
Female	13 (25.0%)	14 (36.8%)	
BMI (kg/m ²)	24.7 (17.6 – 33.4)	26.0 (19.5 – 37.8)	0.003
COPD	1 (1.9%)	0 (0.0%)	1.000
Cardiovascular Disease (Excluding hypertension)	6 (11.5%)	5 (13.2%)	0.817
BCLC Classification			0.605
Very Early	8 (15.4%)	7 (18.4%)	
Early	16 (30.8%)	14 (36.8%)	
Intermediate	21 (40.4%)	15 (39.5%)	
Advanced	7 (13.5%)	2 (5.3%)	
Therapy group			0.730
Resection	36 (69.2%)	25 (65.8%)	
RFA	16 (30.8%)	13 (34.2%)	
WHO Performance Status			0.395
Fully active (0)	47 (90.4%)	37 (97.4%)	
Able to do light activity (1)	5 (9.6%)	1 (2.6%)	
Hepatitis B	9 (17.3%)	6 (15.8%)	0.849
Hepatitis C	14 (26.9%)	8 (21.1%)	0.522
Cirrhosis (Child-Pugh A)	26 (50.0%)	19 (50.0%)	1.000
No. of tumors	1 (1 – 4)	1 (1 – 3)	0.524
Maximum tumor size (cm)	3.5 (0.9 – 18.5)	4.0 (1.0 – 20.0)	0.566
Portal Thrombosis	2 (3.8%)	1 (2.6%)	1.000
α -fetoprotein	13 (2 – 7.582 x 10 ³)	15 (2 – 2.745 x 10 ⁶)	0.325
MA (HU) ^a	34 (14 – 61)	33 (13 – 52)	0.676
Lumbar SMI (cm ² /m ²) ^b	42.5 (21.8 – 51.9)	55.0 (39.8 – 71.7)	< 0.001

a Mean muscle attenuation as assessed at the third lumbar vertebrae.

b Mean cross-sectional area of skeletal muscle mass assessed at the third lumbar vertebrae, and standardized for patient height.

Table 3. Univariate and multivariate Cox regression analysis for overall survival (stratified for gender).

		Univariate			Multivariate		
		HR	95% CI	p	HR	95% CI	p
Age	years	1.036	1.008 – 1.066	0.013	1.029	1.001 – 1.058	0.039
BMI	kg/m ²	0.945	0.881 – 1.014	0.117			
Therapy group	Resection (Reference)						
	RFA	0.915	0.481 – 1.744	0.788			
BCLC ^a Classification		1.513	1.075 – 2.129	0.018			
WHO performance status	Able to do light activity (1)	2.260	0.884 – 5.781	0.089			
Portal thrombosis	yes	2.315	0.712 – 7.524	0.163			
Cirrhosis	yes	1.386	0.750 – 2.560	0.297			
Tumor no.	#	1.312	0.948 – 1.818	0.102			
Max. tumor size	mm	1.007	1.000 – 1.014	0.046	1.013	1.005 – 1.021	0.001
Sarcopenia	yes	2.835	1.414 – 5.686	0.003	3.756	1.778 – 7.932	0.001

a Barcelona-Clinic Liver Cancer staging.

Twenty-six (57.8%) patients with a BMI under 25 were sarcopenic. Nineteen (42.2%) patients with a BMI over 25 were sarcopenic. In patients with a BMI lower than 25, no differences in survival were found between sarcopenic (median: 58 months, range: 20 – 95) and non-sarcopenic patients (median: 46 months, range: 13 – 79; log rank test $p = 0.734$). However, overweight and obese patients (BMI > 25) with sarcopenia had a significantly shorter median survival (median: 17 months, range: 8 – 26) when compared to overweight and obese patients without sarcopenia (median: not reached; log rank test $p < 0.001$).

Figure 2. Overall survival in the sarcopenic vs non-sarcopenic population.

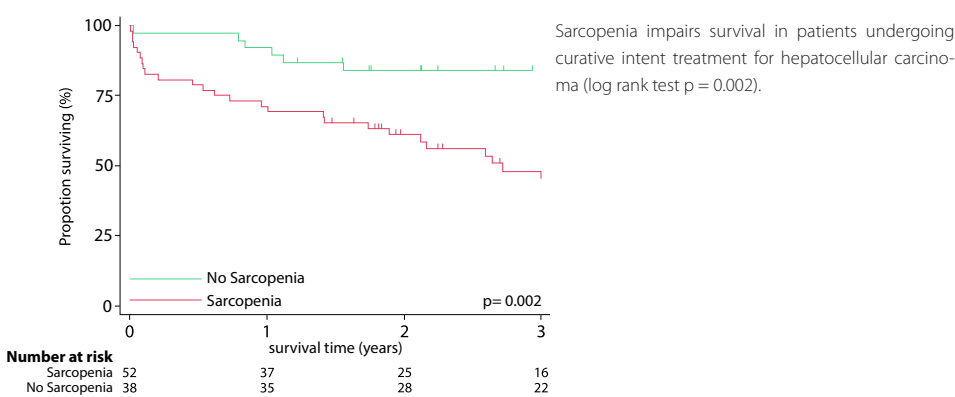
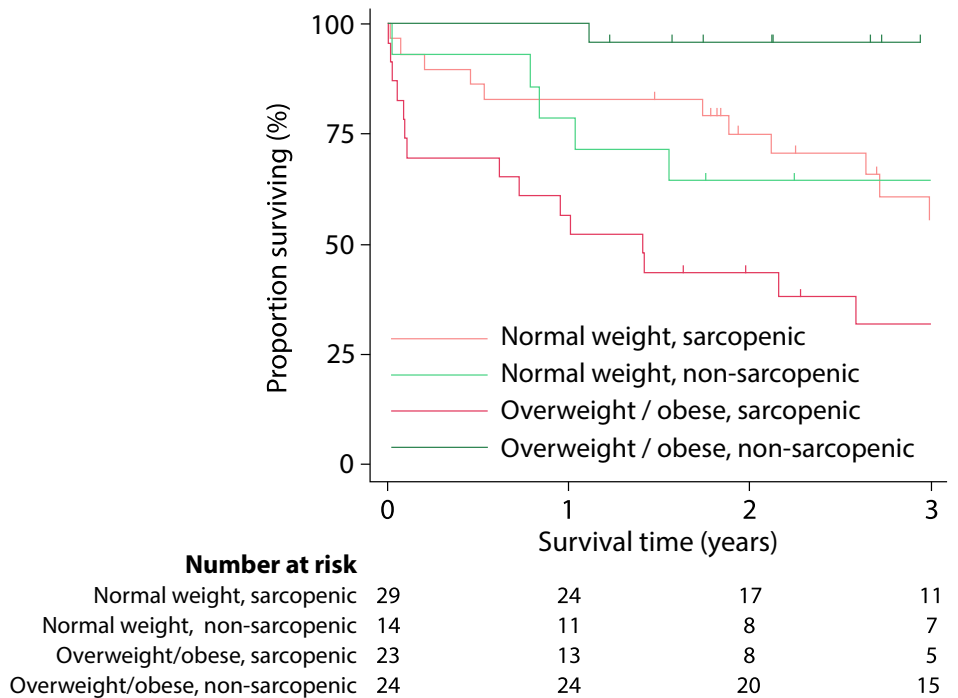


Figure 3. Overall survival in the sarcopenic vs non-sarcopenic, normal weight and overweight/obese population.

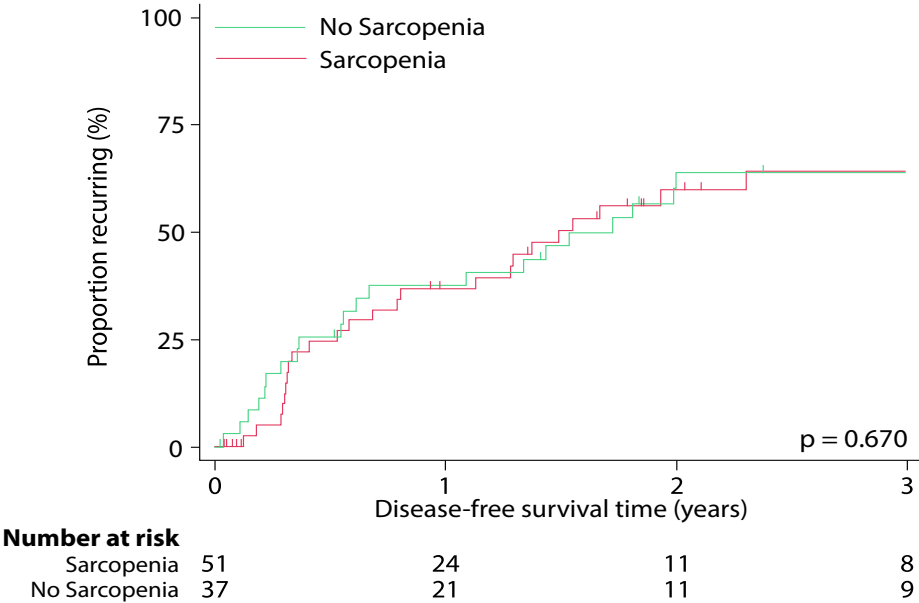


Diminished survival was greatest in sarcopenic overweight and obese patients. Median survival for this selection of patients was 17 months. Survival was reduced compared to non-sarcopenic overweight and obese patients (log-rank $p < 0.001$) as well as normal weight sarcopenic patients (log-rank $p = 0.022$). A trend towards decreased survival when compared with normal weight non-sarcopenic patients was observed.

DISEASE-FREE SURVIVAL

No difference in disease-free survival was found between sarcopenic patients (median: 18 months, range: 12 – 24) and non sarcopenic patients (median: 18 months, range: 11 – 26; log rank test $p=0.670$) (figure 4). After stratification for BMI, no differences in disease-free survival were found between sarcopenic and non sarcopenic patients. In multivariate Cox regression analysis RFA therapy (HR 4.654; 95% CI 1.977 – 10.957; $p < 0.001$), a stepwise increase on the BCLC classification (HR 1.494; 95% CI 1.027 – 2.175; $p = 0.036$) and tumor diameter (HR 1.012; 95% CI 1.003 – 1.021; $p = 0.007$) were associated with decreased disease-free survival.

Figure 4. Disease-free survival in the sarcopenic vs non-sarcopenic population.



Disease-free survival was comparable for patients with and without sarcopenia (log rank test $p = 0.670$).

TREATMENT RELATED COMPLICATIONS AND MORTALITY

A steep decline in survival was noted in survival within the first months after therapy in sarcopenic patients. Seventeen (32.7%) of sarcopenic patients experienced a major complication (Clavien-Dindo grade \geq IIIa) following treatment versus 5 (13.2%) of non-sarcopenic patients ($p = 0.033$). Nine (17.3%) of all sarcopenic patients died during the first 90 days as a result of treatment related complications versus 1 (2.6%) of non-sarcopenic patients ($p = 0.029$). Of the 9 sarcopenic patients who died during the first 90 days as a result of treatment related complications, 3 patients (5.8%) died from liver failure, 5 (9.6%) died from sepsis, and 1 patient (1.9%) died from aspiration. The non-sarcopenic patient died from liver failure. Major complications and treatment-related mortality were observed in the surgical resection group only.

DISCUSSION

In the current study we determined the impact of sarcopenia on survival following treatment with curative intent in patients with hepatocellular carcinoma. Using multivariate Cox-regression analysis the presence of sarcopenia was found to impair overall survival, but not disease-free survival in patients undergoing treatment by resection or RFA. Survival was further diminished in patients who were found to be sarcopenic overweight and obese. This effect was independent of other predictors such as BCLC and the presence of cirrhosis. Sarcopenic patients suffered significantly more from major treatment related complications and treatment-related mortality.

Other studies have recently addressed the impact of sarcopenia on survival in patients with HCC as well. Voron et al. and Harimoto et al. found a decrease in overall and disease-free survival in sarcopenic patients undergoing liver resection for HCC.^{12, 15} Two other studies described a reduction in overall survival in sarcopenic patients with HCC, although there was no association with recurrence-free survival, suggesting other factors associated with poor survival in these patients.^{8, 13, 14} Whether the reduction in survival found in sarcopenic patients is due to an increased risk of recurrent disease or due to an increased risk of treatment-related death remains controversial. Our results give support to the latter, as sarcopenic patients seem to be less fit to recover from the trauma associated with a major surgical procedure. In line with this, the study by Voron et al reported a sharp decline in survival in the first months post-operatively among sarcopenic patients undergoing liver resection.¹⁵ Poor short-term and one-year survival rates as well as prolonged hospital stay have also been reported for sarcopenic patients undergoing liver transplantation.¹⁸⁻²⁰ Although the mechanisms by which sarcopenia affects postoperative recovery are not fully understood, differences in the severity of the underlying liver disease impacting on muscle mass may very well play a part. It is well understood that patients with sarcopenia are more susceptible to nosocomial infection, poor wound healing and prolonged hospital stay.^{10,18,20} Within this study cause of death following treatment could grossly be categorized in infectious complications, predominantly respiratory by nature at first, with subsequent development of sepsis and multi-organ failure or the development of progressive liver failure. In order to improve outcome for these patients, alternative treatment strategies may be required. Ablative therapy with limited treatment related mortality may be a preferential treatment modality in the sarcopenic and overweight patient. Indeed, no treatment related mortality was noted in patients undergoing open or percutaneous ablation in the current study.

Only one study briefly addressed the possible interaction between BMI and sarcopenia on survival, and found that patients with a BMI > 22 were at increased risk of early death after surgery.¹⁴ In the current study, sarcopenia further impaired survival in overweight and obese patients. The dismal effect of sarcopenia with or without obesity on the prognosis of cancer patients has been widely described over recent years.^{1,8} Current understanding on the development of sarcopenic obesity is limited. It is not yet fully understood whether the loss of skeletal muscle mass (i.e. severe sarcopenia) contributes to gain of adipose tissue, or the gain of adipose tissue contributes to the loss of skeletal muscle mass.²¹ However, overweight and obese sarcopenic patients are suggested to represent a distinct group of patients when compared to normal weight sarcopenic patients with regard to performance status, physical activity, underlying chronic inflammation, insulin resistance, hormonal balance and tumor biology.²² Such decreases in performance status and physical activity may contribute to worsened survival.^{23, 24} Although reduced physical activity has been associated with impaired survival in liver transplant candidates²⁵ it is not known whether this applies to HCC patients.

Surprisingly, survival was lower in normal weight patients when compared to non-sarcopenic overweight or obese patients, irrespective of the presence of sarcopenia. Such a beneficial effect of increased BMI could not be observed in the overall overweight and obese population (data not shown). As such, we postulate that these non-sarcopenic overweight or obese patients reflect a subgroup of well-nourished patients who are not affected by wasting disorders commonly seen in elderly, oncological or cirrhotic patients.

Further research is warranted to investigate whether outcome can be improved in sarcopenic patients. Based on findings in this current study, a prospective trial to investigate surgical resection versus (percutaneous) RFA on treatment-related morbidity and mortality, and overall survival, is warranted. The underlying mechanism of muscle wasting in cancer has been thoroughly investigated in recent years^{26, 27} and led to successful inhibition of muscle wasting in experimental models, e.g. by using myostatin inhibitors.^{28, 29} A phase II clinical trial on LY2495655, a humanized monoclonal antibody to myostatin, is currently investigating its effect on overall survival in advanced or metastatic pancreatic cancer patients. This is just one of many current clinical trials investigating the inhibition of muscle wasting in cancer patients.³⁰ Future, prospective trials are required to attest effectivity of such pharmaceutical treatment options in surgical HCC patients, either with or without pre-operative exercise programs.

Several limitations apply to the present study. First, analysis of post-treatment scans may have created a potential bias. Patients with post-treatment scans may have had more complications, which can have a negative impact on long-term outcome. By limiting the window for post-treatment scans to 3 days this potential bias is deemed to be minimal. Fourteen scans were post-treatment scans, eleven of these were day 1 control imaging following RFA treatment. Second, due to the retrospective nature of the current study information regarding possible loss of appetite, anorexia, was not available on a consistent basis. Likewise, physical status and performance of the individual patient was limited to a rapid assessment of physical status according to the World Health Organization performance scale without recorded physical testing.

In conclusion, sarcopenia impairs survival in patients with potentially curable hepatocellular carcinoma mainly due to an increase in treatment-related mortality. Sarcopenia in an overweight or obese patient impairs survival to an even greater extent.

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CHAPTER 7

MUSCLE WASTING AND SURVIVAL FOLLOWING PRE- OPERATIVE CHEMORADIOOTHERAPY FOR LOCALLY ADVANCED RECTAL CARCINOMA

ABSTRACT

BACKGROUND

Neoadjuvant chemoradiotherapy (NACRT) has increased local control in locally advanced rectal cancer. Reduced skeletal muscle mass (sarcopenia), or ongoing muscle wasting, is associated with decreased survival in cancer. This study aims to assess the change in body composition during NACRT and its impact on outcome using computed tomography (CT) imaging in locally advanced rectal cancer (LARC) patients.

METHODS

LARC patients treated with NACRT were selected from a prospectively maintained database and retrospectively analyzed. One-hundred twenty-two patients who received treatment between 2004 and 2012 with available diagnostic CT imaging obtained before and after NACRT were identified. Cross-sectional areas for skeletal muscle was determined, and subsequently normalized for patient height. Differences between skeletal muscle areas before and after NACRT were computed, and their influence on overall and disease-free survival was assessed.

RESULTS

A wide distribution in change of body composition was observed. Loss of skeletal muscle mass during chemoradiotherapy was independently associated with disease-free survival (HR0.971; 95% CI:0.946 – 0.996; $p=0.025$) and distant metastasis-free survival (HR0.942; 95% CI:0.898 – 0.988; $p=0.013$). No relation was observed with overall survival in the current cohort.

CONCLUSIONS

Loss of skeletal muscle mass during NACRT in rectal cancer patients is an independent prognostic factor for disease-free survival and distant metastasis-free survival following curative intent resection.

INTRODUCTION

Colorectal cancer is the third most common malignancy among male and second most common malignancy among female patients worldwide.¹ It is a leading cause of cancer death in more developed countries. Rectal cancer accounts for up to 30% of all colorectal malignancies. For patients with locally advanced rectal cancer, neoadjuvant chemoradiotherapy (NACRT) combined with total mesorectal excision (TME) is considered best available treatment.^{2, 3}

Recently, sarcopenia (muscle wasting) has been described as a potent prognostic marker in gastrointestinal and hepatopancreatobiliary malignancies.⁴⁻¹⁵ Sarcopenic patients, i.e. patients with a lesser quantity of muscle mass, have an increased risk for early death. Age, cancer cachexia and oncological treatment may contribute to this state of low muscle mass.¹⁶⁻¹⁸ Interestingly, NACRT itself has been reported to reduce skeletal muscle mass in esophagogastric cancer patients.¹⁶ Another study confirmed these findings, and furthermore showed that greater loss of muscle mass during neoadjuvant treatment is associated with an increased risk of postoperative mortality.¹⁹ Likewise, in non-resectable colorectal cancer patients, skeletal muscle loss after systemic chemotherapy is an independent, negative prognostic factor.²⁰ Interventions to stop or even reverse progressive muscle wasting in patients undergoing potentially curative anti-cancer therapy are currently being investigated and would, if found, provide new strategies in the management of cancer patients.

To this moment, the impact of NACRT on body composition in patients with locally advanced rectal cancer (LARC) has not yet been described. Therefore, in the current study we aim to (1) investigate whether NACRT induces a change in body composition in LARC patients, (2) assess the impact of change in body composition during NACRT on outcome (i.e. short-term outcome, overall survival, disease-free survival, and development of distant metastases).

METHODS

PATIENTS

All histologically confirmed, LARC patients who underwent NACRT and TME in the Erasmus MC Cancer Institute, a tertiary referral center in the Netherlands for locally advanced and stage IV colorectal cancer, between August 2004 and December 2012 289 patients were enrolled in a prospectively maintained database and retrospectively analyzed. The study protocol was approved by medical ethical committee of the Erasmus MC, University Medical Center, Rotterdam, The Netherlands (MEC-2017-239). LARC was defined as T3 or T4 rectal tumors (i.e. tumors located ≤ 15 cm of the anal verge as determined by MRI and colonoscopy) with clinical suspicion of narrow or involved circumferential resection margins (CRM) with or without potentially malignant lymph nodes, or rectal tumors with potentially malignant lymph nodes outside the TME plane, as previously described.²¹ Collected data included details on patient age, gender, body-mass index (BMI), comorbidities, cancer stage, carcinoembryonic antigen (CEA), surgical and chemoradiotherapeutic treatment, clinical response rate, recurrence and survival. From the initial 289 patients, 122 patients received abdominal computed tomography (CT) imaging before standardized preoperative chemoradiotherapy (preCRT), and a restaging CT scan (postCRT) to identify any possible previously non-detectable distant metastases, according to local protocol.²² Only patients with adequate preCRT and post-CRT scans were considered eligible for inclusion in the current study.

PREOPERATIVE CHEMORADIOTHERAPY AND SURGICAL RESECTION

All patients received preoperative chemoradiation therapy as a long course (50 Gy) delivered in 25 fractions in accordance to the Dutch guidelines, i.e. chemoradiotherapy for rectal cancer classified as LARC. Capecitabine (825 mg/m²) was administered orally twice a day during radiotherapy days, and radiotherapy was administered via a three-field technique, using one posterior and two lateral portals, a four-field box or with five fields using intensity modulated radiotherapy.²³

TME was performed after completing chemoradiation, if considered eligible for resection. A midline laparotomy was carried out in all patients. A primary anastomosis was performed whenever possible. A diverting ileostomy was created at the discretion of the treating physician. In T4 tumors involving the sphincter apparatus after NACRT, an abdominoperineal resection was performed. In T4 tumors involving adjacent structures after NACRT (e.g. prostate, uterus, bladder) these were resected simultaneously. Intraoperative radiotherapy was applied if the circumferential resection margin (CRM, ≤ 2 mm) was considered to be at risk.²⁴

POSTOPERATIVE FOLLOW-UP

Patients follow up was done on an outpatient basis by periodic six months CT imaging or abdominal ultrasonography during the first two postoperative years, followed by yearly imaging for the remainder of the follow-up. Serum CEA determination was done at intervals of three to six months during the first three years of follow-up, and subsequently every six months during the final years of follow-up. Patients were followed up for at least 5 years in case of no recurrence. None of the patients were treated with adjuvant chemotherapy according to the Dutch guidelines. The national civil registry was consulted for definitive survival data.

ASSESSMENT OF BODY COMPOSITION

Body composition was measured on standard diagnostic CT scans with FatSeg version 4.0 (Erasmus MC – BIGR, Rotterdam, Netherlands). Cross-sectional areas (cm²) of skeletal muscle mass was measured at the level of the third lumbar vertebrae as previously described.¹⁵

STATISTICAL ANALYSIS

Continuous data are presented as mean \pm SD or median (IQR) as appropriate. Categorical data are presented as number counts and percentages. The Student's t-test was used for assessment of differences between groups for continuous variables. The χ^2 or Fisher's exact test was used for assessment of differences between groups for categorical variables where appropriate. Skeletal muscle mass was normalized for patient height (skeletal muscle index [SMI]). Paired t-test was used for the between group comparisons of continuous variables for SMI on preCRT and postCRT scans. Relative change in cross-sectional areas (Δ CSA = postCRT / preCRT) were computed for SMI. Gender specific tertiles were determined for Δ SMI. Overall and disease-free survival rates were calculated using the non-parametric Kaplan–Meier method and subsequently compared with the log rank test. Univariate and multi-variable Cox regression analyses were performed to investigate the association between Δ SMI and survival. Hazard Ratios (HR) with 95% confidence intervals (95% CI) were computed. Furthermore, age, gender, diabetes, BMI, tumor location, CEA, surgical procedure, intraoperative radiotherapy, pathologic T-, N- and M- stage, circumferential resection margin, and pathologic complete response were included in the univariate Cox regression analysis. These variables were checked for interaction and confounding. They were subsequently included in the multivariable model if a p-value < 0.05 was found in univariate analysis.

All statistical analyses were performed using SPSS version 21.0 (SPSS, Chicago, Illinois, USA). A p-value < 0.05 was considered statistically significant.

RESULTS

CLINICAL CHARACTERISTICS AND BODY COMPOSITION

One hundred and twenty-two patients, with a median follow-up of 41 months (IQR 26 – 62) were eligible for inclusion (Table 1). During the follow-up period, 50 (41.0%) patients developed recurrent or metastatic disease, and 35 patients (28.7%) died. Forty (32.8%) patients had metastatic disease at onset of NACRT. Twenty-nine (23.8%) patients were treated by liver first approach.^{25, 26} Eleven patients underwent synchronous resection. In the studied population, median length of hospital stay was 8 (IQR: 7 – 11) days.

Abdominal CT-imaging was obtained at median 48 (IQR: 35 – 65) days prior to onset of NACRT. Restaging scans were obtained at a 28 (IQR: 21.5 – 39.5) days after completion of NACRT. Following NACRT, mean skeletal muscle index (SMI) remained unchanged. Despite minimal changes in the mean SMI, a wide distribution in change of body composition was observed.

LOSS OF MUSCLE MASS AND DISEASE STAGE

After NACRT, lower SMI was found in patients with cT4 tumors when compared to patients with cT3 tumors (48.1 ± 8.3 versus 44.7 ± 8.2 , $p = 0.024$). No association between clinical disease stage and Δ SMI was observed.

For analytical purposes, gender-specific tertiles for Δ SMI were created ($< -1.95\%$; $-1.95\% - 1.84\%$; $> 1.84\%$ for male patients and $< -4.53\%$; $-4.53\% - 1.90\%$; $> 1.90\%$ for female patients). Comparing patients in the obtained tertiles for Δ SMI, no differences in patient demographic and clinical characteristics (i.e. age, gender, BMI, clinical TNM staging, CEA, tumor height, surgical procedure, and IORT), pathologic TNM staging, pathologic CRM, and pathologic complete response were observed. There was a weak negative relationship between pre-NACRT SMI and Δ SMI (Pearson's $r: -0.254$; $p = 0.005$), i.e. patients with a higher quantity of muscle mass prior to NACRT experienced greater loss of muscle mass. Vaso-invasion was present in 10 (31.2%) patients in the lower tertile, 3 (8.8%) in the middle tertile, and in 3 (9.4%) patients in the upper tertile for Δ SMI respectively ($p = 0.021$).

Table 1. Baseline Demographic and Clinical Characteristics of the 122 Patients Included in the Study.

		Number of patients	Median (IQR)
Age (years)			61 (53.0 – 66.3)
Gender (M : F)		71 : 51 (58.2% : 41.8%)	
Cardiac comorbidity (excluding hypertension)		10 (8.2%)	
Respiratory comorbidity		19 (15.6%)	
Diabetes		14 (11.5%)	
BMI (kg/m ²)*			24.3 (22.0 – 26.8)
Tumor location (cm)*	< 6	60 (49.6%)	
	≥ 6	61 (50.4%)	
CEA (ng/mL)*	< 5	32 (43.2%)	
	≥ 5	42 (56.8%)	
Clinical T-stage*	T3	65 (53.7%)	
	T4	56 (46.3%)	
Clinical N-stage*	N-	25 (20.7%)	
	N+	96 (79.3%)	
Clinical M-stage*	M0	82 (67.2%)	
	M1	40 (32.8%)	
Time interval between NACRT and resection (days)			70 (62.5 – 84.5)
Pathologic T-stage*	ypT0	25 (20.7%)	
	ypT1	4 (3.3%)	
	ypT2	16 (13.2%)	
	ypT3	52 (43.0%)	
	ypT4	24 (19.8%)	
Pathologic N-stage*	ypN0	84 (69.4%)	
	ypN1	25 (20.7%)	
	ypN2	12 (9.9%)	
Pathologic M-stage	ypM0	83 (68.0%)	
	ypM1	39 (32.0%)	
CRM	R0	100 (82.0%)	
	R1	20 (16.4%)	
	R2	2 (1.6%)	
Vaso-invasion*	No	82 (83.7%)	
	Yes	16 (16.3%)	
Perineural growth*	No	82 (83.7%)	
	Yes	16 (16.3%)	
Lymphoinvasion*	No	60 (95.2%)	
	Yes	3 (4.8%)	

		Number of patients	Median (IQR)
Surgical procedure (all open procedures)	LAR	45 (36.9%)	
	APR	45 (36.9%)	
	Pelvic exenteration	32 (26.2)	
Intraoperative radiotherapy		16 (13.1%)	
SMI pre-NACRT (cm ² /m ²)			46.6 (41.2 – 53.4)
SMI post-NACRT (cm ² /m ²)			46.9 (40.2 – 53.1)

*Data missing for some patients. M : F: Male : Female. BMI: Body-mass index. CEA: Carcinoembryonic antigen. SMI: Skeletal muscle index assessed at the third lumbar vertebrae, and standardized for patient height. NACRT: Neo-adjuvant chemoradiotherapy. CRM: Circumferential resection margin, an R1 resection was defined as a circumferential resection margin < 2mm.

OVERALL SURVIVAL

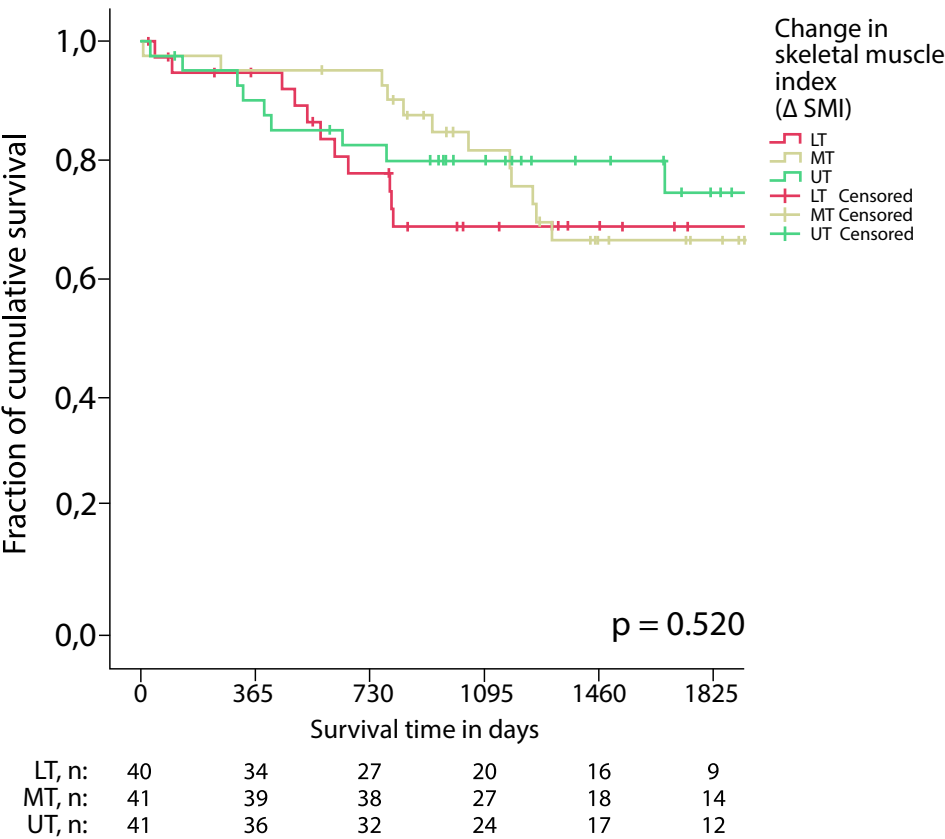
The one-, three-, and five-year overall survival (OS) rates in the current cohort were 93%, 77%, and 69% respectively. A median survival time was not reached. Patients in the lower tertile for Δ SMI had one-, three-, and five-year OS rates of 95%, 68%, and 68% respectively; patients in the middle tertile for Δ SMI had one-, three-, and five-year OS rates of 95%, 82%, and 65%; and patients in the higher tertile for Δ SMI had one-, three-, and five-year OS rates of 90%, 80%, and 74% (figure 1, log-rank $p = 0.520$).

Additionally, gender-specific cut-off values for sarcopenia as previously reported in literature were investigated for their impact on overall survival. [17] No association could be found between sarcopenia pre-operatively (i.e. using the post-NACRT CT scan) and OS (HR: 1.313; 95% CI: 0.675 – 2.551; $p = 0.422$) or sarcopenia pre-NACRT and OS (HR 1.183; 95% CI: 0.607 – 2.305; $p = 0.621$).

DISEASE-FREE SURVIVAL

The one-, two-, and three- -year disease-free survival (DFS) rates were 72%, 62%, and 57% respectively. Eight (6.6%) patients developed local recurrence, and 46 (37.7%) patients developed distant metastases. A median DFS time was not reached. An association was observed between Δ SMI and DFS in log-rank analysis (figure 2) and in multivariable analysis (HR 0.971; 95% CI: 0.946 – 0.996; $p = 0.025$). Moreover, analysis of patients without evidence of metastatic disease at presentation revealed that Δ SMI was an independent predictor for the development of distant metastases following curative intent treatment in multivariable Cox-regression analysis (HR 0.942; 95% CI: 0.898 – 0.988; $p = 0.013$) (Table 3).

Figure 1. Overall survival stratified for change in skeletal muscle index.



Loss of skeletal muscle mass during neoadjuvant chemoradiotherapy does not affect overall survival in rectal patients following surgical resection (log rank test $p = 0.520$).

The one-, three-, and five-year DMFS rates were 74%, 51%, and 51% respectively for patients in the lowest tertile for Δ SMI, compared with 77%, 73%, and 73% respectively for patients in the middle tertile for Δ SMI, and 100%, 92%, and 85% respectively for patients in the upper tertile for Δ SMI (figure 3).

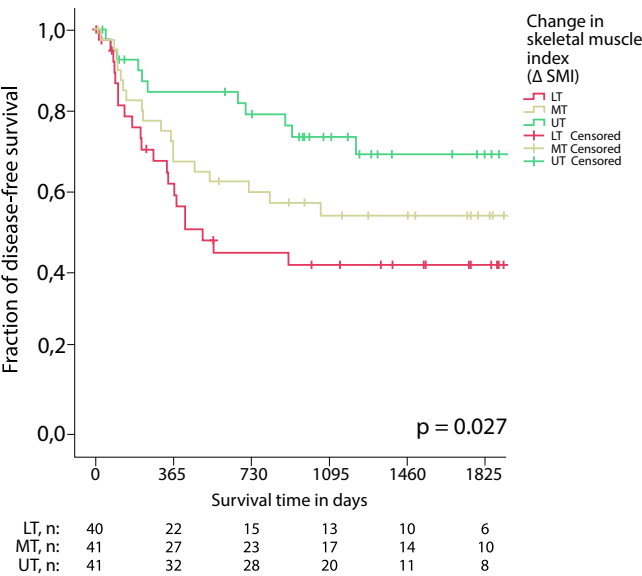
There was no association between pre-operative sarcopenia and DFS using pre-defined cut-off values (HR: 1.153; 95% CI: 0.662 – 2.009; $p = 0.615$). Likewise, there was no association between pre-NACRT sarcopenia and DFS (HR 0.910; 95% CI: 0.521 – 1.592; $p = 0.742$).

Table 2. Univariate and Multivariate Cox Regression Analysis for Disease-Free Survival.

		Univariate analysis		Multivariable analysis	
		Hazard Ratio	P	Hazard Ratio	P
Gender	Male	1.00 (reference)			
	Female	1.04 [0.59 – 1.81]	0.899		
Age	Per year	0.98 [0.96 – 1.00]	0.089		
Diabetes	No	1.00 (reference)			
	Yes	0.52 [0.16 – 1.68]	0.278		
Δ SMI	Per 1% change	0.96 [0.94 – 0.99]	0.004	0.97 [0.95 – 1.00]	0.025
BMI	Per kg/m ²	1.04 [0.97 – 1.12]	0.238		
Tumor location (cm)	< 6	1.00 (reference)			
	\geq 6	0.85 [0.48 – 1.48]	0.557		
CEA (ng/mL)	< 5	1.00 (reference)			
	\geq 5	1.56 [0.76 – 3.20]	0.223		
Surgical procedure	LAR	1.00 (reference)			
	APR	1.38 [0.71 – 2.70]	0.342		
	Pelvic exenteration	1.79 [0.87 – 3.67]	0.111		
Intraoperative radiotherapy	No	1.00 (reference)			
	Yes	2.11 [1.05 – 4.22]	0.035	1.44 [0.47 – 4.39]	0.523
Pathologic T-stage	ypT0 – ypT3	1.00 (reference)			
	ypT4	2.10 [1.13 – 3.89]	0.019	1.23 [0.56 – 2.71]	0.608
Pathologic N-stage	ypN0	1.00 (reference)			
	ypN1 or ypN2	2.44 [1.38 – 4.30]	0.002	1.85 [1.01 – 3.40]	0.047
CRM	R0	1.00 (reference)			
	R1 or R2	2.19 [1.17 – 4.13]	0.015	1.04 [0.37 – 2.94]	0.944
PCR	Yes	1.00 (reference)			
	No	3.72 [1.34 – 10.35]	0.012	2.75 [0.92 – 8.20]	0.069

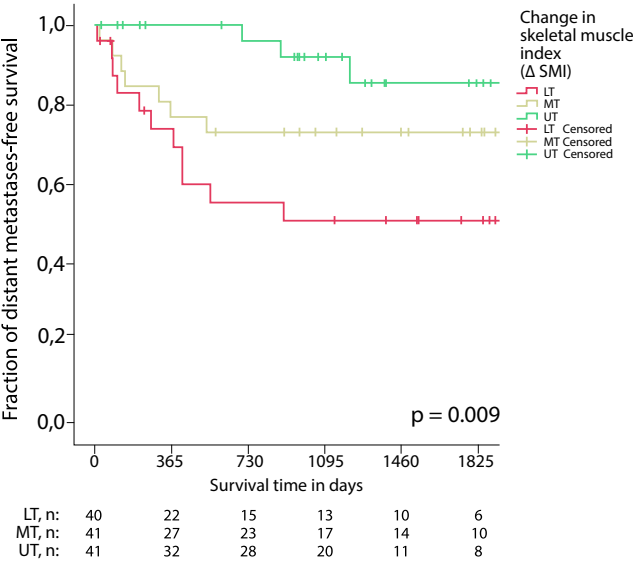
BMI: Body-mass index. CEA: Carcinoembryonic antigen. SMI: Skeletal muscle index assessed at the third lumbar vertebrae, and standardized for patient height. NACRT: Neo-adjuvant chemoradiotherapy. CRM: Circumferential resection margin. PCR: Pathological complete response.

Figure 2. Disease-free survival stratified for change in skeletal muscle index.



Loss of skeletal muscle mass during neoadjuvant chemoradiotherapy is associated with impaired disease-free survival in rectal cancer patients following surgical resection (log-rank $p = 0.027$).

Figure 3. Distant metastases-free survival stratified for change in skeletal muscle index.



Loss of skeletal muscle mass during neoadjuvant chemoradiotherapy is associated with the development of distant metastases following curative intent treatment in patients without evidence of metastatic disease at presentation (log-rank $p = 0.009$).

Table 3. Univariate and Multivariate Cox Regression Analysis for Distant Metastasis-Free Survival in Patients without Evidence of Metastatic Disease at Presentation.

		Univariate analysis		Multivariable analysis	
		Hazard Ratio	P	Hazard Ratio	P
Gender	Male	1.00 (reference)			
	Female	1.38 [0.58 – 3.27]	0.469		
Age	Per year	0.98 [0.95 – 1.02]	0.274		
Diabetes	No	1.00 (reference)			
	Yes	0.04 [0.00 – 21.38]	0.319		
Δ SMI	Per 1% change	0.93 [0.88 – 0.98]	0.007	0.94 [0.90 – 0.99]	0.013
BMI	Per kg/m ²	1.10 [0.99 – 1.22]	0.084		
Tumor location (cm)	< 6	1.00 (reference)			
	≥ 6	0.42 [0.16 – 1.09]	0.073		
CEA (ng/mL)	< 5	1.00 (reference)			
	≥ 5	1.31 [0.42 – 4.10]	0.638		
Surgical procedure	LAR	1.00 (reference)			
	APR	1.80 [0.60 – 5.37]	0.294		
	Pelvic exenteration	2.09 [0.66 – 6.60]	0.209		
Intraoperative radiotherapy	No	1.00 (reference)			
	Yes	2.61 [0.96 – 7.14]	0.061		
Pathologic T-stage	ypT0 – ypT3	1.00 (reference)			
	ypT4	1.90 [0.73 – 4.89]	0.186		
Pathologic N-stage	ypN0	1.00 (reference)			
	ypN1 or ypN2	3.68 [1.56 – 8.69]	0.003	3.49 [1.46 – 8.35]	0.005
CRM	R0	1.00 (reference)			
	R1 or R2	2.32 [0.90 – 5.98]	0.082		
PCR	Yes	1.00 (reference)			
	No	29.94 [0.37 – 2424.34]	0.129		

BMI: Body-mass index. CEA: Carcinoembryonic antigen. SMI: Skeletal muscle index assessed at the third lumbar vertebrae, and standardized for patient height. NACRT: Neo-adjuvant chemoradiotherapy. CRM Circumferential resection margin. PCR Pathological complete response.

DISCUSSION

This study describes the change in body composition which may be observed in patients undergoing NACRT for locally advanced rectal cancer. This is the first study to show that loss of muscle mass during NACRT, assessed by use of routinely obtained diagnostic CT images, has a strong association with disease-free survival and distant metastasis-free survival. This technique is inexpensive, readily available, and may thus help identify patients at risk for detrimental outcome. The results of this study may be used to determine inclusion criteria for future clinical studies investigating treatment regimens aimed at stopping or reversing muscle loss in cancer patients, as well as for future clinical studies investigating follow-up regimens following curative intent rectal cancer surgery.

A wide variation was observed in the amount of muscle loss during NACRT. As such, tumor biology rather than NACRT per se is more likely to be the causative factor inducing this catabolic state. Opposed to what we expected, we did not observe any association between disease-stage and the amount of muscle loss during NACRT. However, we did observe an association between vascular invasion and muscle loss during NACRT. Colorectal cancer is known to be associated with different molecular subtypes, with no association to TNM staging.²⁷ Select molecular subtypes may be associated with a more aggressive tumor biology and stronger systemic catabolic response. A study investigating the association between colorectal cancer genotyping and muscle wasting is currently being undertaken by our research group.

Skeletal muscle loss during NACRT was associated with poor disease-free survival, and a higher risk of developing distant metastasis during follow-up in the current population. These findings are in line with prior literature on esophageal cancer and non-resectable colorectal cancer patients.^{19, 20} Another study showed that loss of muscle mass during NACRT is associated with increased postoperative mortality following surgical resection for esophageal cancer.¹⁹ Yet another study reported non-resectable colorectal cancer patients receiving systemic therapy to have a reduction in both progression-free survival and overall survival if skeletal muscle loss was observed during treatment.²⁰ While loss of muscle mass during NACRT was strongly associated with DFS and DMFS, single time point measurements for sarcopenia that are widely used were not predictive of survival in the current population.

Despite mounting evidence for sarcopenia and muscle wasting to be associated with poor survival and decreased quality of life^{28, 29}, it is still unknown whether targeted treatment of muscle wasting may improve outcome. Over the past decade our understanding of muscle wasting in cancer has greatly increased^{30, 31}, and has led to the initiation of clinical trials investigating interventional strategies aimed at halting or reversing cancer related muscle wasting.³²⁻³⁵ Whether these treatment regimens are efficacious remains to be answered, but if so the interval between chemoradiotherapy and surgery might offer a perfect window of opportunity to improve the overall condition of LARC patients.

There are several limitations to this present study, some of which have already been described. Information regarding change of bodyweight was not gathered routinely in this cohort. Furthermore, information regarding possible lack of appetite, anorexia, was not available on a consistent basis. Likewise, no information regarding physical status and performance was available for these patients. Lastly, although suggestively differences in tumor biology may explain the findings reported within this study, validating this hypothesis was not within the scope of the current study. Data regarding vaso-invasion, perineural growth, and lymphoinvasion was missing for a considerable number of patients. Due to consequential loss of power we did not include these prognostic factors in our multivariable analyses.

This study found loss of skeletal muscle mass during, but not necessarily attributable to, neoadjuvant chemoradiotherapy in resectable rectal cancer patients to be a novel independent prognostic factor for disease-free survival and distant metastasis-free survival following total mesorectal excision. This knowledge may benefit in patient expectation management following curative intent treatment, as well as provide grounds for future clinical studies investigating whether there may be a role for adjuvant therapy in patients showing greatest loss of muscle mass, i.e. who were found to have the highest rate of metastasis development.

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PART THREE

ATTENUATING SKELETAL MUSCLE WASTING IN EXPERIMENTAL CANCER-ASSOCIATED CACHEXIA

Chapter 8

Inhibition of activin-like kinase 4/5 attenuates cancer cachexia associated muscle wasting

Chapter 9

Caloric restriction is associated with preservation of muscle strength in experimental cancer cachexia

Chapter 10

Quercetin supplementation attenuates muscle Wasting in cancer-associated cachexia in mice

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CHAPTER 8

INHIBITION OF ACTIVIN-LIKE KINASE 4/5 ATTENUATES CANCER CACHEXIA ASSOCIATED MUSCLE WASTING

ABSTRACT

Cancer mediated activation of the ActRIIB-ALK4/5 heterodimer by myostatin is strongly associated with muscle wasting. We investigated in vitro and in vivo the efficacy of ALK4/5 receptor blockers SB431542 and GW788388 in preventing muscle wasting, and explored synergy with IGF-I analogue LONG R3 (LR3) IGF-I. In vitro, C2C12 skeletal muscle cells were treated with vehicle, SB431542, GW788388 and LR3 IGF-I. A C26-CD2F1 cachexia model was used to induce cachexia in vivo. Mice were allocated as non-tumor bearing (NTB) or C26 tumor-bearing (C26 TB) vehicle control, treated with SB431542, LR3 IGF-I, SB431542 and LR3 IGF-I, or GW788388 (intraperitoneally or orally). In vitro, differentiation index and mean nuclei count increased using SB431542, GW788388, LR3 IGF-I. In vivo, GW788388 was superior to SB431542 in limiting loss of bodyweight, grip-strength and gastrocnemius weight. and downregulated Atrogin-1 expression comparable to NTB mice. LR3 IGF-I treatment limited loss of muscle mass, but at the expense of accelerated tumor growth. In conclusion, treatment with GW788388 prevented cancer cachexia, and downregulated associated ubiquitin ligase Atrogin-1.

INTRODUCTION

Progressive skeletal muscle wasting, with or without loss of adipose tissue, is observed in up to 50 per cent of all cancer patients.^{1,2} This multifactorial syndrome is known as cachexia, and cannot be fully reversed by conventional nutritional support. Cachexia leads to progressive functional impairment.³ Up to 20 per cent of all cancer-associated deaths may be attributed to cachexia, through the sequelae of immobility and cardiac or respiratory failure.^{2,4} We have shown that skeletal muscle wasting is associated with poor outcome in patients with colorectal and hepatopancreatobiliary malignancies.⁵⁻⁸ Catabolic cytokines released due to the tumor-host interaction⁹ and miRNA cargo bearing microvesicles¹⁰ are key pathogenic mechanisms leading to cancer cachexia, further impacted patient factors, including age and levels of physical activity.^{11,12}

Myostatin, also known as growth and differentiation factor 8 (GDF-8), is a member of the transforming growth factor beta (TGF- β) superfamily¹³ and is an essential regulator of muscle fibre growth and differentiation, i.e. myostatin limits muscle fibre growth.¹⁴⁻¹⁶ Myostatin has a high affinity for the skeletal muscle cell-surface activin IIB receptor (ActRIIB). After binding to myostatin this receptor forms a heteromeric complex with activin-like kinases four (ALK4) and five (ALK5) and activates the myostatin signal transduction pathway,¹⁷⁻¹⁹ including Smad2/3 and MAPK.²⁰ Activation of Smad2/3 not only induces an Akt-mediated FoxO-dependent muscle protein breakdown via the ubiquitin-proteasome system but also decreases muscle protein synthesis via inhibition of Akt.^{21,22} Disruption of the myostatin gene is associated with gross muscle hypertrophy.²³⁻²⁶ Likewise, elevated myostatin levels are associated with progressive muscle wasting in chronic obstructive pulmonary disease (COPD), chronic heart failure (CHF), acquired immunodeficiency syndrome (AIDS), liver cirrhosis, ageing, and experimental cancer models.²⁷⁻³²

Systemic administration of myostatin induces cachexia in mice,³³ whereas inhibition of myostatin using modified RNA oligonucleotides, systemic administration of the activin receptor extracellular domain/Fc fusion protein (ACVR2B-Fc), and soluble ActRIIB receptor preserve skeletal muscle mass in experimental cancer cachexia.³⁴⁻³⁷ Moreover, ALK4 has recently been reported to play a pivotal role in both myogenesis as well as the regulation of protein synthesis and degradation in skeletal muscle cells in mdx muscular dystrophy mice.³⁸ In contrast to myostatin, insulin-like growth factor 1 (IGF-I) is an important anabolic regulator of muscle fibre growth and differentiation.

Via activation of the IGF-I/PI3K/Akt signalling pathway, it not only regulates protein synthesis but also limits upregulation of key mediators of skeletal muscle atrophy, i.e. MuRF1 and FBXO32, more commonly referred to as MAFbx/Atrogin-1.^{39, 40} Such upregulation of MuRF1 and MAFbx/Atrogin-1 has extensively been reported in experimental cancer models. Recently, it has also been reported to be present in patients with malignancies.^{41, 42} Furthermore, decreased serum levels of IGF-I have been reported in experimental cancer cachexia and cachectic gastric cancer patients.^{43, 44} Considering its strong anabolic potential such decrease may aggravate muscle wasting in cancer. Of paramount clinical concern in regard to IGF-I treatment for cancer cachexia is the potential of IGF-I to accelerate tumor growth.^{45, 46} However, in vivo supplementation of similar growth factors, i.e. growth hormone-releasing hormone (GHRH) and a recombinant human IGF-I/insulin-like growth factor binding protein-3 complex (rhIGF-I/IGFBP-3) as a potential treatment for cancer cachexia had no effect on tumor growth.^{47, 48} In contrast, supplementation of insulin-like growth factors attenuates muscle wasting in experimental cancer cachexia models.^{48, 49}

Taking these data into consideration, we sought to determine whether (1) systemic inhibition of ALK4/5, and thus potentially blocking the myostatin signalling pathway, enhances myogenesis in vitro and limits muscle wasting in experimental cancer cachexia in vivo, and (2) whether combined treatment of ALK 4/5 inhibition and IGF-I supplementation would improve treatment outcome without impacting on tumor growth.⁵⁰ Our data shows that both ALK4/5 receptor inhibition and LONG R3 IGF-I analogues enhance C2C12 skeletal muscle cell differentiation in vitro, successfully limit cancer cachexia in vivo, and down-regulates the associated target genes.

MATERIALS & METHODS

MATERIALS

SB431542

SB431542 (Bio-connect BV, Huissen, The Netherlands) is a potent and selective inhibitor of the transforming growth factor- β type I receptors ALK4, ALK5, and ALK7. It has no effect on ERK1, ERK2 or JNK in C2C12 cells in concentrations up to 10 μ M.⁵¹ It does however weakly inhibit MAP kinase p38 α , but not any of the other p38 MAP kinases.⁵¹ C2C12 cells treated with anti-myogenic TGF- β 1 have previously shown full rescue of myogenic effect with the addition of SB431542.⁵² And SB431542 has been shown to inhibit myostatin induced C-terminal Smad2 phosphorylation.⁵³ Dose, schedule and route of administration for in vivo experiments are specified in table 1.

GW788388

GW788388 (Sigma-Aldrich, St. Louis, The United States of America) is a potent and selective inhibitor of the transforming growth factor- β type I receptor ALK5. GW788388 has a dose-dependent inhibition of TGF- β induced Smad activation and it has no effect on ERK1, ERK2 or p38 MAPK⁵⁴. GW788388 has been shown to be orally active⁵⁵. Dose, schedule and route of administration for in vivo experiments are specified in table 1.

LONG R3 IGF-I

LONG R3 IGF-I (Bio-connect BV, Huissen, The Netherlands) is a recombinant analogue of human insulin-like growth factor-I (IGF-I). Dose, schedule and route of administration for in vivo experiments are specified in table 1.

Table 1. Dose, schedule, and route of administration for in vivo experiments.

	Dose	Schedule	Route of administration (volume)
DMSO (control)	-	Daily	IP (1 μ L/g BW)
SB431542	10 mg/kg	Daily	IP (1 μ L/g BW)
LONG R3 IGF-I	200 μ g	Every other day	IM (50 μ L)
GW788388	10 mg/kg	Daily	IP (1 μ L/g BW)
GW788388	10 mg/kg	Daily	PO (250 μ L)

IP Intraperitoneally. IM Intramuscularly. PO Orally.

CELL CULTURES

Colon-26 (C26) adenocarcinoma cells (kindly provided by Dr D.O. McCarthy, Ohio State University, Columbus, OH, USA) were maintained in RPMI 1640 (Westburg BV, Leusden, The Netherlands) supplemented with 10% foetal bovine serum (FBS, Sigma-Aldrich, St. Louis, The United States of America), and 1% penicillin/streptomycin (P/S, Fisher Scientific, Waltham, The United States of America) at 37 °C in a 5% carbon dioxide environment. C2C12 muscle myoblast cells were obtained from American Type Culture Collection (ATCC-CRL-1772, ATCC, Manassas, VA, USA) and maintained in growth medium (GM) consisting of DMEM (glutamine)(Westburg BV, Leusden, The Netherlands) supplemented with 10% FBS, and 1% P/S at 37 °C in a 10% carbon dioxide environment. To induce myogenic differentiation, at near-confluence, GM was substituted with differentiation medium (DM) consisting of DMEM supplemented with 2% horse serum (HS) (Fisher Scientific, Waltham, The United States of America), and 1% P/S. DM was routinely changed every 24 h.

IN VITRO MODEL

C2C12 cells were plated on 0.1% gelatine-coated coverslips in 6-well plates (3x10⁴ cells/cm²) and supplemented with GM. Following overnight attachment, GM was substituted with DM with treatment or vehicle (DMSO 0.1%) (Sigma-Aldrich, St. Louis, The United States of America) for up to 6 days (2, 4, and 6 d). Treatment consisted of SB431542 (dosages: 0.1 µM, 1.0 µM, 2.0 µM, or 5.0 µM), GW788388 (dosages: 1.0 µM, 2.0 µM, 5.0 µM, or 10.0 µM), or LR3-IGF-I (5 ng/mL, 10 ng/mL, 20 ng/mL, or 30 ng/mL). Each treatment was performed in triplicate.

FUSION INDEX

The coverslips were stained with haematoxylin and eosin (H&E) according to standard laboratory protocols. Images were acquired from four predefined fields per well at a magnification of 200x. Differentiation into myotubes was determined by determining the fusion index by manually counting the number of nuclei in multinucleated myotubes, i.e. myotubes with 2 or more nuclei, divided by the total number of nuclei.⁵⁶

ANIMAL ETHICS COMMITTEE APPROVAL

All animal experiments were performed with the approval of the Animal Experiments Committee of the Erasmus University Medical Centre, Rotterdam, the Netherlands and in accordance to the Dutch National Experiments on Animal Act, and complied with the EU adopted Directive 86/609/EEC (1986).

ANIMALS

Male CD2F1 (BALB/c × DBA/2 F1) mice of 8 weeks (~ 25 grams) were obtained from Charles River, Maastricht, the Netherlands. Upon arrival, animals have been housed in individually ventilated cages and maintained at 22 °C under a 12 h light-dark cycle with ad libitum access to CRM (P) chow (Special Diet Services, Witham, Essex, UK) and water (n = 3 – 4 animals per cage). Animals were acclimatized for one week prior to the start of the experiments.

C26 TUMOR-BEARING MICE

Animals allocated in tumor bearing (TB) groups received a subcutaneous (SC) inoculation in the right flank with 0.5×10^6 C26 adenocarcinoma cells in 100 µL sterile PBS under anaesthesia by isoflurane inhalation (5% isoflurane induction), a classic model of cancer cachexia.⁵⁰

ASSESSMENT OF GRIP-STRENGTH

The effect of treatment on muscular strength was quantified via the widely used grip-strength test of Meyer et al.⁵⁷ Combined hind- and forelimb grip strength was measured twice per week by placing the animal on a grid (8 x 8 cm) attached to a force gauge (BIOSEB, Chaville, France). The mice were allowed to grasp on to the grid. Thereafter, the mice were gently pulled by the tail along the sensor axle until grip is released. Grip strength assessment was performed at the same time per day and prior to administration of the investigated treatment agents. Maximum strength produced before releasing the grid was registered in triplicate with one minute rest period for each animal. Obtained values were averaged to provide a mean force measurement for each individual animal and subsequently normalized to each animal's grip-strength respectively on day 0. All measurements were performed blind with respect to treatment.

BODYWEIGHT, MUSCLE MASS, AND TUMOR SIZE

Bodyweight was recorded daily. Bodyweight was normalized to each animal's body weight on day 0. Tumor size was recorded every other day starting on day 9 after tumor inoculation using digital callipers. Tumor mass was estimated via the formula $\text{mass (mg)} = \text{tumor volume (mm}^3\text{)} = \text{width}^2 \times \text{length} / 2$.⁵⁸ Animals were sacrificed by cervical dislocation under isoflurane anaesthesia on day 21 or upon body weight loss exceeding 20%. Gastrocnemius (GCM), tibialis anterior (TA), and soleus (Sol) muscles of both hind legs and tumor were dissected, weighed and immediately snap-frozen in liquid nitrogen and stored at -80 °C until analysis.

RNA ISOLATION AND REAL-TIME POLYMERASE CHAIN REACTION

Cancer-cachexia associated muscle wasting is known to be most pronounced in fast-twitch type II-containing muscles, such as GCM and TA.⁵⁹ Therefore, for gene expression analysis, total RNA was isolated from snap-frozen GCM muscle tissue using Trizol reagent (Invitrogen, Breda, the Netherlands), and subsequently purified by DNase treatment (RQ1 RNase-Free DNase) (Promega Benelux B.V., Leiden, the Netherlands). 1 µg of total RNA was reversed transcribed to cDNA using random hexamer primers (Invitrogen, Breda, the Netherlands), and Superscript II RT (Invitrogen, Breda, the Netherlands). Quantitative real-time polymerase chain reaction (RT-PCR) was performed using an iCycler real-time PCR system (Biorad, California, The United States of America) using SYBR Green (Sigma-Aldrich, St. Louis, The United States of America). Used primer sequences can be found in Table 2. GAPDH, HPRT and HMBS were selected as housekeeping genes for normalization from commonly used housekeeping genes, i.e. ACTB, B2M, GAPDH, HMBS, HPRT, RPL13A, SDHA, TBP, UBC and YHWAZ, after being tested using a gene-stability measure developed by Vandesompele et al.⁶⁰ as previously described.⁶¹ The geometric mean was used to average the control genes.

Table 2. Reverse transcription-polymerase chain reaction primer sequences.

Gene	Forward Primer	Reverse Primer	Genbank Accession Number
Atrogin1	5'-GTTTTTCAGCAGGCAAGAAG	5'-TTGCCAGAGAACACGCTATG	AF_441120
MyoD	5'-AAACCCCAATGCGATTATCAGG	5'-TAAGCTTCATCTTTGGGCGTGA	NM_010866
Myogenin	5'-CACTCCCTTACGTCCATCGT	5'-CAGGACAGCCCCACTTAAAA	NM_031189
Murf1	5'-AGGTGTCAGCGAAAAGCAGT	5'-CCTCCTTTGTCCTCTTGCTG	NM_009066
GAPDH	5'-ATGCATCCTGCACCACCAACT	5'-GCAGTGATGGCATGGACTGTG	NM_008084
Hmbs/Pgbd	5'-GTGCCATTGTCCTGGCTGTG	5'-TGCATTCTCTGGGTGCAAA	-
HPRT	5'-AAGCAGTACAGCCCCAAAATGG	5'-CCAACAAAGTCTGGCTGTATCC	-

STATISTICS

Categorical data are expressed as number (percentage) and continuous variables as mean \pm SEM (normal distribution, visually assessed and by means of the Shapiro-Wilks test) or median and (range). Body weight and grip-strength were normalized to each animal's body weight and grip-strength respectively on day 0. Muscle weight from the left hind leg and right hind leg were averaged to provide a mean muscle weight (GCM, TA, and Sol) for each animal. We tested the difference between healthy animals and untreated, TB animals using an unpaired t-test. Multiple group comparisons were done by one-way ANOVA with a Bonferroni's post hoc test. Spearman-Rho rank correlation coefficient was used for testing bivariate correlations. All analyses were performed using IBM SPSS Statistics for Windows, version 21.0 (IBM Corp., Armonk, NY, USA). A P value < 0.05 was considered statistically significant.

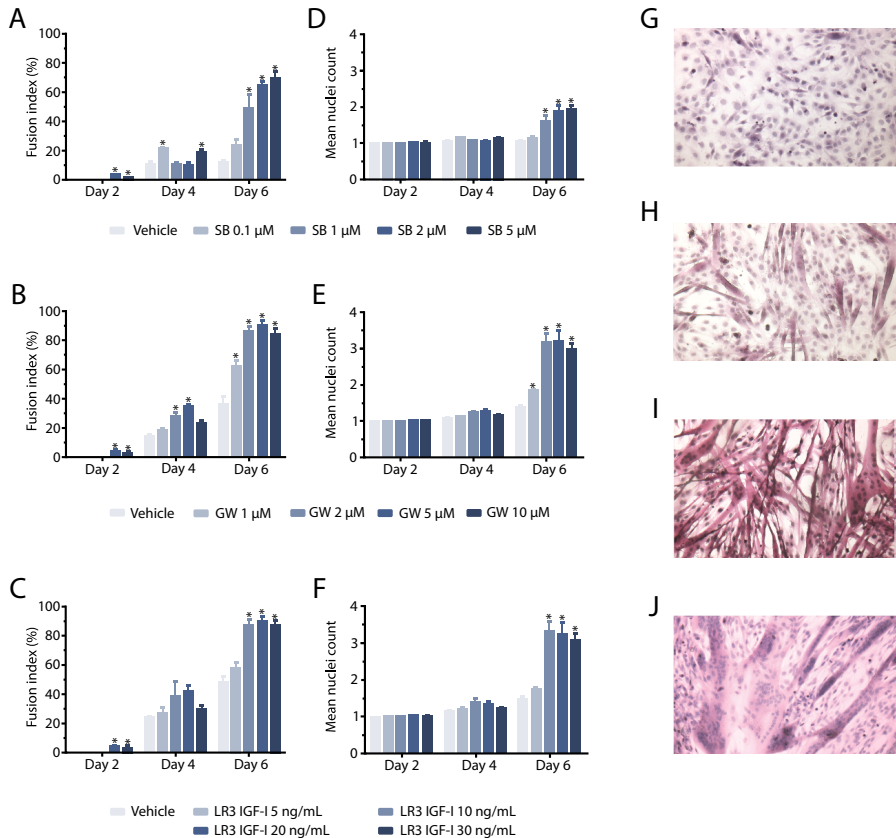
The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

RESULTS

SB431542, GW788388, AND LONG R3 IGF-I ENHANCE MYOGENESIS IN C2C12 CELLS

We investigated the efficacy of ALK4/5 inhibitors SB431542 and GW788388, and the IGF-I analogue LONG R3 IGF-I, in vitro using the C2C12 skeletal muscle cell model. C2C12 myoblasts were cultured in differentiation medium (DM) supplemented with ALK4/5 inhibitors SB431542 (figure 1A), or GW788388 (figure 1B) during six days.

Figure 1. Fusion indices of C2C12 cells treated with SB431542, GW788388 and LONG R3 IGF-I.



Fusion indices of C2C12 cells on days two, four, and six for (A) different concentrations of SB431542 (SB), (B) GW788388 (GW), and (C) LONG R3 IGF-I (LR3 IGF-I) and mean nuclei count for (D) SB431542, (E) GW788388, and (F) LONG R3 IGF-I. * A statistically significant difference ($p < 0.05$ of two-way ANOVA followed by a post hoc Bonferroni test) was observed compared to vehicle samples on the corresponding day. Representative images of H&E stained differentiated (G) vehicle treated C2C12 cells, (H) SB431542 treated cells, (I) GW788388 treated cells, and (J) LONG R3 IGF-I treated C2C12 cells. All acquired images were taken from day 6 samples.

In groups treated with SB431542 1 μ M ($48.8\% \pm 19.6$, $p = 0.001$), 2 μ M ($64.6\% \pm 5.2$, $p < 0.001$), or 5 μ M ($69.8\% \pm 8.4$, $p < 0.001$) differentiation into myotubes as determined by the fusion index on day 6 was significantly higher compared to vehicle treated controls ($11.8\% \pm 2.4$). There were no statistically significant differences between these three treatment concentrations. However, fusion index on day 6 was significantly higher in groups treated with SB431542 1 μ M ($p = 0.042$), 2 μ M ($p = 0.001$), or 5 μ M ($p < 0.001$) compared to treatment with 0.1 μ M ($24.0\% \pm 7.1$). The mean number of nuclei in these cells were 1.1 in the vehicle group, compared to groups treated with SB431542 0.1 μ M, 1.2 ($p > 0.999$), 1 μ M, 1.6 ($p < 0.001$), 2 μ M, 1.9 ($p < 0.001$), and 5 μ M, 1.9 ($p < 0.001$) (figure 1D).

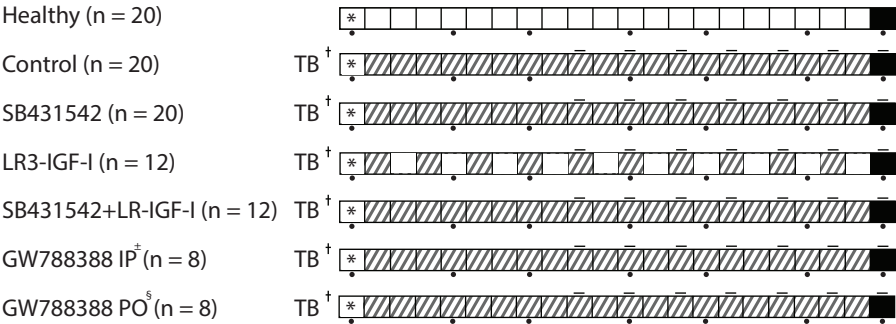
GW788388 enhanced differentiation of C2C12 comparable to, if not better than, SB431542 (figure 1A-B). In groups treated with 1 μ M ($62.5\% \pm 7.8$, $p = 0.004$), 2 μ M ($86.1\% \pm 7.4$, $p < 0.001$), 5 μ M ($90.1\% \pm 6.6$, $p < 0.001$), and 10 μ M GW788388 ($84.3\% \pm 6.7$, $p < 0.001$) the fusion index on day 6 was significantly higher compared to vehicle treated control cells ($36.3\% \pm 11.4$). There were no statistically significant differences between treatment with 2 μ M, 5 μ M, and 10 μ M. However, fusion index on day 6 was significantly higher in groups treated with GW788388 2 μ M ($p = 0.010$), 5 μ M ($p = 0.002$), or 10 μ M ($p = 0.019$) compared to treatment with 1 μ M. The mean number of nuclei in these cells was 1.4 in the vehicle group, compared to groups treated with GW788388 1 μ M, 1.9 ($p = 0.014$), 2 μ M, 3.2 ($p < 0.001$), 5 μ M, 3.2 ($p < 0.001$), and 10 μ M, 3.0 ($p < 0.001$) (figure 1E).

LONG R3 IGF-I gave similar results as GW788388 treatment (figure 1C). In groups treated with LONG R3 IGF-I 10 ng/mL ($87.3\% \pm 7.9$, $p < 0.001$), 20 ng/mL ($90.0\% \pm 6.5$, $p < 0.001$), or 30 ng/mL ($87.0\% \pm 6.4$, $p < 0.001$) the fusion index was significantly higher compared to vehicle treated control cells ($48.0\% \pm 8.9$). There were no statistically significant differences between these three treatment concentrations. However, fusion index on day 6 was significantly higher in groups treated with LONG R3 IGF-I 10 ng/mL ($p = 0.001$), 20 ng/mL ($p < 0.001$), or 30 ng/mL ($p = 0.001$) compared to 5 ng/mL ($58.2\% \pm 6.9$). The mean number of nuclei in these cells was 1.5 in the vehicle group, compared to groups treated with LONG R3 IGF-I 5 ng/mL, 1.6 ($p = 0.513$), 10 ng/mL, 3.3 ($p < 0.001$), 20 ng/mL, 3.2 ($p < 0.001$), and 30 ng/mL, 3.1 ($p < 0.001$) (figure 1F).

The observed differences in fusion index and increase in mean nuclei as a surrogate measurement for muscle hypertrophy suggest enhanced myogenesis in favour of GW788388 and LONG R3 IGF-I compared with SB431542. Representative light microscopy images of the vehicle (0.1% DMSO) treated-, SB431542 treated-, LONG R3 IGF-I treated-, and GW788388 treated C2C12 cells can be found in figure 1G-J.

Figure 2. Experimental groups and timeline.

Efficacy of GW788388, SB431542 ± LR3-IGF-I



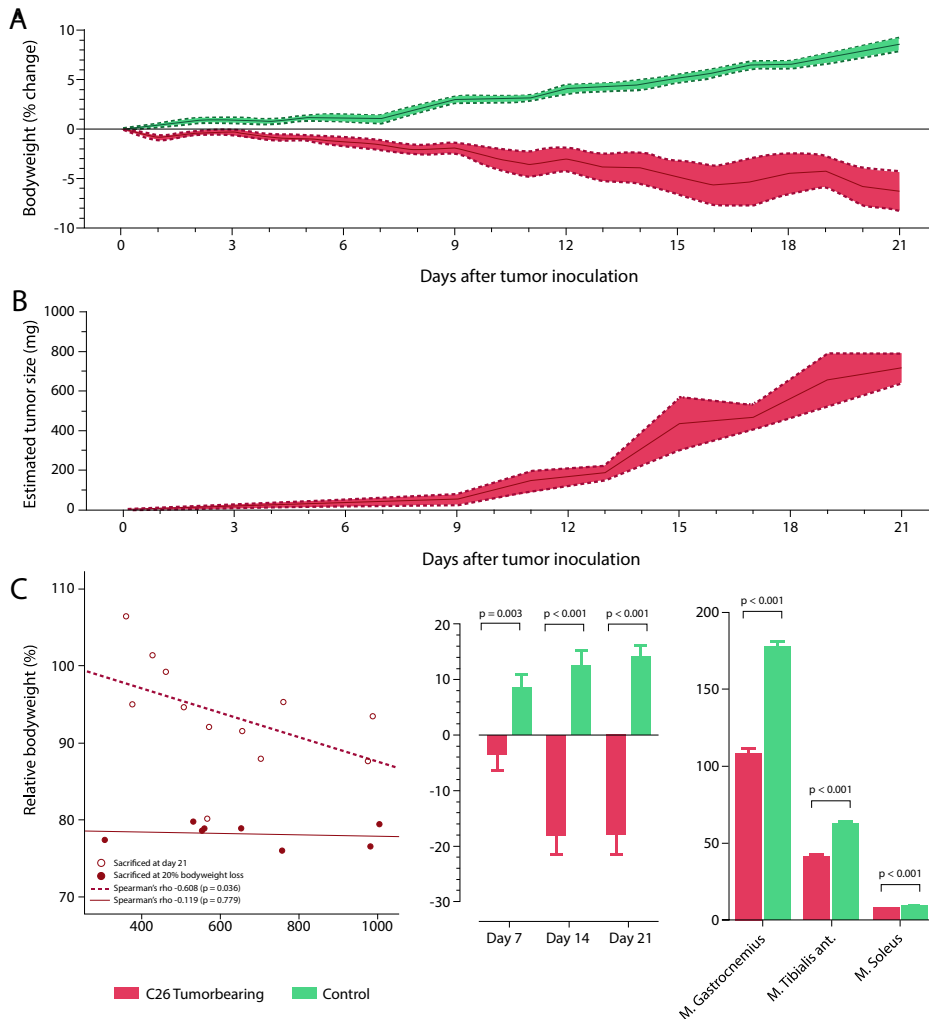
Allocation of male CD2F1 mice to different treatment groups. Each box represents a day, ranging from day 0 to day 21. * Start of the experiment (day 0). † Inoculation on day 0 of C26 cells in Tumor-Bearing animals. ‡ Intraperitoneal. § Orally. Striped boxes indicate day of treatment. Black dots below boxes indicate time points of grip-strength measurement. Lines above boxes indicate time points of tumor size measurement. Black boxes indicate the end of the experiment.

TREATMENT WITH ALK4/5 INHIBITORS LIMITS MUSCLE WASTING AND LOSS OF GRIP-STRENGTH IN C26 TB MICE

Following the favourable *in vitro* results of ALK 4/5 inhibition and LONG R3 IGF-I treatment, we sought to investigate whether treatment with either of these substances alone, or a combination thereof may limit muscle wasting in cancer cachexia. For this purpose, one hundred male CD2F1 mice were allocated to seven groups (figure 2). Mice allocated in tumor-bearing groups were inoculated subcutaneously with 0.5×10^6 C26 adenocarcinoma cells. The tumor-bearing mice receiving vehicle treatment experienced hallmark features of cancer cachexia, including progressive body weight loss, loss of grip strength and muscle weight loss (figure 3). One day after tumor inoculation allocated mice received a daily intraperitoneal injection with ALK 4/5 inhibitors SB431542 (SB, 10 mg/kg) or GW788388 (GW IP, 10 mg/kg). Considering GW788388 is orally active⁵⁵, an additional group of mice received GW788388 via oral gavage (GW PO, 10 mg/kg).

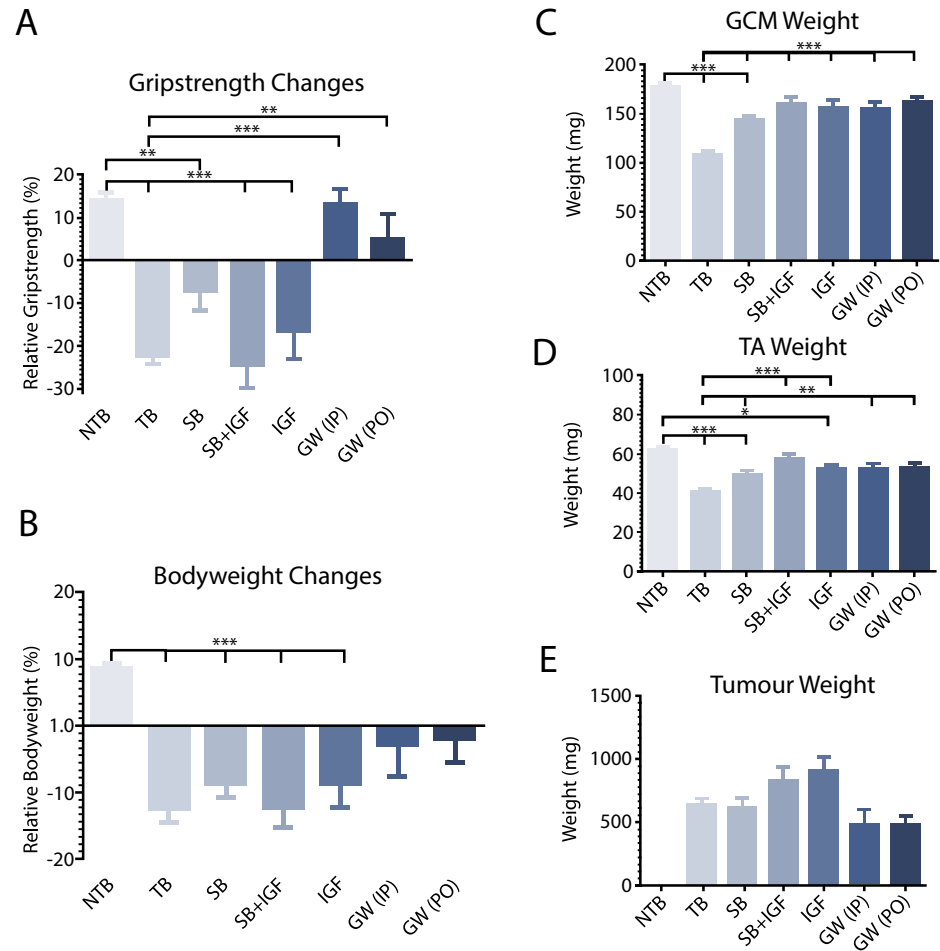
Throughout the experiment, grip-strength of GW PO mice, GW IP mice, and NTB mice was comparable ($p > 0.05$ at all timepoints, figure 4A). SB treated mice and untreated C26 TB mice experienced a statistically significant loss of grip-strength starting on day 14 compared to NTB mice ($p = 0.006$ and $p < 0.001$ respectively) which remained present until sacrifice (figure 4A).

Figure 3. In vivo C26 - CD2F1 cachexia model (impact on body weight, tumor size, grip-strength and muscle mass).



Data depicted in red indicates C26 tumor-bearing, vehicle treated male CD2F1 mice ($n = 20$). Data depicted in green indicates healthy, non-tumor-bearing male CD2F1 mice ($n = 20$). (A) Loss of bodyweight (mean \pm SEM) in vehicle-treated, C26 tumor-bearing (TB) mice ($p < 0.001$). (B) Estimated tumor growth rate as measured every other day, starting on day 9, via digital callipers. (C) Relation between tumor mass and relative bodyweight change. No relation could be observed for animals experiencing rapid bodyweight loss requiring early sacrifice ($n = 8$, Spearman's $\rho = -0.119$, $p = 0.779$). In animals not (yet) experiencing this rapid decline of bodyweight loss prior to day 21, tumor mass was negatively associated with change in bodyweight ($n = 12$, Spearman's $\rho = -0.608$, $p = 0.036$). (D) Untreated C26 TB male CD2F1 mice ($n = 20$) experienced loss of grip-strength throughout the experiments as compared with non-tumor-bearing male CD2F1 mice ($n = 20$). Student's t-test was conducted performed for on the mean differences of grip-strength change. This loss of grip-strength could already be observed on day 7 ($p = 0.003$), but became more apparent on day 14 ($p < 0.001$). (E) Student's t-test was conducted performed for on the mean weight of m. gastrocnemius, m. tibialis anterior and m. soleus. Wet muscle weight of gastrocnemius ($p < 0.001$), tibialis anterior ($p < 0.001$), and soleus ($p < 0.001$) muscles was significantly reduced in untreated, C26 TB male CD2F1 mice ($n = 20$) as compared with non-tumor-bearing male CD2F1 mice ($n = 20$).

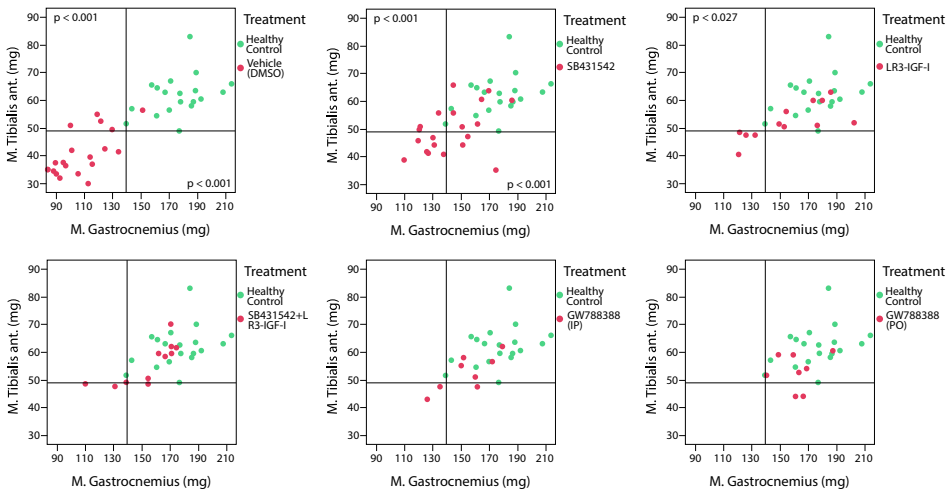
Figure 4. Treatment efficacy of SB431542, GW788388 and LONG R3 IGF-I on bodyweight, grip strength, tumor weight and muscle weight at sacrifice.



Bar graphs depicting the mean \pm SEM (A) relative grip strength immediately prior to sacrifice, (B) relative bodyweight at sacrifice, (C) gastrocnemius (GCM) muscle weight, (D) tibialis anterior (TA) muscle weight, (E) tumor weight for non-tumor bearing (NTB) ($n = 20$); C26 tumor-bearing (TB) vehicle treated ($n = 20$); SB431542 treated (SB, $n = 20$); SB431542 with LONG R3 IGF-I (SB+IGF, $n = 12$); LONG R3 IGF-I treated (IGF, $n = 12$) and GW788388 (GW) treated intraperitoneally (IP, $n = 8$) and orally (PO, $n = 8$) male CD2F1 mice. Multiple group comparisons were done by one-way ANOVA with a Bonferroni's post-hoc test. All groups were compared against NTB mice and TB vehicle treated mice. Asterisk brackets are displayed for significant results only. * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$.

Loss of bodyweight was present in SB mice ($-8.6\% \pm 9.2$, $p < 0.001$) and untreated, C26 TB mice ($-12.5\% \pm 9.4$, $p < 0.001$), but not in GW PO and GW IP treated mice (figure 4B). These differences in efficacy between GW788388 and SB431542 could also be

Figure 5. Scatterplot charts for individual m. gastrocnemius and m. tibialis ant. muscle weight in healthy, non-tumor bearing mice and treated tumor-bearing mice.



Scatterplots of gastrocnemius (GCM) muscle tibialis anterior (TA) muscle weight for all individual tumor-bearing (TB) groups compared with the non-tumor bearing (NTB) male CD2F1 mice. The horizontal reference line indicates the lowest TA muscle weight observed in NTB mice. The vertical reference line indicates the lowest GCM muscle weight observed in NTB mice. Multiple group comparisons were done by one-way ANOVA with a Bonferroni's post-hoc. All groups were compared against NTB male CD2F1 mice ($n = 20$). Differences were observed for (A) untreated, C26 TB ($n = 20$, reduced TA, $p < 0.001$; reduced GCM, $p < 0.001$), (B) SB431542 treated ($n = 20$, reduced TA, $p < 0.001$; reduced GCM, $p < 0.001$) and (C) LONG R3 IGF-I treated male CD2F1 mice ($n = 12$, reduced TA, $p = 0.027$). No differences in muscle weight were observed for (D) combined SB431542 and LONG R3 IGF-I treated ($n = 12$), (E) GW788388 (intraperitoneally, $n = 8$) and (F) GW788388 (orally, $n = 8$) treated male CD2F1 mice.

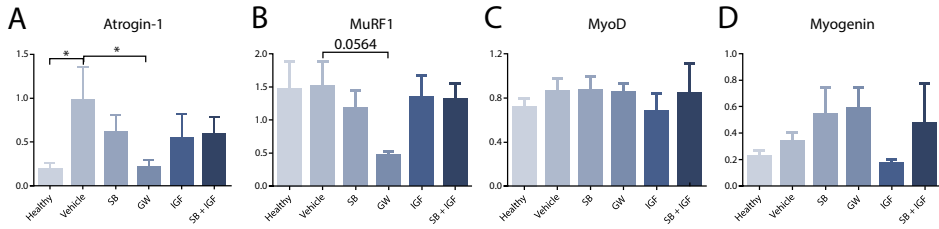
observed in the preservation of muscle mass. GCM muscle mass was significantly higher in mice treated with SB (143.3 ± 20.9 mg, $p < 0.001$), GW IP (154.9 ± 17.8 mg, $p < 0.001$), or GW PO (162.1 ± 13.9 mg, $p < 0.001$) compared to untreated, C26 TB mice (107.3 ± 18.7 mg) (figure 4C, figure 5E-F). The results from the TA data analyses were comparable, e.g. no differences were observed in mice treated with GW788388 when compared with healthy mice (figure 4D, figure 5E-F). No difference in tumor mass was observed between the treatment groups (figure 4E). Collectively, these data show that GW788388 treatment preserves body mass, muscle mass and muscle strength in tumor-bearing cachexia prone mice.

LONG R3 IGF-I TREATMENT PRESERVES MUSCLE MASS IN C26 TB MICE BUT MAY ACCELERATE TUMOR GROWTH

To study the possible synergy between ALK4/5 inhibition and stimulation of muscle growth and differentiation using an IGF-I analogue, mice received the IGF-I analogue LONG R3 IGF-I (200 µg) administered every other day via intramuscular injection, with or without SB431542 (10 mg/kg, i.p.). Compared to NTB mice, LONG R3 IGF-I treated mice experienced a statistically significant loss of grip-strength starting on day 18 ($p = 0.026$, data not shown), which remained present until sacrifice (figure 4A), and loss of body weight ($-8.6\% \pm 13.0$, $p < 0.001$) (figure 4B). LONG R3 IGF-I treated mice had comparable GCM muscle weight (156.4 ± 27.4 mg, $p = 0.174$) (figure 4C, figure 5C), but decreased TA muscle weight (52.3 ± 6.4 mg, $p = 0.027$) (figure 4D, figure 5C). However, LONG R3 IGF-I treatment was superior over TB vehicle-treated animals for both GCM ($p < 0.001$) and TA muscle weight ($p = 0.001$) (figure 4C-D). Combined LONG R3 IGF-I and SB431542 treatment preserved GCM (160.0 ± 25.1 mg, $p = 0.612$) and TA muscle weight (57.2 ± 8.4 mg, $p = 1.000$) compared to NTB mice (figure 4C-D, figure 5D). Despite preserving muscle mass, a statistically significant loss of grip-strength was observed starting on day 14 ($p = 0.004$). This loss of grip-strength remained present until sacrifice (figure 4A). Similar to treatment with either LONG R3 IGF-I or SB431542, mice receiving LONG R3 IGF-I and SB431542 experienced loss of body weight ($-12.2\% \pm 10.6$, $p < 0.001$) (figure 4B). Although the muscle weight data suggest synergism, the observed loss of grip-strength is unfavourable. Moreover, there was a substantial but non-significant increase in tumor growth in mice receiving LONG R3 IGF-I (911 ± 360 mg, $p = 0.08$) or LONG R3 IGF-I with SB431542 (829 ± 371 mg, $p = 0.44$) compared to untreated, C26 TB mice (635 ± 218 mg) (figure 4E). Therefore, possible synergism between LONG R3 IGF-I and GW788388 was not investigated.

TREATMENT WITH ALK4/5 INHIBITORS MODULATES TARGET GENE EXPRESSION

We determined the mRNA expression levels of E3 ubiquitin ligases, MuRF1 and Atrogin-1, and the two myogenic regulatory factors, MyoD and myogenin, in gastrocnemius muscle samples obtained from the mice at sacrifice (figure 6). Atrogin-1 expression was significantly elevated in vehicle-treated animals, but similar to healthy controls in GW788388 treated animals (figure 6A). In contrast to Atrogin-1 expression, MuRF1 expression (figure 6B) did not increase in tumor-bearing vehicle-treated animals. MuRF1 expression decreased in GW788388 treated animals when compared to vehicle treated animals, although did not quite reach significance ($p = 0.056$). MyoD (figure 6C) and Myogenin (figure 6D) expression levels were unaltered by both GW788388 and SB431542 treatment.

Figure 6. mRNA expression levels in cachectic muscle.

Bar graphs depicting the mean \pm SEM mRNA expression levels in gastrocnemius muscle of (A) Atrogin-1, (B) MuRF1, (C) MyoD and (D) Myogenin in non-tumor bearing (NTB, $n = 20$); C26 tumor-bearing (TB) vehicle treated ($n = 20$); tumor-bearing SB431542 treated (SB, $n = 20$); tumor-bearing combined SB431542 and LONG R3 IGF-I treated (SB+IGF, $n = 12$); tumor-bearing LONG R3 IGF-I treated (IGF, $n = 12$) and tumor-bearing GW788388 treated (pooled orally and intraperitoneally GW treated groups, $n = 16$) male CD2F1 mice. Multiple group comparisons were done by one-way ANOVA with a Bonferroni's post-hoc test. All groups were compared against NTB mice and TB vehicle treated mice. Asterisk brackets are displayed for significant results only. * $p < 0.05$.

DISCUSSION

Although cachexia is a common finding in patients suffering from a wide variety of malignancies, with detrimental effects on survival as well as the quality of life^{8,62}, there currently are no treatment modalities available to successfully halt or reverse its progressive muscle wasting. However, an increasing understanding of the underlying pathways associated with cachexia is shaping the building blocks between science and clinical practice. The suggested role of myostatin in the development of cachexia³³, as well as the heteromeric receptor complex it binds to¹⁷⁻¹⁹, have allowed for the first pre-clinical studies aimed to develop and identify treatment modalities to counter progressive muscle loss. Our study is the first to study the role of two ALK 4/5 inhibitors, in the treatment of cancer-associated muscle loss. The present findings show that pharmacological inhibition of ALK 4/5 successfully limits the occurrence of cancer-associated sarcopenia. And although an associated between tumor mass and body weight was observed, no associated between tumor mass and muscle weight could be found. As such, potential differences in muscle weight cannot be explained by observed non-significant differences in tumor mass.

We hypothesized this inhibition to work based on prior research which detailed the myostatin signalling pathway, wherein myostatin has a high affinity for the ActRIIB receptor, after binding this receptor forms a heteromeric complex with ALK4 and ALK5, subsequently activating the myostatin signal transduction pathway,¹⁷⁻¹⁹ including Smad2/3 and MAPK,²⁰ which in turn induces an Akt-mediated FoxO-dependent muscle protein breakdown via the ubiquitin-proteasome system.^{21, 22} In the current study in which we blocked the ALK4 and ALK5 receptors, thus prohibiting the formation of the heteromeric complex, we observed a differential expression of the target genes (i.e. E3 ubiquitin ligases MuRF1 and Atrogin-1) as expected. These findings of preserved muscle mass by targeting the myostatin signalling pathway are in line with previous studies that found muscle mass preservation by using modified RNA oligonucleotides targeting myostatin mRNA, systemic administration of the activin receptor extracellular domain/Fc fusion protein (ACVR2B-Fc), and a soluble ActRIIB receptor.³⁴⁻³⁷ Interestingly, the myostatin pathway might not be the only pathway involved in cancer cachexia. Recent studies found the JAK2/STAT3 pathway to be another candidate for pharmaceutical agents to limit muscle wasting in experimental cancer cachexia.⁶³⁻⁶⁵

In contrast to the successful reduction of muscle wasting by ALK4/5 inhibition, a beneficial synergistic role of the IGF-I analogue LONG R3 IGF-I was not found. Despite a positive effect on wet muscle weight, no effect on muscle strength was found. Despite comparable muscle weight to GW treated mice, LONG R3 IGF-I treated mice had a reduction in body weight. The exact nature of this difference is not known, as no body-composition analysis was performed in this study. Since a trend towards increased tumor growth was observed this was not further investigated. Although this trend was not observed in earlier studies using similar agents^{47, 48}, we consider this finding to be of importance. The possibility of enhanced tumor growth through IGF-I treatment precludes its use in the clinic. The role of the IGF system in cancer has been investigated in depth for approximately half a century, from which the association between IGF-I and oncogenesis became apparent. Certain malignancies are found to be more prone to being driven by IGF-I, e.g. prostate cancer, colon cancer and lung cancer.⁶⁶ Novel treatment strategies are being developed targeting this IGF system, although with mixed results.⁶⁷ Systemic treatment with pharmaceutical agents aimed at inhibiting the IGF system may risk worsening cachexia, by concurrent targeting of the anabolic muscle pathways. Although the impact of inhibiting IGF signalling on cachexia has not purposefully been investigated, muscle weakness is reported as a side-effect for such treatment.⁶⁸ Future development of muscle-specific anabolic agents without a tumor promoting effect might overcome this drawback.

Several limitations apply to the present study. The study was powered on an expected reduction in loss of muscle weight. As such, non-significant differences in secondary outcome parameters (e.g. total body weight, muscle strength and relative mRNA expression levels) may have been subject to type II errors. Furthermore, survival was not included as one of the endpoints due to the strict ethical guidelines associated with the initiation of this study. Although Zhou et al. have previously reported a direct relationship between muscle wasting and survival in a comparable cancer cachexia model³⁵, it is therefore, unknown whether the preservation of muscle mass is associated with increased survival in our study. Moreover, the available inhibitors of ALK 4 and ALK 5 as used in this study are preclinical drugs and cannot be directly validated in humans.

In conclusion, this study found that inhibition of ALK 4 and ALK 5 limited muscle wasting in a mouse model of cancer-associated cachexia and reduced the expression of cachexia associated ubiquitin ligase Atrogin-1. The results obtained in the current study are promising and contribute to a growing body of evidence which suggests that muscle wasting in cancer cachexia might be limited by blocking the myostatin signalling pathway. This knowledge may benefit in the selection and development of drug candidates for clinical trials for the treatment of cancer cachexia.

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CHAPTER 9

CALORIC RESTRICTION IS ASSOCIATED WITH PRESERVATION OF MUSCLE STRENGTH IN EXPERIMENTAL CANCER CACHEXIA

ABSTRACT

Caloric restriction increases lifespan and healthspan, and limits age-associated muscle wasting. In this study, we investigate the impact of 30% caloric restriction (CR) in a murine cancer cachexia model. Forty CD2F1 mice were allocated as C26 tumor-bearing (TB) + ad libitum food intake (dietary reference intake [DRI]), TB CR, non-TB (NTB) CR, or NTB matched intake (MI). TB groups were inoculated subcutaneously with 0.5×10^6 C26 cells 14 days after initiating CR. Bodyweight, food intake, and grip-strength were recorded periodically. Gastrocnemius (GCM) and tibialis anterior (TA) muscles were resected and weighed 3 weeks after tumor inoculation. mRNA expression of MuRF1, Atrogin-1, myogenin, and MyoD was determined. At tumor inoculation, the mean body weight of TB CR was 88.6% of initial body weight and remained stable until sacrifice. TB DRI showed wasting before sacrifice. TB groups experienced muscle wasting compared with NTB MI. Grip-strength change was less severe in TB CR. Expression of MuRF1, Atrogin-1, and MyoD was similar between TB DRI and both CR groups. Expression of myogenin was increased in CR groups. In conclusion, caloric restriction limits loss of muscle strength but has no impact on muscle mass despite significant loss of body weight in an experimental cancer-associated cachexia model.

INTRODUCTION

Cancer cachexia describes a syndrome of progressive weight loss due to muscle wasting with or without the loss of adipose tissue, anorexia, and abnormal metabolism in the presence of underlying cancer.¹ It cannot be reversed by conventional nutritional support and leads to progressive functional impairment.^{1,2} Nearly half of all cancer patients are faced with cachexia in the course of their disease, and it is the cause of death in up to 20 percent.³⁻⁵ Catabolic cytokines and patient-related factors such as age are key pathogenic mechanisms underlying cancer cachexia.⁶⁻⁸ Catabolic pro-inflammatory cytokines associated with cancer cachexia include interleukin-6 (IL-6), interleukin-1 beta (IL-1B), tumor necrosis factor alpha (TNF- α), and interferon gamma (IFN- γ).^{7,9} Particularly IL-6 is found highly upregulated in the final months preceding death.¹⁰ Treatment aimed at reducing the synthesis of pro-inflammatory cytokines or blocking their action may, therefore, contribute to improved physical performance and quality of life.¹¹⁻¹³

Besides novel pharmaceutical strategies to limit the activity of catabolic cytokines in cancer cachexia, dietary interventions have sparked great interest.^{11, 13-19} Thus far dietary interventions for the treatment of cancer cachexia have evaluated supplementation therapy. Long-chain omega-3 fatty acid eicosapentaenoic acid (EPA) is one of the most frequently investigated supplements. Systematic reviews of the literature published since have been unable to support clinical application of EPA for the treatment of cancer cachexia.^{18, 20} Only smaller studies initially reported to limit weight loss in cancer patients.²¹ In contrast to this, β -hydroxy- β -methylbutyrate (HMB), a leucine metabolite, and quercetin have been found to limit experimental muscle wasting in vivo¹⁴⁻¹⁶ as well as in a clinical trial following a 24-week supplementation program.²² Similarly, another study found a strong trend towards the preservation of muscle mass in advanced cancer patients following 8 weeks of HMB supplementation.²³ Although counterintuitive, caloric restriction (CR) may elicit similar effects. The beneficial effects of CR on healthspan and longevity have been thoroughly established in model organisms, and include reduced incidence of cancer, cardiovascular disease, and increased oxidative stress resistance.²⁴⁻³¹ Experiments in our own laboratory have shown that two weeks of 30% CR improves insulin sensitivity, increased insulin/insulin-like growth factor 1 signaling, increases expression of markers of antioxidant defense, and reduces expression of markers of inflammation in mice.²⁹ In rodents and nonhuman primates, CR was able to limit sarcopenia, i.e. the age-related loss of muscle mass.³²⁻³⁵

Similarly as in cancer cachexia, catabolic pro-inflammatory cytokines are suggested to play an important role in the development of sarcopenia.^{36, 37}

Therefore, we questioned whether CR could limit muscle wasting and loss of muscle function in an experimental cancer cachexia model and we examined the impact of CR on body weight, muscle weight, and grip-strength. In addition, the mRNA expression levels of skeletal muscle catabolic E3 ubiquitin ligases and anabolic myogenic regulatory factors were studied.

METHODS

ANIMAL ETHICS COMMITTEE APPROVAL

All animal experiments were performed with the approval of the local Animal Ethics Committee and in accordance with the Dutch National Experiments on Animal Act and complied with the EU adopted Directive 86/609/EEC (1986).

ANIMALS

Male CD2F1 (BALB/c × DBA/2 F1) mice of 8 weeks weighing approximately 25 grams were purchased from Charles River, Maastricht, the Netherlands. All mice were housed in individually ventilated cages under standard conditions with a 12 h light-dark cycle (n = 3 – 4 animals per cage). Animals were acclimatized for one week prior to the start of the experiments.

DIET

All animals had ad libitum access to water and CRM (P) chow (Special Diet Services, Witham, Essex, UK) during the acclimatization period and throughout the full duration of the experiment. At the start of the experiment dietary intake was determined in 3 cages that were randomly allocated as to become tumor-bearing (TB). Twenty-four-hour food consumption in these cages was determined daily by weighing the remnant chow and calculating the difference from the preceding day. This was set as the dietary reference intake (DRI).³⁸ The other cages were randomly allocated as TB, 30% CR animals (i.e. chow weighing 70% of the DRI); non-tumor bearing (NTB), 30% CR animals; and NTB, matched intake animals (i.e. chow weighing 100% of the DRI of the TB animals). All groups consisted of 10 mice. We did not include an AL-NTB group to control for weight loss due to reduced food intake in the TB mice. The pair fed non tumor bearing control group we used compensates for the effects of possible reduced food intake by the tumor bearing animals which allows us to discriminate between the effects of reduced food intake per se, and the effects of the combination of the presence of a tumor and reduced food intake.

CANCER CACHEXIA MODEL

Colon-26 (C26) adenocarcinoma cells were kindly provided by Dr. D.O. McCarthy (Ohio State University, Columbus, OH, USA). These cells were cultured in RPMI 1640 (Westburg BV, Leusden, The Netherlands) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, The United States of America), and 1% penicillin/streptomycin (P/S, Fisher Scientific, Waltham, The United States of America) at 37 °C with 5% CO₂. Animals allocated in TB groups received a subcutaneous inoculation in the right flank with 0.5 x 10⁶ C26 adenocarcinoma cells in 100 µL sterile PBS on the 14th day of the experiment. The inoculation was done under anesthesia by isoflurane inhalation (5% isoflurane induction). This is a well-established model of cancer cachexia in mice.³⁹

GRIP STRENGTH ASSESSMENT

Combined hind- and forelimb grip strength was measured twice per week by placing the animal on a grid attached to a force gauge (BIOSEB, Chaville, France), and steadily pulling the mice by the tail along the sensor axle until grip is released. The maximum strength produced before releasing the grid was registered in triplicate with one minute rest period for each animal. Obtained values were averaged to provide a mean force measurement for each individual animal and subsequently normalized to each animal's grip-strength respectively on day zero.

BODY WEIGHT, MUSCLE MASS, AND TUMOR SIZE

Body weight was recorded daily. Tumor size was recorded every other day starting on day 23 of the experiment, i.e. day 9 after tumor inoculation, using digital calipers. Tumor mass was estimated via the formula $\text{mass (mg)} = \text{tumor volume (mm}^3\text{)} = \text{width}^2 \times \text{length} / 2$.⁴⁰ Animals were sacrificed by cardiac puncture followed by cervical dislocation under isoflurane anesthesia on day 35 of the experiment, i.e. 21 days after tumor inoculation. Immediately following sacrifice the gastrocnemius (GCM), and tibialis anterior (TA) muscles of both hind legs and tumor were dissected, weighed and immediately snap-frozen in liquid nitrogen and stored at -80 °C until analysis.

RNA ISOLATION AND REAL-TIME POLYMERASE CHAIN REACTION

For gene expression analysis, total RNA was isolated from snap-frozen GCM muscle tissue using Trizol reagent (Invitrogen, Breda, the Netherlands), and subsequently purified by DNase treatment (RQ1 RNase-Free DNase) (Promega Benelux B.V., Leiden, the Netherlands). 1 µg of total RNA was reversed transcribed to cDNA using random hexamer primers (Invitrogen, Breda, the Netherlands), and Superscript II RT (Invitrogen, Breda, the Netherlands). Quantitative real-time polymerase chain reaction (RT-PCR) was performed using an iCycler real-time PCR system (Biorad, California, The United States of America) using SYBR Green (Sigma-Aldrich, St. Louis, The United States of America). Used primer sequences can be found in Table 1. GAPDH was used as house-keeping gene for normalization. Relative gene expression was calculated ($2^{-\Delta\Delta Ct}$) / (average $2^{-\Delta\Delta Ct(\text{healthy controls})}$).⁴¹ Each sample was tested in duplicate.

Table 1. Reverse transcription-polymerase chain reaction primer sequences.

Gene	Forward Primer	Reverse Primer	Genbank Accession Number
Atrogin1	5'-GTTTTCAGCAGGCCAAGAAG	5'-TTGCCAGAGAACGCTATG	AF_441120
MyoD	5'-AAACCCCAATGCGATTATCAGG	5'-TAAGCTTCATCTTTGGGCGTGA	NM_010866
Myogenin	5'-CACTCCCTTACGTCCATCGT	5'-CAGGACAGCCCACTTAAAA	NM_031189
Murf1	5'-AGGTGTCAGCGAAAAGCAGT	5'-CCTCCTTTGTCTCTTGCTG	NM_009066
GAPDH	5'-ATGCATCTGCACCACT	5'-CAGTGATGGCATGGACTGTG	NM_008084

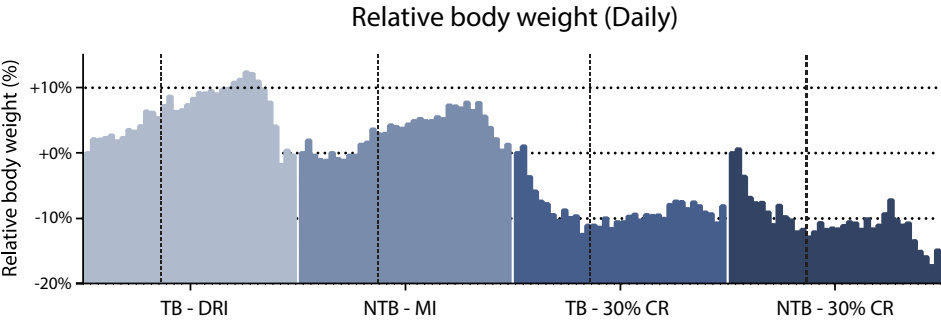
STATISTICS

Categorical data are expressed as number (percentage) and continuous variables as mean ± SEM (normal distribution, visually assessed and by means of the Shapiro-Wilks test). Body weight and grip-strength were normalized to each animal's body weight and grip-strength respectively on day 0. Muscle weight from the left hind leg and right hind leg were averaged to provide a mean GCM and TA muscle weight for each animal. Multiple group comparisons were done by one-way ANOVA with a Bonferroni's post hoc test. For comparison between periodic measurements, the paired-sample t-test was used. Statistical comparison between TB DRI and TB 30% CR mice in tumor weight was done by Student's t-test. All analyses were performed using IBM SPSS Statistics for Windows, version 21.0 (IBM Corp., Armonk, NY, USA). A P value < 0.05 was considered statistically significant.

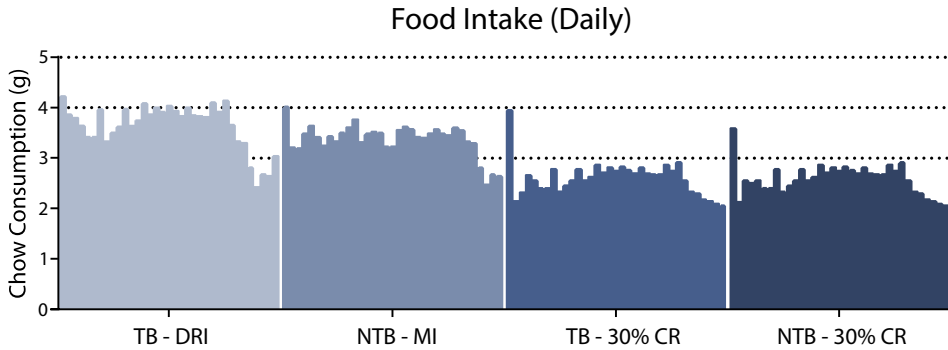
RESULTS

To study the effects of 30% caloric restriction forty male CD2F1 mice were allocated to four groups. Mice allocated to be C26 tumor-bearing (TB) animals with ad libitum access to chow were used as dietary reference intake (DRI) for all other mice in this experiment, i.e. C26 TB mice on a 30% caloric restriction (CR) diet; non-tumor bearing (NTB) mice with matched intake to the TB-DRI group (MI); NTB mice on a 30% caloric restriction diet.

Figure 1. Daily body weight throughout the experiment.



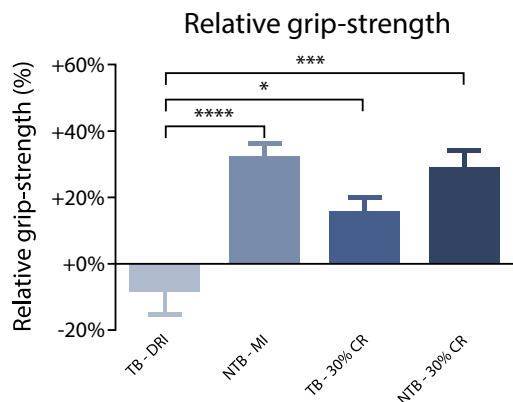
Following initiation of 30% CR, a rapid but similar decline in body weight was observed in both CR groups (NTB-CR and TB 30% CR) (Figure 1). This loss of bodyweight was most apparent in the first week, prior to inoculation of the C26 adenocarcinoma cells. Consequently, this loss of bodyweight was attributable to 30% CR alone. Mice allocated to the NTB MI group had access to an equal amount of food per cage as consumed the day prior by C26 TB DRI mice. Despite this, the NTBI MI group consumed significantly less than the TB DRI mice during the first 7 days of the experiment, i.e. prior to actual tumor inoculation (Figure 2). The mean intake of C26 TB DRI mice was 3.7 g versus 3.4 g in NTB MI mice ($p = 0.03$).

Figure 2. Daily food intake throughout the experiment.

Grouped histograms depicting the mean daily food intake per group in C26 tumor-bearing (TB) male CD2F1 mice with ad libitum access to chow (dietary reference intake [DRI], $n = 10$); C26TB mice on a 30% caloric restriction (CR, $n = 10$); non-tumor bearing (NTB) mice with matched intake (MI, $n = 10$); NTB mice on a 30% caloric restriction ($n = 10$). The vertical bars indicate daily measurements of food intake, ranging from day 0 to 35, for each specified group. Food intake is expressed as grams (g). Food intake of C26 TB DRI mice decreased in the final days preceding sacrifice from 3.8 g to 2.9 g ($p = 0.0002$, paired-sample t-test). Consequently, food intake decreased in the other groups accordingly.

This difference in food intake between the NTB MI and C26 TB DRI groups was associated with a lower maximum increase in bodyweight. At tumor inoculation, mean body weight in TB 30% CR mice was 88.6% of initial body weight compared with 106.9% in the TB DRI mice. Following tumor inoculation, mice in the TB DRI group gained bodyweight until 28 days after the start of the experiment. From day 28 until sacrifice at day 35, animals lost 10.6% of initial body weight ($p = 0.01$). This was associated with a decrease in food intake from 3.8 g to 2.9 g ($p = 0.0002$). Consequently, NTB MI mice too experienced a loss of 6.4% in body weight in these final days of the experiment ($p = 0.002$). Mice in the TB 30% CR group had a stable bodyweight following tumor inoculation, and no further decrease in body weight was observed ($p = 0.186$). Mice in the NTB 30% CR group lost 7.6% in mean body weight in the final days of the experiment ($p = 0.004$). This difference may, in part but not exclusively, be attributed to tumor weight increase in the TB 30% CR group.

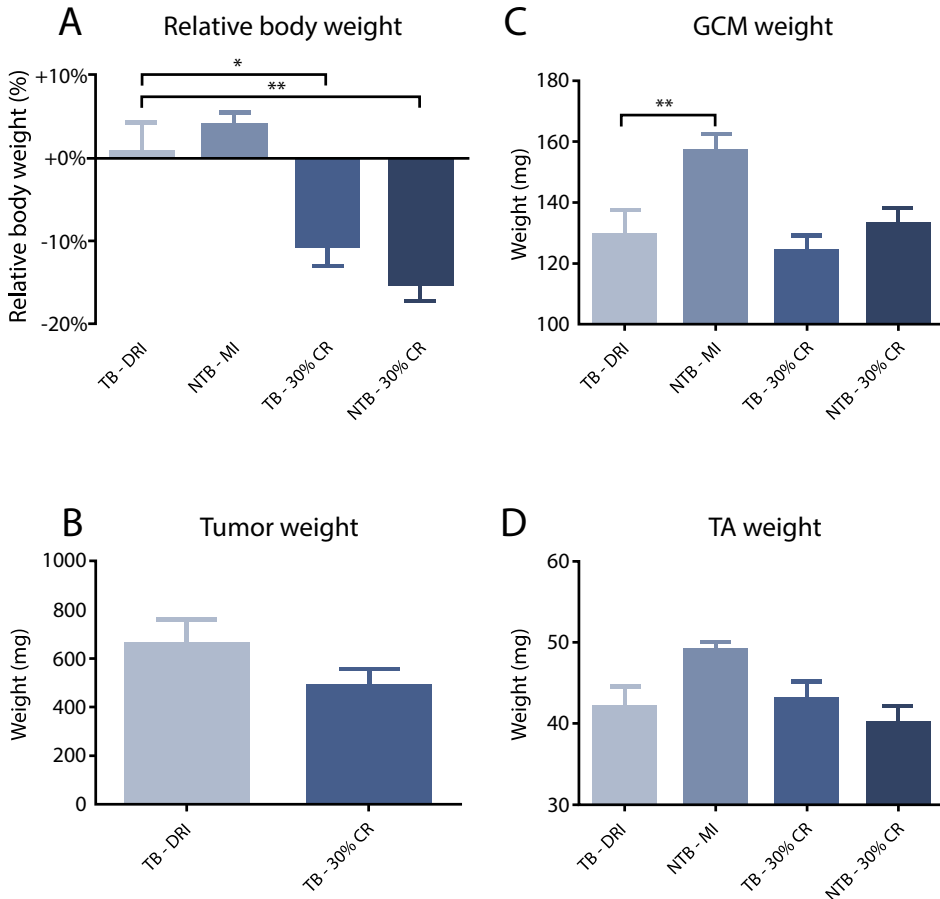
A reduction in grip-strength was observed throughout the follow-up period for TB DRI mice (Figure 3). The final mean loss of grip-strength was 7.9% when compared to starting grip-strength. TB 30% CR mice, on the other hand, experienced an increase of 15.4% in grip-strength throughout the experiment. This difference was significant in comparison to the TB DRI mice ($p = 0.02$). NTB mice, both NTB MI and NTB 30% CR, experienced the greatest increase in grip-strength, which was 31.7% ($p < 0.001$) and 28.6% ($p = 0.0002$) respectively at the end of the experiment.

Figure 3. Relative grip-strength at the end of the experiment.

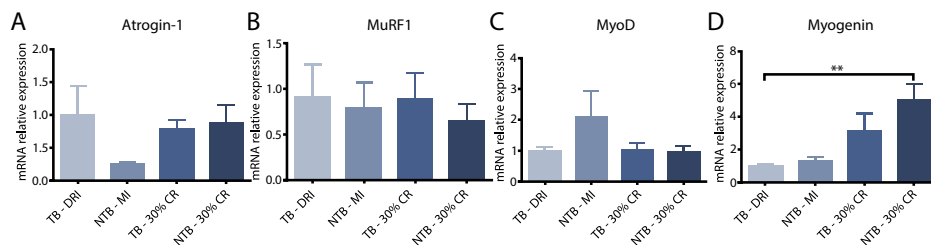
Bar graphs depicting the mean \pm SEM for final grip-strength normalized to starting grip-strength in C26 tumor-bearing (TB) male CD2F1 mice with ad libitum access to chow (dietary reference intake [DRI], $n = 10$); C26 TB mice on a 30% caloric restriction (CR, $n = 10$) diet; non-tumor bearing (NTB) mice with matched intake (MI, $n = 10$); NTB mice on a 30% caloric restriction ($n = 10$). Multiple group comparisons were done by one-way ANOVA with a Bonferroni's post hoc test. All groups were compared against TB - DRI mice. Asterisk brackets are displayed for significant results only. * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$.

All animals were sacrificed at 21 days following tumor inoculation, i.e. 35 days after onset of the experiment. At sacrifice, the final decrease in bodyweight was greatest in TB 30% CR and NTB 30% CR mice, 10.5% and 14.0% respectively (Figure 4A). As expected, NTB MI mice had an increase in body weight of 4.0%. TB DRI mice experienced a rapid decline in body weight in the final days preceding sacrifice by 10.6%. Tumor mass increased until day 21, when resected mean tumor weight was 662 ± 316 mg in TB DRI mice versus 480 ± 249 mg in TB 30% CR mice. This trend towards reduced tumor growth in CR mice was not significant ($p = 0.17$) (Figure 4B). Furthermore, no association between tumor weight and body weight loss was observed. Directly following sacrifice, the gastrocnemius and tibialis anterior muscles were resected and weighed. Mean gastrocnemius muscle weight in NTB MI mice was 158.3 ± 18.3 mg versus 128.7 ± 25.3 mg in TB DRI mice, $p = 0.008$ (Figure 4C). Mean gastrocnemius muscle weight for C26 TB 30% CR mice was 124.4 ± 15.5 mg, comparable to C26 TB DRI mice ($p > 0.99$). Similarly, mean gastrocnemius muscle weights for NTB 30% CR mice were 132.5 ± 15.4 mg, comparable to C26 TB DRI mice ($p > 0.99$). Mean tibialis anterior muscle weight in NTB MI mice was 48.9 ± 3.4 mg versus 42.1 ± 8.5 mg in the C26 TB DRI mice ($p = 0.08$) (Figure 4D). Mean tibialis anterior muscle weights for C26 TB 30% CR mice were 42.6 ± 5.4 mg, comparable to C26 TB DRI mice ($p > 0.99$). Similarly, mean tibialis anterior muscle weights for NTB 30% CR mice were 40.0 ± 4.9 mg, comparable to C26 TB DRI mice ($p > 0.99$).

Figure 4. Body weight, muscle weight and tumor mass at sacrifice.



Bar graphs depicting the mean \pm SEM for (A) final bodyweight normalized to starting bodyweight, (B) tumor weight, (C) gastrocnemius muscle weight and (D) tibialis anterior muscle weight in C26 tumor-bearing (TB) male CD2F1 mice with ad libitum access to chow (dietary reference intake [DRI], $n = 10$); C26 TB mice on a 30% caloric restriction (CR, $n = 10$) diet; non-tumor bearing (NTB) mice with matched intake (MI, $n = 10$); NTB mice on a 30% caloric restriction ($n = 10$). Multiple group comparisons were done by one-way ANOVA with a Bonferroni's post hoc test. All groups were compared against TB - DRI mice. Asterisk brackets are displayed for significant results only. * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$. Statistical comparison between TB DRI and TB 30% CR mice in tumor weight was done by Student's t-test ($p = 0.17$).

Figure 5. mRNA expression levels in cachectic muscle.

Bar graphs depicting the mean \pm SEM mRNA expression levels in gastrocnemius muscle of (A) Atrogin-1, (B) MuRF1, (C) MyoD and (D) Myogenin in C26 tumor-bearing (TB) male CD2F1 mice with ad libitum access to chow (dietary reference intake [DRI], $n = 10$); C26 TB mice on a 30% caloric restriction (CR, $n = 10$) diet; non-tumor bearing (NTB) mice with matched intake (MI, $n = 10$); NTB mice on a 30% caloric restriction ($n = 10$). Multiple group comparisons were done by one-way ANOVA with a Bonferroni's post hoc test. All groups were compared against TB – DRI mice. Asterisk brackets are displayed for significant results only. * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$.

Skeletal muscle E3 ubiquitin ligases and myogenic regulatory factors mRNA expression profiles were determined in gastrocnemius muscle samples. A substantial, non-significant difference in E3 ubiquitin ligase atrogin-1 expression was observed between C26 TB DRI and NTB MI (Figure 5A). No difference was observed between C26 TB DRI, C26 TB 30% CR and NTB 30% CR. Expression of the second E3 ubiquitin ligase MuRF1 and myogenic regulatory factor MyoD were comparable between all four groups (Figure 5B, 5C). Finally, and perhaps most interesting, there was increased expression of the myogenic regulatory factor myogenin in the NTB 30% CR group ($p = 0.002$) as well as a substantial, non-significant elevation in the TB 30% CR group (Figure 5D).

DISCUSSION

Cancer-associated cachexia is a common finding in patients affected by numerous types of malignancies.^{42, 43} Unfortunately, there are still no treatment modalities to halt or reverse this process of muscle wasting. Previously it was shown that caloric restriction may decrease age-related sarcopenia.³²⁻³⁴ Our study investigated whether caloric restriction might protect against muscle wasting and loss of muscle function. Although counterintuitive, our findings show that in the C26 cancer cachexia model, caloric restriction had no impact on muscle wasting when compared to ad libitum fed TB mice. Moreover, the mRNA expression of E3 ubiquitin ligases MuRF1 and Atrogin-1 expression was unaffected by 30% caloric restriction. This suggests a protective mechanism by which CR prevents aggravated muscle wasting. This was also reflected in grip-strength. The final grip-strength in the TB 30% CR group was greater than the final grip-strength in TB DRI mice. Nonetheless, this grip-strength was still decreased compared to both NTB MI as well as NTB 30% CR mice. CR alone had no impact on grip strength in non-tumor-bearing mice. Similar findings have been previously reported.⁴⁴ Discrepancies between muscle mass and muscle strength have also been noted in human populations.⁴⁵⁻⁴⁷ Taken together, these findings show a limited protective effect on the functional outcome of CR in tumor-bearing mice, which is not powerful enough to prevent loss of muscle strength. This protective effect may be attributed to the enhanced expression of myogenin in mice on a 30% caloric restriction diet. Similar effects of myogenin have previously been described following myogenin gene transfer in an ALS model.⁴⁸ In that study, myogenin gene transfer lead to increased rotarod performance, whilst the bodyweight loss profile remained unaffected.

In addition, mice allocated to receive CR, both tumor-bearing and non-tumor bearing, showed enhanced activity throughout the experiment, e.g. increased running and climbing, as well as being found frequently hanging from the top of the cage. Although we did not quantify these findings, similar results have been reported in an age-related sarcopenia caloric restriction rodent model.⁴⁴ The increased activity of animals on CR may have contributed to the preservation of grip-strength as well as to myogenin upregulation.

Furthermore, non-tumor-bearing mice on caloric restriction demonstrated a higher mean body weight loss than tumor-bearing on caloric restriction. This difference may in part, but not exclusively, be attributed to tumor weight. Increased organ weight, i.e. liver and spleen, has been reported in C26-bearing mice^{49, 50} and is likely to have

contributed to these difference in body weight. Moreover, considering fluid intake was not monitored a possible contribution of water weight is unknown. Lastly, despite energy intake being fixed, energy expenditure is not. Possible differences in physical activity may too have contributed to these differences.

Studies employing caloric restriction have been primarily aimed at investigating its role in improving the efficacy of anti-cancer therapies⁵¹⁻⁵⁴, protecting against anti-cancer therapy side-effects^{55,56}, as well as preventing oncogenesis.⁵² Although the difference in tumor mass was non-significant between the C26 TB DRI and C26 TB 30% CR mice in the current study, an earlier meta-analysis has shown that caloric restriction may reduce tumor growth.⁵⁷ This anti-cancer effect has also been described after short-term fasting and fasting cycles.⁵⁸ Even though in the current study we did not seek to investigate the anti-cancer effects of caloric restriction, the observed trend towards reduced tumor growth can be regarded as an additional benefit of caloric restriction.

Several limitations apply to the present study. The study was powered on an expected reduction in loss of muscle weight. As such, non-significant differences in secondary outcome parameters (e.g. relative mRNA expression levels) may have been subject to type II errors. Furthermore, survival was not included as one of the endpoints due to the strict ethical guidelines associated with the initiation of this study. Another important consideration is timing of caloric restriction. For this study mice were put on a calorie restricted diet prior to inoculation of cancer cells. This may limit direct translation of these findings to clinical patients, who have established cancer, and may already suffer from anorexia. Moreover, a recent study by Boldrin et al. reports that changes induced by caloric restriction in an age-related sarcopenia model do not persist with time, and, perhaps even more important, are dependent on mouse strain and gender differences.⁵⁹ Taking our own findings into account we concur with the authors of the aforementioned study to be cautious in applying caloric restriction to improve skeletal muscle function in humans.

In conclusion, we found that caloric restriction limits the loss of muscle strength *in vivo* in an experimental cancer-associated cachexia model. Caloric restriction did not aggravate the loss of cachexia associated muscle mass despite significant body weight loss. These findings suggest that although caloric restriction does not fully protect against the detrimental effects of cancer-associated cachexia, it does limit muscle strength loss. This suggests that caloric restriction might be safely utilized in improving the efficacy of-, and protect against the adverse side effects of anti-cancer therapies. Further research is warranted to confirm these findings upon initiation of caloric restriction in early and late-stage cancer.

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CHAPTER 10

QUERCETIN SUPPLEMENTATION ATTENUATES MUSCLE WASTING IN CANCER-ASSOCIATED CACHEXIA IN MICE

ABSTRACT

BACKGROUND

Quercetin is a flavonoid with reported antioxidant, anti-inflammatory and anti-aging effects, and may limit muscle wasting in cancer cachexia. We set out to investigate the effect of quercetin on muscle wasting in the murine C26 cancer-cachexia model and assess the feasibility of non-invasive micro-CT analysis of skeletal muscle in addition to grip strength and muscle weight.

METHODS

Custom CRM(P) diets supplemented with 250 mg/kg quercetin (Q) were obtained. Thirty CD2F1 mice were allocated randomly into three groups: non-tumor-bearing (NTB), tumor-bearing (TB), TB+Q. TB(+Q) mice were inoculated subcutaneously with 0.5×10^6 C26 cells to induce cachexia. Simultaneously, all groups started their allocated diet and underwent hindlimb micro-CT. Bodyweight, food intake, and grip-strength were recorded periodically. After 21 days, repeat micro-CT was performed. Gastrocnemius (GCM) and tibialis anterior (TA) muscles were resected and weighed. mRNA expression of MuRF1, Atrogin-1, myogenin, and MyoD was determined.

RESULTS

NTB mice and TB+Q mice gained 9.4% and 5.3% bodyweight respectively, TB mice lost 3.9 %. Hindlimb skeletal muscle volume measurement by micro-CT was comparable for all groups on day 1 and remained stable for NTB and TB+Q mice. TB mice hindlimb skeletal muscle volume dropped from 242.0 mm³ to 212.8 mm³. A high positive correlation was observed between skeletal muscle volume resected muscle weight. Mean GCM muscle weight was 175.2mg (NTB), 171.3mg (TB+Q) versus 125.5mg (TB). Mean TA muscle weight was 64.1mg (NTB), 63.7mg (TB+Q) versus 48.9 mg (TB). A substantial, non-significant decrease in expression of atrogin-1 was observed in TB+Q mice, as well as to a lesser extent in the expression of MuRF1. Expression of MyoD and Myogenin was comparable between all groups.

CONCLUSIONS

Dietary quercetin supplementation limits bodyweight loss and muscle wasting in the C26-cancer-associated cachexia model.

INTRODUCTION

Progressive muscle wasting is a characteristic feature of cachexia commonly observed in cancer patients.¹ This multifactorial syndrome cannot be fully reversed by the conventional nutritional support and leads to progressive functional impairment.^{1,2} Up to 50 percent of all cancer patients are faced with cachexia during the course of their disease and up to 20 percent of all cancer-associated deaths may be attributed to cachexia.³⁻⁵ The etiology of cancer cachexia is as of yet not fully understood, however catabolic pro-inflammatory cytokines are suggested to be of key importance. The most frequently described cytokines associated with cachexia include interleukin-6 (IL-6), tumor necrosis factor alpha (TNF- α), interferon gamma (IFN- γ), and interleukin-1-beta (IL-1B).⁶⁻⁸

Treatment of cancer cachexia may be directed at reduction of synthesis or blocking the action of the aforementioned pro-inflammatory cytokines. By doing so, it may contribute to improving the patient quality of life and, possibly, even prolong survival.⁹ Not just the development of novel pharmaceutical strategies has been suggested to limit the activity of catabolic cytokines in cancer cachexia, but also dietary interventions have been proposed.⁹ For instance dietary supplementation with long-chain omega-3 fatty acid eicosapentaenoic acid (EPA) in cachectic patients appeared promising in initial studies.¹⁰ Later studies, however, conclude that there is as of yet insufficient evidence to support the clinical application of EPA for the treatment of cancer cachexia.^{11,12} In contrast, the leucine metabolite β -hydroxy- β -methyl-butyrate (HMB) limits muscle wasting in experimental cachexia¹³⁻¹⁵ and has been found to attenuate muscle loss in a clinical trial.^{16,17} Additionally, quercetin has recently been described as to limit muscle wasting in vivo.¹⁴ Quercetin is a plant pigment (flavonoid). It is found in many vegetables, herbs, and fruits such as capers, dill, cilantro, red onion, broccoli, berries, and apples.¹⁸ Its antioxidant, anti-inflammatory, and anti-aging effects have previously been described.¹⁹⁻²² In both an APC knockout cachexia model as well as an obesity model quercetin supplementation limited associated loss of muscle mass.^{14,23}

In this current study, we sought to investigate the potential of quercetin in the C26 adenocarcinoma cancer cachexia model, a well-established model of cancer cachexia in mice²⁴, and assess the feasibility of micro-CT analysis as a novel non-invasive and potentially supplemental technique of skeletal muscle measurement.

METHODS

ANIMAL ETHICS COMMITTEE APPROVAL

All animal experiments were performed with the approval of the Erasmus MC, Rotterdam, the Netherlands Animal Ethics Committee and in accordance to the Dutch National Experiments on Animal Act, and complied with the EU adopted Directive 86/609/EEC (1986).

ANIMALS

Thirty male CD2F1 (BALB/c × DBA/2 F1) mice of 8 weeks (~ 25 grams) were obtained from Charles River, Maastricht, the Netherlands. Upon arrival, animals have been housed in individually ventilated cages and maintained at 22 °C under a 12 h light-dark cycle with ad libitum access to CRM (P) chow (Special Diet Services, Witham, Essex, UK) and water (n = 3 – 4 animals per cage). Animals were acclimatized for one week prior to the start of the experiments.

QUERCETIN

Quercetin was obtained from Sigma-Aldrich, Zwijndrecht, the Netherlands (product code Q4951 SIGMA) and after arrival at our institution immediately shipped through to Special Diet Services for the purpose of manufacturing a custom quercetin supplemented diet.

CRM (P) CHOW

CRM (P) chow with and without supplemented quercetin was obtained from Special Diet Services, Witham, Essex, UK. Diet composition is detailed in Table 1. A concentration of 250 mg quercetin per kg chow was manufactured for purpose of this study. This concentration corresponds with an expected daily intake of 35 mg per kg of body weight per mouse per day.

Table 1. Rat and mouse breeder and grower diet, pelleted (CRM(P)).

Nutrients		Total	Of which added*
Proximate analysis			
Moisture (l)	%	10.00	
Crude oil	%	3.36	
Crude protein	%	18.35	
Crude fiber	%	4.23	
Ash	%	6.27	
Nitrogen free extract	%	57.39	
Digestibility coefficients			
Digestible crude oil	%	3.05	
Digestible crude protein	%	16.44	
Carbohydrates, fiber and non-starch polysaccharides (NSP)			
Total dietary fiber	%	15.06	
Pectin	%	1.40	
Hemicellulose	%	8.85	
Cellulose	%	3.89	
Lignin	%	14.0	
Starch	%	42.37	
Sugar	%	3.90	
Energy			
Gross energy	Mj/kg	15.01	
Digestible energy	Mj/kg	12.27	
Metabolizable energy	Mj/kg	11.19	
Atwater fuel energy (AFE)	Mj/kg	13.93	
AFE from oil	%	9.08	
AFE from protein	%	42.37	
AFE from carbohydrate	%	68.90	
Fatty acids			
Saturated fatty acids			
C12:0 Lauric	%	0.03	
C14:0 Myristic	%	0.14	
C16:0 Palmitic	%	0.33	
C18:0 Stearic	%	0.06	
Monosaturated fatty acids			
C14:1 Myristoleic	%	0.02	
C16:1 Palmitoleic	%	0.10	
C18:1 Oleic	%	0.87	
Polyunsaturated fatty acids			
C18:2(ω6) Linoleic	%	0.96	

Nutrients		Total	Of which
Proximate analysis			added*
C18:3(ω 3) Linolenic	%	0.11	
C20:4(ω 6) Arachidonic	%	0.11	
C22:5(ω 3) Clupanodonic	%		
Amino acids			
Arginine	%	1.19	
Lysine	%	1.04	0.17
Methionine	%	0.28	0.02
Cystine	%	0.29	
Tryptophan	%	0.22	
Histidine	%	0.46	
Threonine	%	0.69	
Isoleucine	%	0.77	
Leucine	%	1.46	
Phenylalanine	%	0.96	
Valine	%	0.91	
Tyrosine	%	0.69	
Taurine	%		
Glycine	%	1.55	
Aspartic acid	%	1.00	
Glutamic acid	%	3.72	
Proline	%	1.34	
Serine	%	0.78	
Hydroxyproline	%		
Hydroxylysine	%		
Alanine	%	0.21	
Macro minerals			
Calcium	%	0.83	0.72
Total phosphorus	%	0.64	0.19
Phytate phosphorus	%	0.23	
Available phosphorus	%	0.41	0.19
Sodium	%	0.27	0.22
Chloride	%	0.40	0.35
Potassium	%	0.69	
Magnesium	%	0.22	0.01
Micro minerals			
Iron	mg/kg	130.65	60.21
Copper	mg/kg	16.42	6.90
Manganese	mg/kg	91.05	44.90

Nutrients		Total	Of which
Proximate analysis			added*
Zinc	mg/kg	86.59	52.86
Cobalt	µg/kg	494.92	420.30
Iodine	µg/kg	390.43	310.17
Selenium	µg/kg	265.49	100.34
Fluorine	mg/kg	9.63	
Vitamins			
β-Carotene	mg/kg	1.28	
Retinol	µg/kg	5218.35	4500.38
Vitamin A	iu/kg	17376.38	15001.26
Cholecalciferol	µg/kg	76.94	75.00
Vitamin D	iu/kg	3077.42	3000.00
α-Tocopherol	mg/kg	93.03	72.81
Vitamin E	iu/kg	102.81	80.09
Vitamin B1 (Thiamine)	mg/kg	15.84	9.83
Vitamin B2 (Riboflavin)	mg/kg	13.28	11.76
Vitamin B6 (Pyridoxine)	mg/kg	17.65	13.74
Vitamin B12 (Cyanocobalamin)	µg/kg	78.17	75.00
Vitamin C (Ascorbic acid)	mg/kg	1.80	
Vitamin K (Menadione)	mg/kg	185.05	180.00
Folic acid (Vitamin B9)	mg/kg	4.30	2.94
Nicotinic acid (Vitamin PP)	mg/kg	78.92	27.65
Pantothenic acid (Vitamin B3/5)	mg/kg	25.24	11.56
Choline (Vitamin B4/7)	mg/kg	899.51	75.63
Inositol	mg/kg	2253.88	12.78
Biotin (Vitamin H)	µg/kg	488.74	230.85

The control diet used in the current study is the rat and mouse breeder and grower diet, pelleted (CRM(P)), obtained from Special Diet Services (SDS). * Added nutrients from manufactured and mined sources. Ingredients (wheat, wheatfeed, barley, de-hulled extracted toasted soya, maize, macro minerals, soya oil, potato protein, hydrolyzed wheat gluten, full fat soya, maize gluten meal, vitamins, micro minerals, amino acids) and the calculated analysis in the table above are retrieved from the SDS CRM(P) product specification sheet.⁵¹ The quercetin supplemented diet was manufactured with the addition of 250 mg quercetin per kg chow to the base diet detailed in this table.

C26 TUMOR-BEARING MICE

Colon-26 (C26) adenocarcinoma cells (kindly provided by Dr. D.O. McCarthy, Ohio State University, Columbus, OH, USA) were maintained in RPMI 1640 (Westburg BV, Leusden, The Netherlands) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, The United States of America), and 1% penicillin/streptomycin (P/S, Fisher Scientific, Waltham, The United States of America) at 37 °C in a 5% carbon dioxide environment. On the 14th first day of the experiment, animals allocated to tumor-bearing (TB) groups received a subcutaneous (SC) inoculation in the right flank with 0.5×10^6 C26 adenocarcinoma cells in 100 μ L sterile PBS under anesthesia by isoflurane inhalation (5% isoflurane induction). This is a well-established model of cancer cachexia in mice.²⁴

ASSESSMENT OF GRIP-STRENGTH

Combined hind- and forelimb grip strength was measured twice per week by placing the animal on a grid attached to a force gauge (BIOSEB, Chaville, France), and steadily pulling the mice by the tail along the sensor axle until grip is released. Maximum strength produced before releasing the grid was registered in triplicate with one minute rest period for each animal. Obtained values were averaged to provide a mean force measurement for each individual animal and subsequently normalized to each animal's grip-strength respectively on day 0. All measurements were performed blind with respect to treatment.

SKELETAL MUSCLE VOLUMETRIC MEASUREMENT BY MICRO-CT

In clinical cachexia imaging modalities such as CT are frequently used to determine muscle mass.^{25,26} For rodents micro-CT is available as a non-invasive method for screening anatomical change.²⁷ High spatial resolution and temporal resolution allow capture of detailed anatomical imaging and monitor disease progression in rodents.^{27,28} The absorbed radiation dose from serial micro-CT imaging is low and likely below the threshold for carcinogenesis.²⁹ This makes micro-CT analysis an interesting novel approach in the assessment of muscle weight loss in experimental cancer cachexia.

Hindlimb imaging was performed in all animals prior to the start of the experiment, and once more directly preceding sacrifice. Under anesthesia by isoflurane inhalation animals were positioned on the left flank, and had their right hindlimb fixed in extended position. Images were obtained using a Quantum FX (Perkin Elmer, Waltham, MA,

USA) low-dose microcomputed tomography (μ CT) scanner which scanned at 90 kV of peak voltage, with 160 μ A of current, 40 mm field of view, and 4.5-minute scan time. Obtained scans were subsequently analyzed using Analyze 11.0 (AnalyzeDirect, Inc., Lenexa, KS, USA). This was done by manually delineating the skeletal muscle of the right hindlimb from the tibial surface proximally to the malleoli distally and segmenting the tissue of interest based on HU thresholds (-30 HU to + 150 HU for skeletal muscle²⁵).

BODYWEIGHT, MUSCLE MASS, AND TUMOR SIZE

Bodyweight was recorded daily. Bodyweight was normalized to each animal's body weight on day 0. Tumor size was recorded every other day starting on day 9 after tumor inoculation using digital calipers. Tumor mass was estimated via the formula mass (mg) = tumor volume (mm^3) = width² x length/2.³⁰ Animals were sacrificed by cervical dislocation under isoflurane anesthesia on day 21 or upon body weight loss exceeding 20%. Gastrocnemius (GCM), tibialis anterior (TA) and soleus (Sol) muscles of both hind legs and tumor were dissected and weighed. Muscle weight from the left and right hind leg were averaged to provide a mean muscle weight (GCM, TA, and Sol) for each animal. Muscle samples were immediately snap-frozen in liquid nitrogen and stored at -80 °C until analysis.

RNA ISOLATION AND REAL-TIME POLYMERASE CHAIN REACTION

Cancer-cachexia associated muscle wasting is known to be most pronounced in fast-twitch type II-containing muscles, such as GCM and TA. [31] Therefore, for gene expression analysis, total RNA was isolated from snap-frozen GCM muscle tissue using Trizol reagent (Invitrogen, Breda, the Netherlands), and subsequently purified by DNase treatment (RQ1 RNase-Free DNase) (Promega Benelux B.V., Leiden, the Netherlands). 1 μ g of total RNA was reversed transcribed to cDNA using random hexamer primers (Invitrogen, Breda, the Netherlands), and Superscript II RT (Invitrogen, Breda, the Netherlands). Quantitative real-time polymerase chain reaction (RT-PCR) was performed using an iCycler real-time PCR system (Biorad, California, The United States of America) using SYBR Green (Sigma-Aldrich, St. Louis, The United States of America). Used primer sequences can be found in Table 2. GAPDH was used as housekeeping gene for normalization. Relative gene expression fold change was calculated with the comparative delta-delta Ct method ($2^{-\Delta\Delta\text{Ct}}$)/(average $2^{-\Delta\Delta\text{Ct}}$ (healthy controls)).³² Each sample was tested in duplicate.

Table 2. Reverse transcription-polymerase chain reaction primer sequences.

Gene	Forward Primer	Reverse Primer	Genbank Accession Number
Atrogin1	5'-GTTTTTCAGCAGGCCAAGAAG	5'-TTGCCAGAGAACACGCTATG	AF_441120
MyoD	5'-AAACCCCAATGCGATTATCAGG	5'-TAAGCTTCATCTTTGGGCGTGA	NM_010866
Myogenin	5'-CACTCCCTTACGTCCATCGT	5'-CAGGACAGCCCCACTTAAAA	NM_031189
Murf1	5'-AGGTGTCAGCGAAAAGCAGT	5'-CCTCCTTTGTCCTCTTGCTG	NM_009066
GAPDH	5'-ATGCATCCTGCACCACTCACT	5'-CAGTGATGGCATGGACTGTG	NM_008084

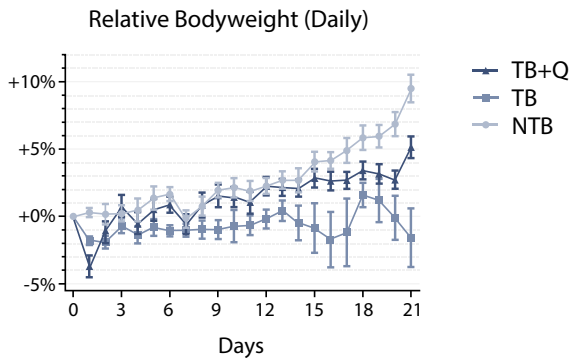
STATISTICS

Categorical data are expressed as number (percentage) and continuous variables as mean \pm SEM (normal distribution, visually assessed and by means of the Shapiro-Wilks test). Body weight and grip-strength were normalized to each animal's body weight and grip-strength respectively on day 0. Muscle weight from the left and right hind leg were averaged to provide a mean GCM and TA muscle weight for each animal. Multiple group comparisons were done by one-way ANOVA with a Bonferroni's post hoc test. Spearman-Rho rank correlation coefficient was used for testing bivariate correlations. All analyses were performed using IBM SPSS Statistics for Windows, version 21.0 (IBM Corp., Armonk, NY, USA). A P value < 0.05 was considered statistically significant.

RESULTS

To study the effects of a quercetin supplemented diet, ten CD2F1 mice were randomly allocated to three groups, i.e. non-tumor bearing (NTB) with access to regular chow, tumor-bearing (TB) with access to regular chow, and tumor-bearing with access to chow supplemented with 250 mg quercetin per kg chow (TB+Q), starting on the first day of the experiment. Simultaneously, mice allocated to tumor-bearing groups were inoculated subcutaneously with 0.5×10^6 C26 adenocarcinoma cells. Body weight was recorded daily.

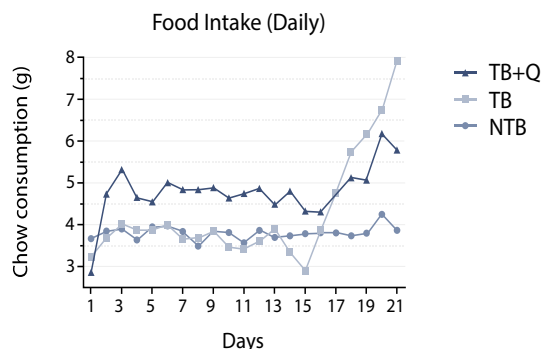
Figure 1. Daily body weight throughout the experiment.



Line chart depicting the mean \pm SEM daily bodyweights per group in non-tumor-bearing male CD2F1 mice (NTB, $n = 10$), C26 tumor-bearing (TB, $n = 10$) mice with ad libitum access to regular chow, and C26 TB mice with ad libitum access to quercetin supplemented chow (TB+Q, $n = 10$). Bodyweight was normalized to each animal's body weight on day 0 and is expressed as the percental difference. Mice in the TB group experienced a significant loss of body weight in comparison to both TB+Q and NTB mice.

During the experiment NTB mice gained 9.4 % bodyweight, whereas TB mice lost 3.9 % body weight ($p < 0.0001$) (Figure 1). Tumor-bearing mice showed a decrease in body weight on the first day after changing the diet to quercetin supplemented, likely as a result of the different taste of this diet. Thereafter, however, they gained 5.3 % body weight, which was significant when compared to tumor-bearing mice on a regular diet ($p = 0.0024$).

Daily food intake was relatively constant for non-tumor-bearing mice throughout the experiment with a mean daily intake of 3.8 g chow per mouse (Figure 2). For tumor-bearing mice, on a regular diet, comparable food intake was observed during the first two weeks of the experiment. Throughout the last 7 days of the experiment, these mice showed a gradual increase in daily food intake up to a mean of 7.9 g chow per mouse on the last time point. Mice on the quercetin diet had an increased intake throughout the experiment, starting rapidly after initiation of this diet, resulting in a mean daily intake of 4.7 g chow per mouse during the first two weeks of the experiment.

Figure 2. Daily food intake throughout the experiment.

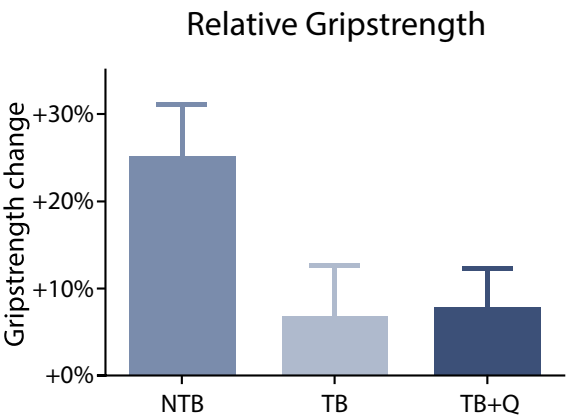
Line chart depicting the mean daily food intake over the prior 24 hours per group in non-tumor-bearing male CD2F1 mice (NTB, $n = 10$), C26 tumor-bearing (TB) mice with ad libitum access to regular chow (C26 TB, $n = 10$) and mice with ad libitum access to quercetin supplemented chow (TB+Q, $n = 10$). Food intake was measured per cage and subsequently averaged per mouse. Food intake is expressed in grams (g). Food intake of TB mice was comparable to NTB mice throughout the first two weeks, subsequently, daily chow consumption more than doubled. TB+Q mice had an overall higher chow consumption throughout the first two weeks, subsequently, a minor increase in chow consumption was observed in the final days of the experiment.

These tumor-bearing mice on a quercetin diet too were observed to increase their daily food intake during the last 7 days of the experiment, up to a maximum mean of 6.2 g chow per mouse on the 20th day of the experiment.

Grip-strength was assessed twice per week. NTB mice showed an increase in grip-strength over the course of the experiment. This increase registered up to 24.7% at the end of the experiment (Figure 3). TB mice, on the other hand, showed a limited increase of 6.7% at the end of the experiment, this difference did not reach significance ($p = 0.06$). Tumor-bearing mice on a quercetin supplemented diet had a comparable grip-strength to the tumor-bearing mice on a regular chow diet. These mice too showed a limited increase in grip-strength of 7.6%.

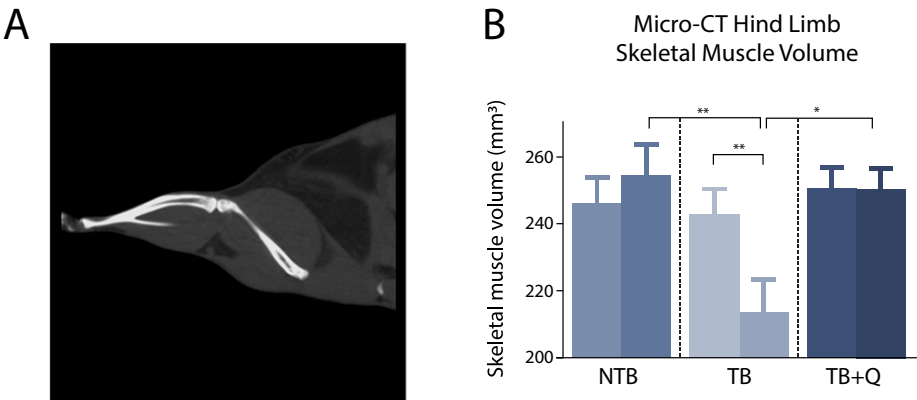
Hindlimb skeletal muscle volumetric measurements by micro-CT (Figure 4A) on day 1 were comparable for all three groups; 245.6 mm³ for NTB mice, 242.0 mm³ for TB mice, and 250.1 mm³ for TB+Q mice (Figure 4B). No significant change was observed in skeletal muscle volume on day 21 for NTB mice and TB+Q mice; 253.8 mm³ for NTB mice, and 249.6 mm³ for TB+Q mice. Tumor-bearing mice on a regular chow diet experienced a decrease in skeletal muscle volume to 212.8 mm³ on day 21 ($p = 0.006$). These differences between NTB and TB, as well as TB+Q and TB, were significant with respective p -values of 0.0078 and 0.0172. A high positive correlation is observed between skeletal muscle volume on day 21 and combined right hindlimb muscle weight (GCM, TA, and Sol) at sacrifice (Spearman's $\rho = 0.718$, $p < 0.0001$).

Figure 3. Relative grip-strength at the end of the experiment.



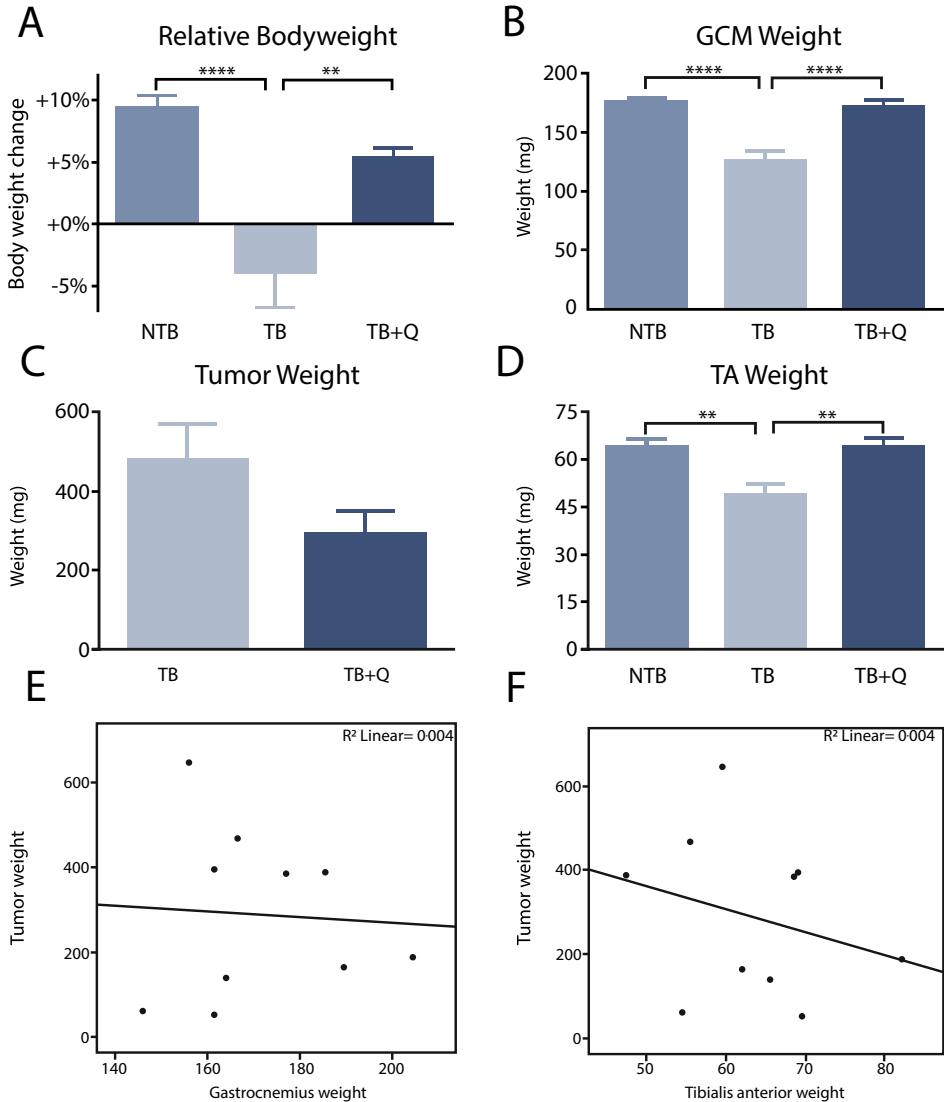
Bar graphs depicting the mean \pm SEM for final grip-strength normalized to starting grip-strength in chow (TB+Q, $n = 10$). Multiple group comparisons were done by one-way ANOVA with a Bonferroni's post hoc test. All groups were compared against TB mice. A substantial, non-significant difference in relative grip-strength was observed non-tumor-bearing male CD2F1 mice (NTB, $n = 10$), C26 tumor-bearing (TB) mice with ad libitum access to regular chow (C26 TB, $n = 10$) and mice with ad libitum access to quercetin supplemented between TB and NTB mice. TB+Q relative grip-strength was comparable to TB mice.

Figure 4. Micro-CT hindlimb skeletal muscle mass volumetric.

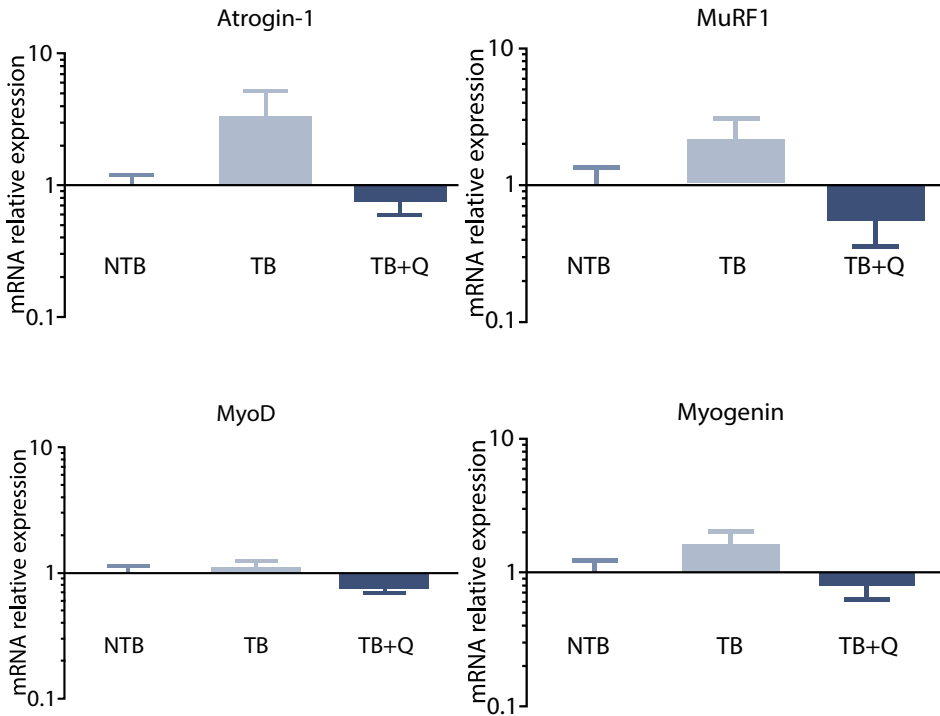


(A) Micro-CT image depicting the hindlimb musculature. (B) Bar graphs depicting the mean \pm SEM for micro-CT skeletal muscle volume on day 1 and day 21 respectively in non-tumor-bearing male CD2F1 mice (NTB, $n = 10$), C26 tumor-bearing (TB) animals with ad libitum access to regular chow (C26 TB, $n = 10$) and mice with ad libitum access to quercetin supplemented chow (TB+Q, $n = 10$). Multiple group comparisons were done by one-way ANOVA with a Bonferroni's post hoc test. All groups were compared against TB mice. Comparisons between multiple time-points were done by paired samples t-test. * $p < 0.05$ ** $p < 0.01$.

Figure 5. Bodyweight, muscle weight and tumor mass at sacrifice.



Bar graphs depicting the mean \pm SEM for (A) final bodyweight normalized to starting bodyweight, (B) gastrocnemius muscle weight, (C) tumor weight, and (D) tibialis anterior muscle weight in non-tumor-bearing male CD2F1 mice (NTB, $n = 10$), C26 tumor-bearing (TB) mice with ad libitum access to regular chow (C26 TB, $n = 10$) and mice with ad libitum access to quercetin supplemented chow (TB+Q, $n = 10$). Multiple group comparisons were done by one-way ANOVA with a Bonferroni's post hoc test. All groups were compared against TB mice. Asterisk brackets are displayed for significant results only. ** $p < 0.01$ **** $p < 0.0001$. A possible tumor weight reduction in quercetin treated mice was noted, therefore the relationship between gastrocnemius muscle weight and tumor weight, as well as tibialis anterior muscle weight and tumor weight, were assessed to demonstrate that a possible reduction in tumor burden did not contribute to the attenuation in muscle atrophy. Scatter-dot plots depict no relationship between (E) gastrocnemius muscle mass and tumor mass (Spearman's $\rho = 0.091$, $p = 0.80$) and (F) tibialis anterior muscle mass and tumor mass (Spearman's $\rho = -0.261$, $p = 0.47$) in quercetin-treated mice. Considering these statistics, the attenuation in muscle atrophy cannot be sufficiently explained by differences in tumor burden.

Figure 6. mRNA expression levels in cachectic muscle.

Bar graphs depicting the mean \pm SEM mRNA expression levels in gastrocnemius muscle of (A) Atrogin-1, (B) MuRF1, (C) MyoD and (D) Myogenin in non-tumor-bearing male CD2F1 mice (NTB, $n = 10$), C26 tumor-bearing (TB) mice with ad libitum access to regular chow (C26 TB, $n = 10$) and mice with ad libitum access to quercetin supplemented chow (TB+Q, $n = 10$). Multiple group comparisons were done by one-way ANOVA with a Bonferroni's post hoc test. All groups were compared against TB mice. A substantial but non-significant difference in expression of E3 ubiquitin ligase atrogin-1, and to a lesser extent MuRF1, was observed between TB and TB+Q mice.

All animals were sacrificed at 21 days following tumor inoculation. The gastrocnemius and tibialis anterior muscles were resected and weighed. Mean gastrocnemius muscle weight in NTB mice was 175.2 ± 12.4 mg versus 125.5 ± 27.3 in TB mice ($p < 0.0001$, Figure 5B). Mean gastrocnemius muscle weight in TB+Q mice was 171.3 ± 17.8 mg ($p < 0.0001$ compared to TB mice). Mean tibialis anterior muscle weight in NTB mice was 64.1 ± 7.1 mg versus 48.9 ± 11.1 in TB mice ($p = 0.0024$, Figure 5D). Mean tibialis anterior muscle weight in TB+Q mice was 63.7 ± 9.6 mg ($p = 0.0031$ compared to TB mice). Tumor mass increased until at day 21 a mean tumor weight of 478.3 ± 288.7 mg was recorded for TB mice and 288.7 ± 196.3 mg for TB+Q mice ($p = 0.14$) (Figure 5C). Tumor mass was not correlated with tibialis anterior muscle weight (Spearman's $\rho = -0.261$, $p = 0.47$) and gastrocnemius muscle weight (Spearman's $\rho = 0.091$, $p = 0.80$) at sacrifice for TB+Q mice (Figure 5E, 5F).

Skeletal muscle E3 ubiquitin ligases and myogenic regulatory factors mRNA expression profiles were determined in gastrocnemius muscle samples. A substantial, non-significant difference in expression of E3 ubiquitin ligase atrogin-1 was observed between TB and TB+Q mice, as well as to a lesser extent in the expression of MuRF1 (Figure 6A, 6B). Expression of MyoD and Myogenin was comparable between all groups (Figure 6C, 6D).

DISCUSSION

Cancer-associated cachexia is a common finding in patients afflicted by multiple types of malignancies.^{26, 33} It has detrimental effects on survival as well as the quality of life.^{26, 33} Unfortunately, there are no validated treatment modalities to halt or reverse the associated progressive muscle wasting. Our study investigated whether quercetin supplementation could attenuate muscle wasting in the murine C26 cancer-cachexia model. We found this to be true, C26 tumor-bearing mice on a diet supplemented with quercetin showed a preservation of gastrocnemius muscle weight as well as tibialis anterior muscle weight. In this group of mice, bodyweight was also preserved. These findings were accompanied by a substantial, non-significant difference in expression of E3 ubiquitin ligases atrogin-1 and MuRF1 between tumor-bearing mice on a regular chow diet and tumor-bearing mice on a quercetin supplemented diet. As this study was not powered to detect differences in mRNA expression we consider it likely this lack of significance is due to a type II error. Despite preservation of muscle mass and body weight, we did not observe a difference in grip-strength between TB and TB+Q mice. Our findings are in line with the study of Velázquez et al.¹⁴ They showed quercetin supplementation to help preserve muscle mass and limit bodyweight loss in an ApcMin/+ mice model. Furthermore, a recent study reported similar muscle preservation by quercetin treatment in nude mice with cachexia induction via A459 cells, a human alveolar basal epithelial adenocarcinoma cell line.³⁴ In contrast, C26 tumor-bearing mice given a cocktail of antioxidants, including quercetin, showed accelerated development of cachexia and even expedited death.³⁵ In our study food intake was higher in TB+Q mice compared to mice receiving control chow. Velázquez et al. found no difference in chow consumption between the quercetin-treated groups.¹⁴ This difference in food intake is not readily explained. Although food intake of TB mice was comparable to NTB mice throughout the first two weeks, daily chow consumption more than doubled during the final days of the experiment. Despite the increased nutritional intake, a significant loss of body weight was observed. This suggests a hypermetabolic state for these mice, but was not quantified in this study.

Quercetin is a natural, bioactive and readily available flavonoid found in a selection of fruits, vegetables and herbs¹⁸, as well as over the counter dietary supplement. The quercetin dose in the current study equates to a human equivalent dose³⁶ of 2.85 mg/kg. For an average European or Northern American adult³⁷, this would equate to 202 mg or 230 mg quercetin respectively. Well within the limits of what has been safely used in prior

studies^{19, 38, 39}, and below the threshold of what is commercially available in over the counter supplements in various western countries. Quercetin has previously been used in studies, including athletes, military personnel, and elderly persons, to investigate its potential as a performance-enhancing supplement. These studies yielded ambiguous results with regard to lean body mass, basal metabolic rate, total energy expenditure and VO₂max.^{19, 38-40} Moreover, quercetin has been investigated for its possible role as a senolytic compound with inconsistent results.^{41, 42} Although quercetin has been suggested as a therapeutic candidate in cachexia⁴³, no studies have been published on quercetin treatment countering muscle wasting in humans.⁴⁴

A substantial, non-significant difference in tumor burden was observed between tumor-bearing mice on a regular chow diet and tumor-bearing mice on a quercetin supplemented diet. Anti-cancer effects of quercetin have been previously reported, although available evidence is limited.⁴⁵⁻⁵⁰ In our study we found a negligible, non-significant correlation between tumor weight and muscle mass. However, we cannot exclude that the anti-tumor effect has impacted on other outcome parameters, e.g. by selective down-regulation of tumor-derived mediators of cachexia.

Using resected hindlimb muscle tissue at the end of the experiment as gold standard we found a highly positive correlation with micro-CT hindlimb skeletal muscle volume measurement. This technique was found to be feasible and relevant in the current study and may allow for quantitative assessment of individual muscles, as well as allowing for tissue composition quantification, i.e. assessment of fatty infiltration of muscle.

Several limitations apply to the present study. The study was powered on an expected reduction in loss of muscle weight. As such, non-significant differences in secondary outcome parameters (e.g. relative mRNA expression levels) may have been subject to type II errors. Furthermore, survival was not included as one of the endpoints due to the strict ethical guidelines associated with the initiation of this study.

In conclusion, dietary quercetin supplementation limits bodyweight loss and prevents muscle wasting in a murine C26-cancer-associated cachexia model. These data add to a body of evidence supporting the use of quercetin to halt muscle wasting in experimental cancer-associated cachexia models and pave the way for clinical research on the efficacy of quercetin in the attenuation of muscle wasting in humans.

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S. Levolger contributed to the research design, performed the research, analysed the data and wrote the paper. S. van den Engel and G. Ambagtsheer performed the research, aided in analysis of the mRNA expression data and in writing the paper. J.N.M. IJzermans and R.W.F. de Bruin contributed to the research design and writing the paper. This work was supported by the Applied Molecular Imaging Erasmus MC (AMIE) facility by providing the imaging equipment. We would like to thank R. Ridwan for the technical support of the micro-CT. Furthermore, we would like to thank Dr. G.M. van Woerden and prof.dr. Y. Elgersma from the dept. of neuroscience (Erasmus MC University Medical Center) allowing us to make use of the grip-strength meter. And lastly, we would like to thank prof.dr. D.O. McCarthy (Ohio State University, OH, USA) for kindly providing us with the C26 cell-line.

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PART FOUR

SUMMARY, CONCLUSIONS AND FUTURE DIRECTIONS

Chapter 11

Summary, general discussion and future directions

Chapter 12

Nederlandse samenvatting

CHAPTER 11

SUMMARY, GENERAL DISCUSSION AND FUTURE DIRECTIONS

SUMMARY AND GENERAL DISCUSSION

Skeletal muscle wasting has long been recognized to be indicative for poor outcome, one of the first recorded accounts dating back to Hippocrates who wrote “the flesh is consumed and becomes water, the abdomen fills with water, the feet and legs swell, the shoulders, clavicles, chest and thighs melt away, the illness is fatal”.¹ In the current era, medical imaging technologies may help in the detection of muscle wasting early during the course of disease, prior to the hallmark clinical characteristics as aforementioned.²⁻⁵ This may be particularly relevant in the identification of patients at risk for poor outcome following invasive surgical procedures as well as selection of patients for clinical trials. In this thesis, we have investigated the use of diagnostic computed tomography (CT) imaging² to measure skeletal muscle mass and determined the impact of low skeletal muscle mass in patients undergoing curative intent treatment for gastrointestinal and hepatopancreatobiliary malignancies, and patients considered for solid organ transplantation. Being able to identify muscle wasting early on in the course of disease, it prompts the question whether intervention to attenuate the loss of muscle mass is possible and beneficial. For this reason, we have investigated various treatment strategies in an experimental cancer-cachexia model, expanding on an increasing body of knowledge on this subject reported over the last years.⁶⁻¹⁴

SARCOPENIA AND SURGERY: MORBIDITY AND SURVIVAL

Over the last years, sarcopenia has become a focal point of attention as a possible indicator for worsened outcome following invasive treatment procedures (e.g. surgery), physically demanding treatment procedures (e.g. chemotherapy), and possibly disease alone irrespective of treatment.^{2, 3, 15} In many of these studies the use of routinely obtained diagnostic computed tomography imaging has given us a valuable insight on the prognostic impact muscle wasting in various pathologic conditions has on either overall survival, disease-free survival, short-term outcome, quality of life, and/or physical functioning.^{2, 3, 15-30}

In **part one** of this thesis we assessed the impact of muscle wasting in patients undergoing surgical resection for a variety of malignancies and for solid organ transplantation. In **chapter 2** a systematic search of Embase, PubMed and Web of Science was performed to identify studies which assessed the impact of CT-assessed sarcopenia on short- and long-term outcomes in patients undergoing surgical resection

of gastrointestinal and hepatopancreatobiliary malignancies. Thirteen observational studies with a total of 2884 patients were included in the analysis.²¹⁻³³ The malignancies of affected patients included in these cohorts encompassed esophageal cancer, gastric cancer, colorectal cancer including hepatic metastatic disease, hepatocellular cancer, and pancreatic cancer. Due to great heterogeneity between studies no meta-analysis was performed. A wide variation in prevalence of sarcopenia was observed, ranging from 17 per cent in a cohort of patients with hepatic colorectal metastases to 79 per cent in a cohort of esophageal and gastric cancer patients.^{26, 31} Less variation in the prevalence of sarcopenia was observed when grouping malignancies by type.^{21-23, 26, 27, 30, 33} Post-operative morbidity rates were found to be increased in all studies investigating this outcome in colorectal cancer and hepatic colorectal metastases cohorts.^{26, 27, 29, 30} No differences in post-operative morbidity rates were reported in other malignancies. Sarcopenia was reported to be associated with disease-free survival in four of nine studies, with no distinct difference between cancer site or tumor origin.^{21-24, 26, 28, 29, 32, 33} On the other hand, most authors did report a significant decrease in overall survival in patients with sarcopenia, irrespective of cancer site or tumor origin.^{21-25, 28, 29, 33-35} It needs to be taken into consideration however that there has been no standard definition for sarcopenia used in these studies. Not only were distinct (sex-specific) cut-off values used between studies using comparable methods for skeletal muscle mass assessment, some studies made use of different techniques entirely.

In **chapter 3** a systematic review and meta-analysis on the impact of computed tomography assessed skeletal muscle mass on outcome in liver transplant candidates was performed. In this systematic review a total of nineteen studies with a total of 3803 patients were included in the analysis.³⁶⁻⁵⁴ The prevalence of sarcopenia varied between 22.2% and 70% in the included patient cohorts.^{36-38, 40-52, 54} For all studies reporting distinct prevalence rates for male and female patients, a higher prevalence amongst male patients was observed.^{36, 38, 41, 45-50, 54} Sarcopenia was found to be a significant predictor of waiting list mortality, independent of the model for end-stage liver disease (MELD) score, which is regarded as a reliable measure of mortality risk and is used for stratification of liver transplant candidates by Eurotransplant. Seven of eleven enrolled studies describing overall survival reported sarcopenia to be negatively associated with overall survival.^{37, 38, 40, 42, 44, 45, 53} In meta-analysis sarcopenia as dichotomous variable, cross-sectional muscle area, and psoas muscle area were similarly associated with overall survival in the enrolled studies. Two studies reported overall postoperative complications, in both of these studies low skeletal muscle mass was associated with increased risk of postoperative complications.^{44, 52}

Furthermore, severe postoperative complications were found to have occurred exclusively in sarcopenic patients. Moreover, sarcopenia was reported to be associated with increased rates of sepsis and bacterial infections following surgery.

Chapter 4 investigates the inter- and intra-observer correlations as well as intra-software correlations of 4 frequently described software applications, i.e. ImageJ , OsiriX, FatSeg, and sliceOmatic.^{2, 55-57} This study showed an excellent agreement for cross-sectional muscle area, visceral adipose tissue area and subcutaneous adipose tissue area for all included software applications. Furthermore, excellent inter- and intra-observer agreement were achieved. Therefore, we concluded that the results of studies using these different software applications may reliably be compared.

A comparable, negative impact of sarcopenia on outcome was observed in our own center. In **chapter 5** we assess the impact of body composition, i.e. sarcopenia and visceral obesity, in patients undergoing surgical resection for hepatic liver metastases in a cohort of 196 patients. Cross-sectional muscle index was determined by measuring the total surface area of skeletal muscle at the level of the third lumbar vertebrae, i.e. rectus abdominis, external and internal obliques, transversus abdominis, quadratus lumborum, erector spinae and psoas muscles and subsequently normalizing it for squared patient height. Subsequently, gender-specific cut-off values at which the survival difference between the two groups were most pronounced were determined using optimal stratification. Computed thresholds for sarcopenia were $41.1 \text{ cm}^2/\text{m}^2$ for female patients and $43.75 \text{ cm}^2/\text{m}^2$ for male patients. Sarcopenia was found to be associated with significantly diminished long term outcome. Moreover, when adjusting for well known risk-factors, sarcopenia was found to be an independent predictor of worse disease-free and overall survival. Likewise, the impact of visceral obesity on overall survival was assessed. The computed threshold for visceral obesity was determined to be $94 \text{ cm}^2/\text{m}^2$ for male patients and was associated with an increased risk of recurrence. No threshold for visceral obesity could be determined in female patients. Others have also linked visceral adiposity to reduced disease-free survival or increased incidence of (pre-)malignant tumors of the gastro-intestinal tract.^{58, 59}

In **chapter 6** we discuss the presence and impact of sarcopenia in patients undergoing surgical resection or radiofrequency ablation therapy (limited to lesions $\leq 3 \text{ cm}$) for hepatocellular carcinoma in a cohort of 90 patients. Patients with Child-Pugh B or Child-Pugh C liver cirrhosis were not included in this study due to insufficient patient numbers. Cross-sectional muscle index and subsequent computation of sarcopenia thresholds were done in accordance to methods as described in the previous chapter.

Computed thresholds for sarcopenia were $39.5 \text{ cm}^2/\text{m}^2$ for female patients and $52.0 \text{ cm}^2/\text{m}^2$ for male patients. Patients with sarcopenia had a reduced overall survival, independent of available known risk factors. A steep decline in survival was noted in the first months following therapy in patients with sarcopenia associated with an increased incidence of major complications. Liver failure, sepsis and aspiration attributed to the cause of death in these patients. This in line with prior results where sarcopenic patients have been reported to be more susceptible to nosocomial infection, poor wound healing and prolonged hospital stay.^{17,43,48} Furthermore, a strong interaction was found between sarcopenia and BMI. In patients with a BMI lower than 25, no differences in survival were found between patients with and without sarcopenia. However, in overweight or obese patients those with sarcopenia had a markedly reduced overall survival when compared to overweight or obese patients without sarcopenia. Whether the reduction in survival found in sarcopenic patients is due to an increased risk of recurrent disease or due to an increased risk of treatment-related death remains controversial, as the impact of sarcopenia on disease-free survival in HCC is ambiguous.¹⁸⁻²¹

As recent studies suggested that development of body composition throughout treatment or the course of disease might be a more sensitive prognostic factor than single time-point assessment of skeletal muscle index⁶⁰⁻⁶², **chapter 7** investigates not only single time-point body composition as performed in the aforementioned chapters, but also the change of body composition during neoadjuvant chemoradiotherapy for locally advanced rectal carcinoma followed by surgical resection in a cohort of 122 patients. Although following neoadjuvant chemoradiotherapy, mean skeletal muscle index (SMI) remained unchanged. A wide distribution in change of body composition was observed. An association was observed between Δ SMI and disease-free survival, as well as the development of distant metastases, following curative intent treatment. We did not observe any association between disease-stage and Δ SMI. There was however an association between vascular invasion and Δ SMI. The behavior of colorectal cancer is associated with different molecular subtypes, independent of TNM staging.⁶³ Selected molecular subtypes may be associated with a more aggressive tumor biology and stronger systemic catabolic response. While loss of muscle mass during neo-adjuvant chemoradiotherapy was strongly associated with disease-free survival and distant metastases free survival, single time-point measurements for sarcopenia that are widely used in literature were not predictive of survival in the current population.

LIMITING MUSCLE WASTING IN VIVO

In **part two** of this thesis we have explored a variety of treatment strategies to attenuate muscle wasting in a cancer-associated cachexia mouse model. We have first investigated the efficacy of activin-like kinase 4 and 5 inhibitors in the attenuation of skeletal muscle wasting in **chapter 8**. In this study we found that the inhibition of ALK 4 and ALK 5 limited muscle wasting and reduced the relative expression of the cachexia associated ubiquitin ligase MURF1. It also showed a clear trend to reduced Atrogin-1 expression, and favorably altered the miRNA expression profile of cachectic muscle. These results are promising and contribute to a growing body of evidence which suggests that muscle wasting in cancer cachexia in animal models might be limited by blocking the myostatin signaling pathway.^{6, 64-70} This knowledge may benefit in the selection and development of drug candidates for clinical trials for the treatment of cancer cachexia. Furthermore, we assessed whether simultaneous treatment with an anabolic agent, the IGF-1 analogue LONG R3 IGF-I, could elicit a beneficial, synergetic effect. Unfortunately, a beneficial synergistic role of LONG R3 IGF-I was not found in combination with ALK 4 and ALK 5 inhibitor SB431542. Moreover, a trend towards enhanced tumor-growth was observed.

In addition to potential new drug candidates for the treatment of skeletal muscle wasting, dietary interventions may elicit similar effects. Dietary intervention studies for the treatment of cancer cachexia have evaluated various supplements already, e.g. Long-chain omega-3 fatty acid, eicosapentaenoic acid (EPA) and β -hydroxy- β -methylbutyrate (HMB), a leucine metabolite, with mixed results.^{7, 71-77} Caloric restriction (CR) poses another interesting treatment strategy which may elicit similar effects. The beneficial effects of CR on healthspan and longevity have been thoroughly established in model organisms, and have been reported to include reduced incidence of cancer, cardiovascular disease, increased oxidative stress resistance⁷⁸⁻⁸⁵, and it is reported to limit sarcopenia in rodents and nonhuman primates.⁸⁶⁻⁸⁹ **Chapter 9** describes the impact of caloric restriction in an experimental cancer-associated cachexia model. It was found to limit the loss of muscle strength and did not aggravate the loss of cachexia associated muscle mass, despite significant body weight loss. As such we have to conclude that caloric restriction does not fully protect against the detrimental effects of cancer-associated cachexia. However, considering it did limit muscle strength loss and did not aggravate the loss of muscle mass, caloric restriction may possibly be safely utilized in improving the efficacy of-, and protect against the adverse side effects of anti-cancer therapies. However, further research is warranted to confirm these findings upon initiation of caloric restriction in early and late-stage cancer.

Lastly, in **chapter 10** we explored the potential of quercetin, a flavonoid and commercially available as over-the-counter product in various western countries, in the attenuation of cancer-associated cachexia. Its antioxidant, anti-inflammatory, and anti-aging effects have been described.⁹⁰⁻⁹³ And in both an APC knockout cachexia model as well as an obesity model quercetin supplementation attenuated the loss of muscle mass.^{7, 44} We found dietary quercetin supplementation to limit bodyweight loss and prevent muscle wasting in the C26 colon tumor cancer-associated cachexia model, adding to a body of evidence supporting the use of quercetin to halt muscle wasting in experimental cancer-associated cachexia models.

FUTURE DIRECTIONS

Throughout the last decade our understanding of the impact of muscle wasting on outcome in cancer patients and candidates for liver transplant surgery has vastly improved. Multiple studies have now unequivocally shown muscle wasting to be associated with a detrimental outcome in cancer patients⁴, liver transplant candidates⁵, patients with heart failure^{94, 95}, COPD⁹⁶, and chronic kidney disease⁹⁷. Awareness has been raised amongst the medical profession⁹⁸ as well as in mass media.^{99, 100}

However, its importance in clinical decision making is still often neglected. In part, this may be explained by limited awareness of available guidelines and lack of clinical consequence as sarcopenia is not often referenced in international treatment guidelines. The EWGSOP2 2018 updated definition on sarcopenia recommends to assess sarcopenia by testing for low muscle strength¹⁰¹ (hand dynamometer or chair-stand-test)¹⁰²⁻¹⁰⁴, subsequently confirming by quantifying low muscle quantity or quality, and lastly assess severity by testing for low physical performance (gait speed, short physical performance battery, timed-up-and-go test or 400 m walk test)¹⁰¹. Low muscle quantity or quality may be quantified by CT, MRI, DXA, ultrasound or bioelectrical impedance analysis (BIA). High-resolution imaging quantification via such as CT is likely to be more widely used in the future¹⁰¹. There are however several limitations to this technique. Radiation exposure, lack of automatic segmentation, the multitude of cut-off points currently available^{4, 5} and impact of scan parameters such as tube potential¹⁰⁸ and contrast timing¹⁰⁹ are important limitations to consider. Current available tools allow for manual or semi-automatic delineation of skeletal muscle mass on exported DICOM images, a tedious task which limits widespread adaptation. Automatic skeletal muscle mass assessment, readily integrated into available mainstream PACS viewers will be essential and need to be developed to make wide-spread adaptation of sarcopenia detection possible in daily clinical practice. Available cutoff points are often determined in different pathologies and it is not well known whether they are interchangeable. It is paramount to define population-specific values which may be used interchangeably irrelevant of pathology, adjusted for scan parameters, comparable to for instance the DXA population-specific reference standards. Current reference values in healthy populations are limited^{106, 107}, and may be influenced by not only gender and age, but also e.g. ethnicity and body mass index.¹⁰⁵

In recent years multiple pre-clinical studies have investigated various potential treatment strategies to counter cancer-associated cachexia, with mixed results. Some of these, including within the current thesis ALK4/5 inhibitors or quercetin, show promising results in a pre-clinical setting. Unfortunately, not all of these are readily translatable to a clinical trial, in part due to clinical cachexia's multifactorial etiology¹¹⁰⁻¹¹⁴, impacted by aging, disease, inactivity and malnutrition in varying extent.¹⁰¹ Considering the positive effects of quercetin in both this thesis and other studies^{7,44}, and its safety level as demonstrated by the over the counter availability as dietary supplement, a quercetin supplemented diet should be considered in clinical trial, comparing standard clinical care versus standard clinical plus multimodal intervention consisting of physical therapy, nutritional supplementation in the case of macronutrient deficiencies with or without dietary quercetin supplementation in a daily dose of 2.85 mg/kg body weight. Ideally, such therapy would be performed in a population at the time least influenced by concurrent therapy. Surgically treated rectal cancer patients with skeletal muscle wasting exceeding 1.95% for male patients, and 4.53% for female patients, fit this requirement. With a high proportion of disease recurrence in the first year postoperatively and no adjuvant therapy indicated, these patients could be enrolled 6 weeks post-surgery following initial post-operative recovery. The impact of short-term intervention could be assessed at a 6-week and 12-week timepoint. Long-term impact could be assessed at 12-months. All enrolled study candidates would need to be followed up for a period of 30 months to assess for detectable tumor recurrence and appropriate categorization in analysis.

Despite our increased understanding of the impact of muscle wasting on outcome in e.g. oncological patients and liver transplant candidates the underlying pathogenesis and molecular mechanisms are as yet not fully understood. Recent studies have further investigated the pathogenesis of muscle wasting in various experimental models as well as differences throughout the course of disease.¹¹⁵⁻¹¹⁸ There is not only a difference in gene regulation throughout the course of the development of muscle wasting, but also a difference depending on the tumor model used. Distinct cachexia phenotypes in cancer patients have also been described.¹¹⁹ It is important to also investigate the extent of heterogenicity in pathogenesis and molecular pathways involved in different patients groups. This may be further influenced by tumor characteristics, such as specific tumor mutations, comorbidity and age. Possibly requiring a tailor-made treatment approach for effective pharmaceutical treatment of muscle wasting.

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CHAPTER 12

NEDERLANDSE SAMENVATTING

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Skeletspierweefsel verval is reeds lange tijd beschreven als determinant van een slechte uitkomst, daterend terug tot Hippocrates. Hij omschreef het als “het vlees is geconsumeerd en wordt water, het abdomen vult zich met water, de voeten en benen zwellen op, de schouders, claviculae, borst en dijbenen smelten weg, deze ziekte is fataal”. Hedendaags kan medische beeldvorming een rol spelen in de detectie van skeletspierweefsel verval nog voordat deze tot klinische uiting komt. Dit kan met name relevant zijn in de detectie van patiënten waarbij een slechte postoperatieve uitkomst te verwachten is, dan wel als selectie criterium voor patiënten binnen klinische trials. In dit proefschrift hebben wij de rol van skeletspiermassa gemeten op diagnostische ‘computed tomography’ (CT) beeldvorming bepaald op postoperatieve uitkomst in patiënten die een behandeling met curatieve intentie hebben ondergaan voor gastro-intestinale en hepato-pancreato-biliaire maligniteiten, alsmede patiënten in aanmerking komend voor orgaan transplantatie. Met het in staat zijn tot vroegtijdige detectie, rijst de vraag of het mogelijk is interventies toe te passen gericht op het beperken van skeletspierweefsel verlies én of dit gepaard gaat met gezondheidswinst. Om dit te onderzoeken hebben wij meerdere behandelstrategieën onderzocht in een experimenteel kanker cachexie model, hierbij bijdragende aan de toenemende kennisontwikkeling omtrent dit vraagstuk.

SARCOPENIE EN CHIRURGIE: MORBIDITEIT EN OVERLEVING

Gedurende de laatste jaren is sarcopenie toenemend in de belangstelling komen te staan als mogelijke voorspeller van een verslechterde uitkomst na invasieve behandeling (bijv. chirurgie), fysiek veeleisende behandelingen (bijv. chemotherapie) en mogelijk ziekte op zich, onafhankelijk van de ingeslagen behandeling. Deze studies hebben de prognostische waarde van op CT gemeten spieroppervlakte aangetoond op algehele overleving, ziektevrije overleving, korte termijn uitkomsten, kwaliteit van leven en of fysiek functioneren in een brede variatie aan onderliggende pathologie.

In **deel één** van dit proefschrift hebben wij de impact van skeletspierweefselverlies beoordeeld in patiënten die een chirurgische behandeling in het kader van een variëteit aan maligniteiten ondergaat en patiënten die in aanmerking komen voor een levertransplantatie. In **hoofdstuk 2** is een systematische zoekstrategie binnen Embase, Pubmed en Web of Science uitgevoerd met als doel het identificeren van de beschikbare

studies waarin de invloed van aan de hand van CT-gemeten sarcopenie de impact of korte- en lange-termijn uitkomsten is bepaald in patiënten die een chirurgische behandeling hebben ondergaan voor een gastro-intestinale of hepato-pancreatico-biliaire maligniteit. Dertien observationele studies met een totaal van 2884 patiënten zijn geïnccludeerd in deze analyse. De maligniteiten van de aangedane patiënten binnen deze patiëntenpopulaties omvatten oesophaguscarcinomen, maagcarcinomen, colorectale carcinomen inclusief colorectale levermetastasen, hepatocellulair carcinoom en pancreascarcinoom. Gezien de grote heterogeniteit binnen de diverse studies is er geen meta-analyse verricht. Een brede variatie in prevalentie van sarcopenie werd bevonden, variërend van 17 procent in een cohort van patiënten met colorectale levermetastasen tot 79 procent in een cohort van patiënten met oesophagus- en maagcarcinomen. De variatie in prevalentie van sarcopenie was kleiner tussen de groepen met maligniteit van hetzelfde type. Postoperatieve morbiditeit was verhoogd voor sarcopenie patiënten in alle studies die deze uitkomst maat hebben gerapporteerd, omvattende patiëntenpopulaties met colorectale carcinomen en colorectale levermetastasen. Voor de overige maligniteiten is postoperatieve morbiditeit niet gerapporteerd. Sarcopenie was geassocieerd met ziektevrije overleving in vier van negen studies, zonder invloed van tumor lokalisatie dan wel origine. Daarentegen rapporteren de meeste auteurs een significante afname in algehele overleving in sarcopenie patiënten, zonder invloed van tumor lokalisatie dan wel origine. Er dient in acht genomen te worden dat er geen standaard definitie is gehanteerd om patiënten te classificeren als sarcopenie binnen deze studies. Niet enkel zijn er verschillende (geslachtsafhankelijke) afkapwaarden gebruikt in soortgelijke methoden voor skeletspiermassa oppervlakte bepalingen, maar zijn er in enkele studies andere methoden toegepast.

In **hoofdstuk 3** is een systematische review en meta-analyse omtrent de invloed van CT-gemeten sarcopenie op uitkomst in levertransplantatie kandidaten uitgevoerd. Negentien observationele studies met een totaal van 3803 patiënten zijn geïnccludeerd in deze analyse. De prevalentie van sarcopenie varieert tussen 22,2% en 70% in de geïnccludeerde patiëntenpopulaties. Alle studies die de prevalentiecijfers per geslacht vermelden beschrijven een hogere prevalentie in het mannelijk geslacht. Sarcopenie blijkt uit deze resultaten een onafhankelijke voorspeller voor wachtlijst mortaliteit, onafhankelijk van de 'Model for End-stage Liver Disease' (MELD) score, wat wordt beschouwd als een betrouwbare maat voor wachtlijst mortaliteit risico en toegepast wordt in de stratificatie voor levertransplantatie kandidaten door Eurotransplant. Zeven van elf geïnccludeerde studies die algehele overleving beschrijven rapporteren dat sarcopenie een negatieve invloed heeft op de algehele overleving. In de meta-analyse zijn sarcopenie als

dichotome variabele, skeletspiermassa oppervlakte en psoas spier oppervlakte soortgelijk geassocieerd met algehele overleving. Twee studies rapporteerden de postoperatieve complicaties, in beide studies was sarcopenie geassocieerd met een verhoogd risico op postoperatieve complicaties. Bovendien, ernstige postoperatieve complicaties werden enkel waargenomen in sarcopenie patiënten. Daarnaast werd sarcopenie geassocieerd met een verhoogd risico op sepsis en bacteriële infecties na chirurgie.

Hoofdstuk 4 onderzoekt de inter- en intra-observer correlaties alsmede ook de intra-software correlaties van 4 frequent gebruikte software toepassingen, i.e. ImageJ, OsiriX, FatSeg en sliceOmatic. Dit onderzoek toont een uitstekende overeenkomst voor cross-sectionele skeletspiermassa oppervlakte, viscerale vetweefsel oppervlakte en subcutaan vetweefsel oppervlakte metingen voor de diverse software toepassingen. Bovendien werd ook een uitstekende inter- en intra-observer overeenkomst bereikt. Dientengevolge konden wij concluderen dat de resultaten van studies die verschillende software toepassingen gebruiken betrouwbaar vergeleken kunnen worden.

Een soortgelijke, negatieve invloed van sarcopenie op uitkomst werd ook in ons eigen centrum waargenomen. In **hoofdstuk 5** beschrijven wij de invloed van lichaamssamenstelling, dat wil zeggen sarcopenie en viscerale obesitas, op uitkomst in 196 patiënten met colorectale levermetastasen die een chirurgische behandeling hebben ondergaan. Cross-sectionele skeletspiermassa index was bepaald door het meten van het totale skeletspiermassa oppervlakte op het niveau van de derde lumbale wervel (rectus abdominis, obliquus externus en internus, transversus abdominis, quadratus lumborum, erector spinae en psoas musculatuur) en dit te normaliseren voor lengte in het kwadraat. Vervolgens zijn geslachtsafhankelijke afkapwaarden waarop het verschil in overleving tussen de twee groepen de grootste significantie vertoonde bepaald gebruik makend van optimale stratificatie. Berekende afkapwaarden voor sarcopenie waren $41.1 \text{ cm}^2/\text{m}^2$ voor vrouwen en $43.75 \text{ cm}^2/\text{m}^2$ voor mannen. Sarcopenie was geassocieerd met een significante verslechtering van de lange-termijn uitkomst. Gecorrigeerd voor bekende risicofactoren was sarcopenie een onafhankelijke voorspeller voor een verslechterde ziektevrije en algehele overleving. Op soortgelijke wijze is ook de invloed van viscerale obesitas bepaald. De berekende afkapwaarde was $94 \text{ cm}^2/\text{m}^2$ voor mannen en was geassocieerd met een afgenomen ziektevrije overleving. Voor vrouwen kon geen afkapwaarde worden bevonden. Andere auteurs hebben ook een negatieve associatie beschreven tussen ziektevrije overleving of verhoogde incidentie van (pre-) maligne tumoren van het gastro-intestinale systeem.

In **hoofdstuk 6** hebben wij de invloed van sarcopenie onderzocht in 90 patiënten die een chirurgische resectie of radiofrequente ablatie (laesies beperkt tot ≤ 3 cm) van een hepatocellulair carcinoom hebben ondergaan. Patiënten met een Child-Pugh B of Child-Pugh C levercirrose zijn niet geïnccludeerd binnen dit onderzoek op basis van te kleine aantallen. Cross-sectionele skeletspiermassa index en hieropvolgende berekening van sarcopenie afkapwaarden is gedaan zoals beschreven in het voorgaande hoofdstuk. Berekende afkapwaarden voor sarcopenie waren $39.5 \text{ cm}^2/\text{m}^2$ voor vrouwen en $52.0 \text{ cm}^2/\text{m}^2$ voor mannen. Patiënten met sarcopenie hadden een afname in algehele overleving, onafhankelijk van bekende risicofactoren. Een sterke afname in overleving werd waargenomen in de eerste maanden na behandeling, geassocieerd met een toegenomen incidentie aan majeure complicaties. Leverfalen, sepsis en aspiratie droegen bij aan de doodsoorzaak van deze patiënten. Dit in lijn met eerdere resultaten waarin beschreven wordt dat sarcopene patiënten vatbaarder zijn voor ziekenhuisinfecties, slechte wondgenezing en verlengde opnameduur. Er werd een sterke interactie bevonden tussen sarcopenie en BMI. In patiënten met een BMI kleiner dan 25 werd geen verschil in overleving bevonden tussen sarcopene en niet-sarcopene patiënten. Daarentegen werd er een groot verschil in overleving bevonden tussen sarcopene en niet-sarcopene patiënten met overgewicht of obesitas. Of het verschil in mortaliteit tussen sarcopene en niet-sarcopene patiënten toegeschreven kan worden aan ziekte recidief of therapie-geassocieerde mortaliteit blijft controversieel, gezien de invloed van sarcopenie op ziektevrije overleving niet eenduidig is.

Recente onderzoeken suggereren dat de ontwikkeling van lichaamssamenstelling tijdens de behandeling of aanwezigheid van de ziekte mogelijk een betere prognostische indicator is dan een skeletspiermassa oppervlakte index meting op een enkel tijdstip. In lijn met deze kennis onderzochten wij in **hoofdstuk 7** niet alleen de invloed van een enkel tijdstip meting, maar ook het verschil in lichaamssamenstelling tijdens neo-adjuvante chemoradiotherapie in een patiënten populatie van 122 patiënten met een lokaal geavanceerd rectumcarcinoom. Hoewel tijdens neo-adjuvante chemoradiotherapie op populatie niveau de mean skeletspiermassa oppervlakte index onveranderd bleef, werd er een brede variatie in ontwikkeling van skeletspiermassa oppervlakte index (ΔSMI) bij de individuele patiënt waargenomen. Er was een associatie tussen ΔSMI en ziektevrije overleving, alsmede ook de ontwikkeling van afstandsmetastasen, na behandeling met curatieve intentie. Er werd geen associatie waargenomen tussen traditionele ziekte stadiëring en ΔSMI . Er was echter een associatie met vasculaire invasie. Het gedrag van colorectale carcinomen is geassocieerd met een diversiteit in moleculaire subtypes, onafhankelijk van TNM stadiëring. Bepaalde moleculaire subtypes

zijn mogelijkwijs geassocieerd met een meer agressieve tumor biologie en sterkere systemische katabole respons. Hoewel het verlies aan spiermassa tijdens neo-adjuvante chemoradiotherapie sterk geassocieerd was met ziektevrije overleving en afstands-metastasen vrije overleving, waren enkel tijdstip tot op heden frequent gebruikt worden binnen de literatuur niet voorspellend voor overleving binnen deze populatie.

REMMEN VAN SKELETSPIERMASSAVERLIES IN VIVO

In **deel twee** van dit proefschrift hebben wij verscheidende behandelstrategieën onderzocht met als doel het beperken van skeletspiermassaverlies in een kanker geassocieerde cachexie muizenmodel. Allereerst hebben wij de effectiviteit van activin-like kinase 4 en 5 remmers in het beperken van skeletspiermassaverlies onderzocht in **hoofdstuk 8**. In dit onderzoek hebben wij aangetoond dat remming van ALK 4 en ALK 5 het spiermassaverlies beperkt en de relatieve expressie van de met cachexie geassocieerde ubiquitine ligase MuRF1 reduceert. Tevens was er een duidelijke trend tot reductie van Atrogin-1 expressie en waren de miRNA expressie profielen ten faveure van de behandelde muizen. Deze resultaten zijn veelbelovend en dragen bij aan een toenemende hoeveelheid bewijs dat aantoont dat kanker geassocieerde cachexie in diermodellen kan worden geremd door de inhibitie van de myostatine signaalcascade. Deze kennis kan gebruikt worden in de selectie en ontwikkeling van nieuwe medicamenten voor toepassing in klinische trials voor de behandeld van kanker geassocieerde cachexie. Daarnaast hebben wij gekeken of simultane behandeling met een anabolisch medicament, de IGF-1 analoog LONG R3 IGF-I, een bijdragend, synergistisch effect teweeg kon brengen. Er bleek echter geen sprake van een bijdragend, synergistisch effect. Daarentegen werd er wel een trend tot toegenomen tumorgroei waargenomen.

Naast potentieel nieuwe medicamenten voor de behandeling van skeletspiermassaverlies kunnen soortgelijke effecten mogelijk bereikt worden met dieet interventies. Dieet interventie studies voor de behandeling van kanker geassocieerde cachexie hebben reeds multi-pele supplementen geëvalueerd, e.g. Long-chain omega-3 fatty acid, eicosapentaenoic acid (EPA) and β -hydroxy- β -methylbutyrate (HMB), een leucine metaboliet, met wisselvallige resultaten. Calorische restrictie (CR) vormt een andere interessante behandelstrategie waarmee soortgelijke effecten mogelijk behaald kunnen worden. De gunstige effecten van CR op levensduur zijn reeds beschreven en mede-gerapporteerd te leiden tot een afgenomen incidentie van kanker, hart- en vaat-ziekten en toegenomen oxidatieve stress resistentie. Bovendien is er een afname van

leeftijdsgebonden skeletspiermassaverlies beschreven in knaagdieren en non-humane primaten. Hoofdstuk 9 beschrijft de invloed van calorische restrictie in een experimenteel kanker geassocieerd cachexie model. Hier hebben wij aangetoond dat CR het verlies aan spierkracht beperkt en het verlies aan skeletspiermassa niet doet toenemen, ondanks een significante gewichtsreductie. Derhalve hebben wij moeten concluderen dat CR geen volledige bescherming biedt tegen de nadelige effecten van kanker geassocieerde cachexie. Echter, gezien het bevonden beperken van krachtsverlies en het niet doen toenemen van spiermassaverlies, kan CR wel veilig worden toegepast in het mogelijksterwijs doen verbeteren van de effectiviteit en doen afnemen van bijwerkingen van systemische behandelingen. Er is echter aanvullend onderzoek noodzakelijk om te bevestigen dat deze resultaten ook standhouden bij inductie van CR in laat- en eindstadium kanker. Tot slot hebben wij in hoofdstuk 10 quercetine, een commercieel verkrijgbaar flavonoïde, onderzocht als voedingssupplement om skeletspiermassaverlies te beperken. De antioxidantia, anti-inflammatoire en anti-veroudering effecten van quercetine zijn reeds beschreven. En in zowel een APC knockout cachexie model als een obesitas model is reeds een reductie aan skeletspiermassaverlies aangetoond bij quercetine behandeling. Wij hebben aangetoond dat quercetine suppletie gewichtsreductie beperkt en ook skeletspiermassaverlies beperkt in het C26 colon carcinoom geïnduceerde cachexie model, dit in aanvulling op de reeds bestaande literatuur.

APPENDICES

DANKWOORD
LIST OF PUBLICATIONS
CONTRIBUTING AUTHORS
CURRICULUM VITAE
PHD PORTFOLIO

DANKWOORD

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LIST OF PUBLICATIONS

PART OF THIS THESIS:

S. Levolger, S. van den Engel, S. Ambagtsheer, J.N.M. IJzermans, R.W.F. de Bruin. Quercetin supplementation attenuates muscle wasting in cancer-associated cachexia in mice. Submitted.

S. Levolger, E.A.C. Wiemer, J.L.A. van Vugt, S.A. Huisman, M.G. van Vledder, S. van den Engel, G. Ambagtsheer, J.N.M. IJzermans, R.W.F. de Bruin. Inhibition of activin-like kinase 4/5 attenuates cancer cachexia associated muscle wasting. *Scientific Reports*. 2019 JUL; 9:9826.

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J.L.A. van Vugt, **S. Levolger**, A. Gharbharan, M. Koek, W.J. Niessen, J.W. Burger, S.P. Willemsen, R.W.F. de Bruin, J.N.M. IJzermans. A comparative study of software programmes for cross-sectional muscle and adipose tissue measurements on abdominal computed tomography scans of rectal cancer patients. *J Cachexia Sarcopenia Muscle*. 2017 Apr;8(2):285-297.

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S. Levolger, M.G. van Vledder, R. Muslem, M. Koek, W.J. Niessen, R.A. de Man, R.W.F. de Bruin, J.N.M. IJzermans. Sarcopenia impairs survival in patients with potentially curable hepatocellular carcinoma. *J Surg Oncol*. 2015 Aug; 112(2):208-13.

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OTHER PUBLICATIONS:

K.F. Ma, B. Berends, I.R. Vedder, **S. Levolger**, M. Gupta, R.C.L. Schuurman, J.P.M. de Vries, R.P.H. Bokkers. Quantification of muscle mass in the legs of patients with peripheral arterial occlusive disease; associations between volumetric and cross-sectional single-slice measurements for identification of atrophy and focal sarcopenia. Submitted.

I.R. Vedder, **S. Levolger**, R.A.J.O. Dierckx, C.J. Zeebregts, J.P.M. de Vries, A.R. Viddeler, R.P.H. Bokkers. Effect of muscle depletion on survival in peripheral arterial occlusive disease: quality over quantity. Submitted.

J.L.A. van Vugt, M.P. Gaspersz, J. Vugts, S. Buettner, **S. Levolger**, R.W.F. de Bruin, W.G. Polak, J. de Jonge, F.E.J.A. Willemsen, B. Groot Koerkamp, J.N.M. IJzermans. Low Skeletal Muscle Density Is Associated with Early Death in Patients with Perihilar Cholangiocarcinoma Regardless of Subsequent Treatment. *Dig. Surg*. 2019 ;36(2):144-152.

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CURRICULUM VITAE

Stef Levolger, was born on April 14th 1987 in Rotterdam, the Netherlands. He graduated at the RSG Goeree-Overflakkee in Middelharnis, the Netherlands in 2005. Subsequently, he started his medical studies in Rotterdam, the Netherlands at the Erasmus University Rotterdam. One of the early chapters in this thesis was done as part of his scientific internship in 2010 – 2011 on body composition and outcome in patients undergoing liver resection for colorectal liver metastases. He obtained his medical degree on the 11th of November 2011 and started as a resident not in training (ANIOS) at the Department of Surgery at Erasmus MC, Rotterdam, the Netherlands whilst continuing his research activities.

September 2012 he got a full-time position as PhD candidate at the Department of Surgery at Erasmus MC, Rotterdam, the Netherlands at which he continued working on this thesis till September 2015 under the supervision of Prof. Dr. J.N.M. IJzermans and dr. R.W.F. de Bruin. After this date he continued his scientific research as part of this thesis whilst continuing his clinical career. Until October 2016 as a resident not in training (ANIOS) at the Department of Surgery at the IJsselland Hospital, Capelle aan den IJssel, the Netherlands. It was during this period that his ambitions changed, his interest for radiology exceeded his surgical interest. He is currently working as an interventional radiology resident in training (AIOS) at the Department of Radiology at the University Medical Center Groningen, Groningen, the Netherlands and ZiekenhuisGroep Twente, Almelo, the Netherlands.

PHD PORTFOLIO

Name PhD student:	S. Levolger	Promotor:	Prof. Dr. J.N.M. IJzermans
Erasmus MC department:	Surgery	Supervisors:	Dr. R.W.F. de Bruin
PhD period:	2012 - 2015		

	Year	Workload (ECTS)
Courses		
Laboratory animal science, Erasmus MC, Rotterdam, Netherlands	2012	5.7
Basiscursus Regelgeving en Organisatie (BROK), Erasmus MC, Rotterdam, Netherlands	2013	1.5
Basic introduction in SPSS, Erasmus MC, Rotterdam, Netherlands	2013	0.3
Attended conferences & poster presentations		
European Society for Surgical Research (ESSR), Aachen, Germany	2011	1.0
Symposium Experimenteel Onderzoek Heelkundige Specialismen (SEOHS), Amsterdam, Netherlands	2012	1.0
Nederlandse Vereniging voor Heelkunde (NVvH) Chirurgendagen, Veldhoven, Netherlands	2011 – 2014	4.0
European Society for Surgical Research (ESSR), Budapest, Hungary	2014	1.0
American Association for Cancer Research (AACR), San Diego, United States of America	2014	1.0
Podium presentations		
European Society for Surgical Research (ESSR), Aachen, Germany	2011	1.0
European Society for Surgical Research (ESSR), Budapest, Hungary	2014	1.0
Seminars and workshops		
Lof der Geneeskunst, Rotterdam, Netherlands	2012 – 2015	1.2
Molecular Medicine Day, Rotterdam, Netherlands	2013 – 2015	0.9
Wetenschapsdag Heelkunde Erasmus MC, Rotterdam, Netherlands	2014 – 2015	0.6
Teaching		
Examination of Basic Life support of medical students, Erasmus MC, Rotterdam, Netherlands	2012 – 2015	1.2

Appendix

	Year	Workload (ECTS)
Supervising medicine master student dhr. R. Muslem, Erasmus MC, Rotterdam, Netherlands	2013 – 2014	1.5
Supervising Life Sciences (HLO) bachelor thesis mw. Y.D.G. Reigina, Erasmus MC, Rotterdam, Netherlands	2013 – 2014	5.0
Supervising Life Sciences (HLO) bachelor thesis dhr. G. Dickens, Erasmus MC, Rotterdam, Netherlands	2014 – 2015	5.0
Supervising Life Sciences (HLO) bachelor thesis dhr. R. Porrazzo, Erasmus MC, Rotterdam, Netherlands	2014 – 2015	5.0
Other		
Reviewer for scientific journals (Journal of cachexia, sarcopenia and muscle; Journal of gastroenterology and hepatology; Liver International; Nutrition)		1.2

